

Full-length article

Effects of (-)-stepholidine on NMDA receptors: comparison with haloperidol and clozapine¹Wei-hua GU², Shen YANG², Wei-xing SHI³, Xue-chu ZHEN², Guo-zhang JIN^{2,4}²Department of Pharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes of Biological Sciences, Graduate School of the Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 201203, China;³Neuropsychopharmacological Research Unit, Department of Psychiatry, Yale University School of Medicine, New Haven, Connecticut 06511, USA**Key words**

(-)-stepholidine; haloperidol; clozapine; NMDA receptor; calcium imaging; neuroleptics

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Abstract

Aim: To examine whether (-)-stepholidine (SPD) has a direct effect on the *N*-methyl-*D*-aspartic acid receptors (NMDAR) containing the NMDA receptor subunits NR2A or NR2B and to compare its effect with those of haloperidol (Hal) and clozapine (Cloz). **Methods:** NMDAR was transiently expressed in human embryonic kidney 293 (HEK293) cells. Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) induced by NMDAR activation were monitored with Fura-2 ratio imaging techniques. **Results:** SPD had no significant effects on either subunit of NMDAR at a concentration of less than 100 μ mol/L. Hal selectively inhibited NMDAR containing the NR2B subunit, whereas Cloz inhibited both subunits of NMDAR. Although both Hal and Cloz inhibited NR1a/NR2B receptor-mediated Ca^{2+} influx, their effects were different. Hal was more potent and had a faster peak effect than Cloz. **Conclusion:** Both Hal and Cloz inhibit NMDAR-mediated function, whereas SPD produced only a little inhibition at a high concentration. Based on our other studies, the modulation of SPD on NMDAR function may be via D₁ receptor action underlying an indirect mechanism.

Introduction

Animal and imaging data support a novel model of schizophrenia that putative brain dopamine (DA) function imbalance, that is, the subcortical D₂ receptor (D₂R) hyperfunction and prefrontal cortical (PFC) D₁ receptor (D₁R) dysfunction, may be sequential to NMDA receptor (NMDAR) hypofunction in the PFC^[1,2]. NMDAR are ligandgated ion channels and are composed of multiple subunits (NR1, NR2A-D, and NR3A-C)^[3]. A remarkable property of NMDAR is its high permeability to the calcium ion (Ca^{2+}). Several studies have shown that neuroleptics may affect influence NMDAR directly or indirectly. Haloperidol (Hal), a typical neuroleptic, is a potent antagonist of D₂R. The cyclic AMP (cAMP) pathway is enhanced by Hal via its D₂R blockage and it can phosphorylate the NR1 subunit of the NMDAR, which is necessary for neuroleptic-mediated gene expression and may contribute to the therapeutic bene-

fits as well as side effects of neuroleptics treatment^[4]. Clozapine (Cloz), an atypical neuroleptic, is effective in treating positive and negative syndromes of schizophrenia and refractory schizophrenia. In contrast to Hal, Cloz treatment induces relatively few extrapyramidal side effects^[5]. A number of neurotransmitters and receptors, including Glu, DA, could contribute to Cloz's atypical profile and clinical efficacy^[6]. Cloz, but not Hal, can facilitate Glu receptor-mediated neurotransmission in the PFC^[7].

A direct interaction of neuroleptics with NMDAR has been demonstrated. Hal has been shown to inhibit NMDAR function in a subunit-selective manner with NR2B-containing receptors displaying more sensitivity to the drug than non-NR2B-containing receptors^[8]. Cloz, on the other hand, inhibits both NR2A and NR2B-containing NMDAR^[9]. It is noted that these effects of Hal and Cloz would be counteractive to their therapeutic effects since blockade of NMDAR exacerbates symptoms in schizophrenia^[10].

(-)-Stepholidine (SPD) is a tetrahydroprotoberberine alkaloid isolated from the Chinese herb *Stephania*. Our previous studies have shown that SPD is an agonist at D₁R, but acts as antagonist at D₂R^[2]. SPD exhibits a profile similar to that of atypical neuroleptics^[2,11–13]. More recently, it has been shown that SPD possesses agonist action on D₁R in the PFC pyramidal glutamic (Glu) neuron, by which it modulates firing activity of nucleus accumbens neurons^[14]; it mimics the effect of Cloz, preferentially increasing *c-fos* expression in the PFC via DA and non-DA systems^[15]. Moreover, it has been found that SPD (0.1–10 μmol/L) can potently enhance synaptically-evoked NMDA EPSC (excitatory postsynaptic currents) in PFC neurons recorded in brain slices^[16]; SPD (50 μmol/L) also increased the amplitude under the similar brain slices experiments^[17]. However, little is known about the effect of SPD on NMDA receptors by direct or indirect mechanisms at the molecular level.

This study is intended to elucidate whether SPD has a similar pharmacological property to NMDAR as Cloz or whether it is different from Cloz and Hal, using Ca²⁺ imaging techniques for studying NMDAR function. The calcium imaging technique has an advantage in studying NMDAR function without change of intracellular conditions, which is different from patch clamp recording. The latter might cause alterations in the concentrations of intracellular constituents, such as protein kinase or phosphatase (important constituents in modulating NMDAR activity). The direct effects of SPD were compared with Hal and Cloz on cloned NMDAR transiently expressed in HEK293 cells.

Materials and methods

Materials Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA), newborn calf serum (Si Ji Qing, Hangzhou, China), penicillin and streptomycin (Watson Biotechnologies, Shanghai, China), Fura-2 acetoxymethyl ester (Fura-2 AM, Molecular Probes, Eugene, OR, USA), pluronic-127 (Sigma, St Louis, MO, USA), *L*-Glu acid sodium and glycine (Gly, Sino-American Biotechnology, Beijing, China), and Hal and Cloz (RBI, Natick, MA, USA) were used in the experiments. (-)-Stepholidine was obtained from Shanghai Institute of Materia Medica (Chinese Academy of Sciences, Shanghai, China). (-)-Stepholidine and Hal were dissolved in DMSO at a final concentration of 100 mmol/L, and Cloz in 0.1 mol/L HCl at a final concentration of 100 mmol/L. All stock solutions were stored under -20 °C and diluted with extracellular solution immediately before use.

Cell culture and transfection The HEK293 cells, a generous gift from Dr Gang PEI (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences,

Chinese Academy of Sciences, Shanghai, China), were maintained in DMEM supplemented with 10% newborn calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin. The cells were plated at 2.5×10⁴ onto acid-washed, poly-*L*-lysine coated glass coverslips (20 mm×20 mm). Transfections were performed when cell confluence reached about 80%. Rat NR1a, NR2A, or NR2B (generous gifts from Dr John WOODWARD, Medical University of South Carolina, Charleston, SC, USA), and enhanced green fluorescent protein (EGFP) plasmids were delivered at a ratio of 1:1:1 by the calcium-phosphated precipitation method with the modification of an additional 1 mmol/L ketamine to prevent excitotoxicity during transfections^[18]. After transfection and incubation for 24 h, the cells were used for the experiments. EGFP was used to identify the transfected cells.

Calcium imaging^[19] The transfected cells were incubated with Fura-2 AM (4 μmol/L with 0.025% pluronic-127) for 45 min at 37 °C in extracellular solution (135 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl₂, 10 mmol/L glucose, and 5 mmol/L HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid); pH (6.8). After washing 3 times with Fura-free extracellular solution (pH 7.2), the dishes were mounted on the stage of an Olympus BX51WI upright microscope (Tokyo, Japan) and perfused continuously with modified Ringer's solution at a flow rate of 1.2–1.5 mL/min. *L*-Glu/Gly were applied via a computer-controlled Y-tube (outer φ=100 μm) situated 80 μm above the cells. The cells were exposed to the alternating 340 nm and 380 nm light from a xenon lamp via DG4 (Sutter Instrument Company, Novato, CA, USA) every 2 s during and immediately after Glu/Gly application, and the images were acquired via a charge coupled device (CCD) camera (CoolSNAP HQ, Roper Scientific, Duluth, GA, USA). To minimize UV exposure, images were taken every 15–60 s between drug applications. Ratio images were generated using MetaFlour software from Universal Imaging (West Chester, PA, USA). Intracellular calcium concentrations ([Ca²⁺]_i) were determined by the ratio of the intensity of the wavelengths at 340 and 380 nm. NMDA-dependent increases in [Ca²⁺]_i were calculated by subtracting the average baseline value from the peak value obtained during Glu/Gly application. SPD, Hal, and Cloz were applied throughout the bath.

Data analysis Data were analyzed using Origin 6.0 (OriginLab Corporation, Northampton, MA, USA) and expressed as mean±SEM. The statistical significance of a drug's effect was determined by comparing measures before and after drug injection using ANOVA followed by a *post-hoc* Tukey test^[20].

Results

Calcium imaging study of NMDA receptor function To establish stable Ca^{2+} response induced by *l*-Glu/Gly, and to protect cells from potential damage due to UV exposure, $[Ca^{2+}]_i$ induced by Glu/Gly was recorded at 2 s intervals and basal $[Ca^{2+}]_i$ were recorded at 15–60 s intervals. Pseudocolour ratio images at wavelengths of 340 and 380 nm were taken (Figure 1A). Cells were locally perfused with Glu/Gly (100/10 μ mol/L) for 3 s. An increase of $[Ca^{2+}]_i$ was detected in cells expressing NR1a/NR2A or NR1a/NR2B receptors. The peak of $[Ca^{2+}]_i$ was 3–7 s after Glu/Gly treatment and then returned to basal levels following washout for 300 s.

The local perfusion of Glu/Gly for 3 s could induce the reproducible increases in $[Ca^{2+}]_i$ performed every 400 s duration (Figure 1B). Bath applications of (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine (MK-801) (1 μ mol/L), a specific NMDAR antagonist, significantly blocked Glu/Gly-induced Ca^{2+} influx (80.3% \pm 1.4% of inhibition, $P<0.01$, $n=15$; Figure 1C).

Effects of SPD, Hal, and Cloz on the NR2B subunit of NMDA receptors In the cells expressing the NR1a/NR2B receptor, both Hal and Cloz significantly inhibited the increase in $[Ca^{2+}]_i$ induced by Glu/Gly. The inhibition induced by the 2 drugs could be observed at concentrations as low as 0.1–1 μ mol/L (Figure 2). In contrast, SPD elicited only

limited inhibition at a concentration of 100 μ mol/L (18.1% \pm 2.4% inhibition, $P<0.01$, $n=26$; Figures 2, 3). SPD produced no significant effect at less than 100 μ mol/L (0.1 μ mol/L, $P=0.97$, $n=12$; 1 μ mol/L, $P=0.52$, $n=7$; and 10 μ mol/L, $P=0.15$, $n=10$).

Although both Hal and Cloz inhibited the function of the NR1a/NR2B receptor, their effects were not entirely identified. Hal was much more potent than Cloz. Hal (0.1 μ mol/L) inhibited Glu/Gly-induced Ca^{2+} influx ($P<0.01$, $n=21$), while Cloz produced no inhibition at the same concentration. However, when the concentration reached 10 μ mol/L, Cloz inhibited the Glu/Gly-induced Ca^{2+} influx (Figure 3). The speed for Hal and Cloz operation also differed. Hal produced its peak effect at about 5 min after onset, while the maximal inhibition induced by Cloz required a 30 min perfusion. However, the effects of both Hal and Cloz were much less potent than that of MK-801 (Figure 2C).

Effects of SPD, Hal, and Cloz on the NR2A subunit of NMDA receptors Cloz, not SPD (100 μ mol/L) or Hal (50 μ mol/L), significantly inhibited the function of the NR1a/NR2A receptor (Figure 4A, 4B). Cloz produced an average of 31.2% \pm 4.2% ($P<0.01$, $n=9$) and 59.2% \pm 5.8% ($P<0.01$, $n=9$) inhibition at 10 μ mol/L and 100 μ mol/L, respectively. A similar result was observed with the NR1a/NR2B receptors. Cloz also showed a slow onset in its effect on the NR1a/NR2A receptors (Figure 4A, lower section), which took 30 min to

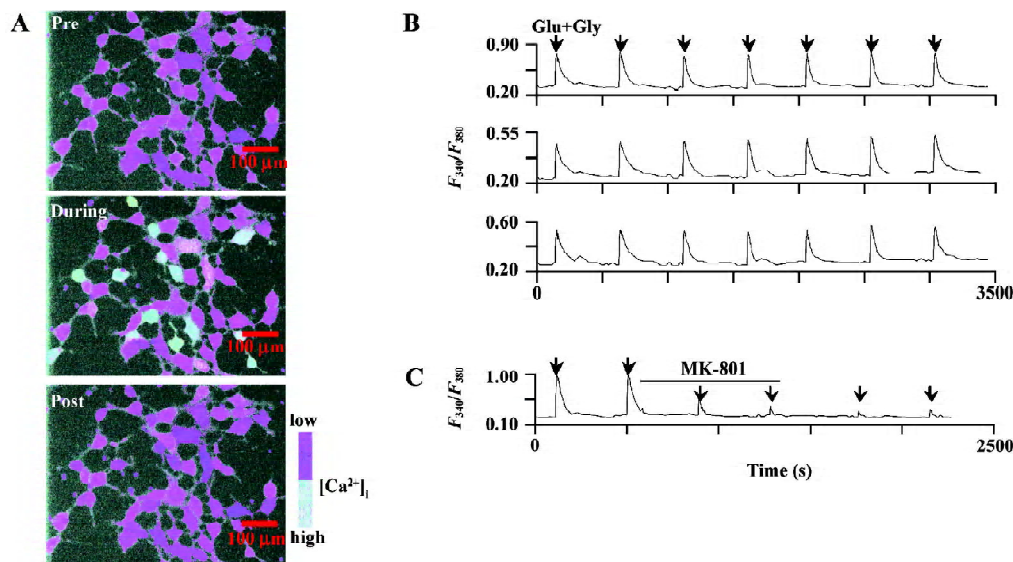


Figure 1. Calcium image of NMDA receptor function. (A) pseudocolour ratio images at wavelengths of 340 and 380 nm. Images pre-, during, and post-Glu/Gly application are shown. Increasing $[Ca^{2+}]_i$ was detected (middle section) and returned to the basal level after washout (lower section). Scale bar = 50 μ m. (B) representative tracing of Glu/Gly-induced Ca^{2+} influx in cells expressing NR1a/NR2B receptors. Glu/Gly-induced Ca^{2+} influx was highly reproducible when Glu/Gly was applied every 400 s (tested for 7 paradigms). (C) MK-801 (1 μ mol/L), a specific NMDA receptor antagonist, blocked Glu/Gly-induced Ca^{2+} influx.

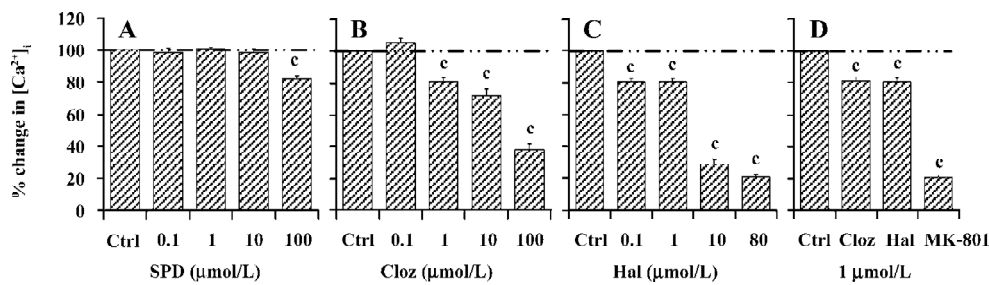


Figure 2. Dose-dependent response of SPD, Hal, and Cloz on the NR2B subunit of NMDA receptors and comparison with the effects of MK-801. Effects of both Hal and Cloz were much less potent than that of MK-801. Responses are expressed as a percentage of control values. ^c*P*<0.01 vs control.

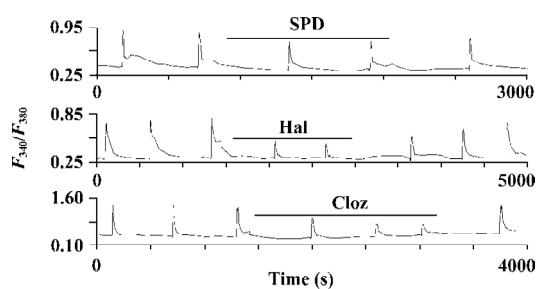


Figure 3. Representative trace of SPD, Hal, and Cloz on the NR2B subunit of NMDA receptors. SPD had a weak inhibition on the NR1a/NR2B receptors at high concentration (100 μmol/L, upper section). Cloz (100 μmol/L, lower section) had a gradually increasing inhibition on the NR1a/NR2B receptors. Hal effects (10 μmol/L, middle section) were longer lasting than Cloz (100 μmol/L).

reach the maximum effect.

Discussion

The results of the present study found that SPD did not interact with the NR1a/NR2A or NR1a/NR2B receptors at low concentrations, unless 100 μmol/L SPD elicited certain inhibitory effects on NR1a/NR2B receptor-mediated Ca²⁺ influx. It appeared that SPD did not interact directly with NMDAR. In contrast with SPD, Hal and Cloz produced significant inhibitory effect on NMDAR-mediated Ca²⁺ influx. However, Hal selectively blocked the NR1a/NR2B receptors, whereas Cloz inhibited both NR1a/NR2B- and NR1a/NR2A-mediated responses.

In a previous study^[8], Hal inhibited the function of NMDAR in a subunit-selective manner, with the NR2B-containing receptor more sensitive to the drug. However, Cloz inhibited Ca²⁺ influx mediated by both the NR2A and NR2B subunits of NMDAR^[9]. Our results of Hal and Cloz were similar to the above mentioned both reports. Therefore, the different effects of the 3 drugs on NMDAR function may

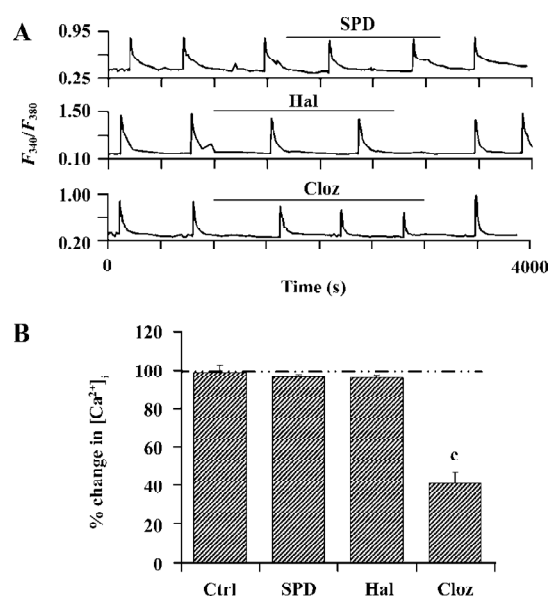


Figure 4. Effects of SPD, Hal, and Cloz on the NR2A subunit of NMDA receptors. (A) representative tracing of the effects of neuroleptics on Glu/Gly-induced Ca²⁺ influx in cells expressing NR1a/NR2A receptors. SPD, 100 μmol/L; Hal, 50 μmol/L; Cloz, 100 μmol/L. (B) summary of the effects. ^c*P*<0.01 vs control. *n*=13.

imply that SPD, Hal, and Cloz have different mechanisms in its antipsychotic use. In other words, Hal and Cloz have direct interaction with NMDAR, while SPD has an indirect one^[21].

Our results showed that the significant inhibitory effect on Ca²⁺ influx by Hal was observed at 0.1 μmol/L, while Cloz was at 1 μmol/L. However, the clinic plasma concentration of Hal and Cloz could reach 8–35 ng/mL (0.021–0.093 μmol/L)^[22] and 350 ng/mL (1.07 μmol/L)^[23,24], respectively, at which the 2 drugs may display their inhibitory effects on NMDAR-mediated Ca²⁺ influx. So what is the meaning of this inhibitory effect? There are two aspects with a contradiction. The inhibitory effect induced by Hal or Cloz is

implicated to be beneficial for the negative symptoms of schizophrenia^[25,26]. Alternatively, the inhibition of Hal and Cloz on NMDAR would limit their clinical therapeutic effect^[27,28]. It may be suggested that the inhibition induced by Hal or the Cloz D₂R blockade is the result of the inhibitory effect on the PFC NMDAR; subsequently, the inhibition would deteriorate the therapeutic effect, based on the DA function imbalance hypothesis^[1,2].

As to the indirect interaction between SPD and NMDAR, we obtained definite evidence requiring postsynaptic density-95 (PSD-95)^[21]. In an *in vitro* study, when PSD-95 was co-expressed with D₁R and NMDAR in HEK293 cells, the activation of D₁R could enhance NMDAR-mediated Ca²⁺ influx^[21]. SPD hence could not display its enhancing effect on NMDAR under such experimental conditions without PSD-95. In the present study, SPD also could not affect NMDAR directly in this *in vitro* expression system for lack of D₁R and other components.

On the other hand, in the experiment of brain PFC slices, including endogenous D₁R and PSD-95, the patch clamp study recently demonstrated that SPD could increase the amplitude^[17] or frequency^[16] of sEPSC recording from the NMDAR of PFC pyramidal cells, respectively. Furthermore, the D₁R agonist of SPD is mediated via intracellular protein kinase A and protein kinase C signaling pathways^[16]. These results also strongly support that the D₁R agonist of SPD enhances the NMDAR effect. Nevertheless, the enhancement of SPD on the NMDAR is obviously different from the inhibition elicited by Hal or Cloz on the NMDAR. According to these findings, 3 possible pathways have been proposed to explain how SPD can enhance NMDAR-mediated neurotransmission in the PFC. First, SPD possesses a unique D₁R agonist effect^[2], and the activation of D₁R has been shown to enhance NMDAR-mediated responses in PFC neurons^[16,29]. Second, SPD may act through the D₁R to either enhance synaptic Glu release or to increase the response of the cell to NMDAR activation^[16,17], as Cloz has been shown to increase spike-dependent presynaptic Glu release^[30]. Third, SPD could result in a part effect through non-DA systems^[15]. However, which class of non-DA receptors is responsible for the enhancing effect of SPD on NMDAR is still unknown.

Our results indicate that unlike Hal and Cloz, SPD did not directly affect the function of NMDAR at a low concentration. It is presumed that the modulatory effect of SPD on NMDAR function is an indirect effect via the D₁R^[16,21].

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