Molecular Pharmacology Fast Forward. Published on August 18, 2006 as doi:10.1124/mol.106.028332
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MOL #28332

Voltage-dependent Block of NMDA Receptors by Dopamine D1 Receptor Ligands

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Molecular Pharmacology Fast Forward. Published on August 18, 2006 as DOI: 10.1124/mol.106.028332
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Running Title: Blocking NMDA receptors by dopamine D1 ligands

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Number of Text pages: 31
Number of Table: 1
Number of Figures: 6
Number of References: 40
Number of words in the *Abstract*: 162
Number of words in the *Introduction:* 577
Number of words in the *Discussion:* 1469

Nonstandard abbreviations: NMDA, N-methyl D-Aspartic Acid; NR1A, NMDA receptor 1A; NR2A, NMDA receptor 2A; HEK cell, Humane Embryonic Kidney cell; EGFP, enhanced green fluorescent protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EBBS, Earle's Balanced Salt Solution; CsBAPTA, cesium salt of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetra acetic acid, Rp-cAMPs, adenosine- 3',5'-cyclic monophosphorothioate;

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Abstract

Accumulating evidence indicates that dopamine and D1 receptor ligands modulate NMDA receptors through a variety of D1 receptor-dependent mechanisms. Here, we reveal a distinct D1 receptor-independent mechanism by which NMDA receptors are modulated. Using the HEK cell recombinant system and dissociated neurons, we have discovered that dopamine and several D1 ligands act as voltage-dependent open channel blockers for NMDA receptors, regardless of whether they are agonists or antagonists for D1 receptors. Analysis of structural and functional relationships of D1 ligands revealed the elements that are critical for their binding to NMDA receptors. Furthermore, using D1 receptor knockout mice we verified that this channel blocking effect was independent of D1 receptors. Finally, we demonstrated that D1 ligands functionally interact with Mg^{2+} block through multiple sites, implying a possible role of the direct channel block under physiological conditions. Our results suggest that the direct inhibition of NMDA receptors by dopamine D1 receptor ligands is due to the channel pore block rather than receptor-receptor interactions.

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Functional integration of glutamate and dopamine neurotransmitter systems has been implicated in playing crucial roles in cognition, motor control, and a variety of neurological disorders (Cepeda and Levine, 2006; Greengard, 2001). Accumulating evidence demonstrates that dopamine or dopamine receptors modulate excitatory glutamatergic synaptic transmission in various brain regions through either presynaptic or postsynaptic mechanisms (Nicola et al., 2000). Despite the fact that these discoveries have greatly advanced our understanding of functional modulations of glutamate receptors by dopamine receptors, many studies on this topic provide conflicting results, particularly on D1 receptor modulation of NMDA receptors.

Some studies report the potentiation of NMDA receptor-mediated currents by D1 agonists (Cepeda et al., 1998; Chen et al., 2004; Flores-Hernandez et al., 2002; Seamans et al., 2001), while others suggest inhibition or no effects (Harvey and Lacey, 1997; Lee et al., 2002; Nicola et al., 1996; Otmakhova and Lisman, 1999). These conflicting results raise an intriguing question as to what accounts for these discrepancies. It is possible that they are due to differences in methodology and/or brain region specific effects. However, it is also possible that there are as yet undiscovered mechanisms underlying these contradictions. Noteworthy evidence exists which demonstrated some D1 agonists and antagonists had the same effect (Downes and Waddington, 1993; Wachtel and White, 1995), and dopamine could have both excitatory and inhibitory effects on neuronal activities depending on the concentration (Downes and Waddington, 1993). All these converging lines of evidence suggest that there is a missing piece of the puzzle in understanding the functional modulations of NMDA receptors by dopamine D1 receptors, which may arise from unrevealed properties of D1 ligands.

As a major subfamily of glutamate receptors, NMDA receptors perform essential roles in excitatory synaptic transmission and plasticity via both postsynaptic and presynaptic terminals (Dingledine et al., 1999). Functions of NMDA receptors are influenced by a variety of intracellular and extracellular modulators (Dingledine et al., 1999). One distinct property of NMDA receptors is the voltage-dependent block by physiological concentrations of Mg^{2+} ions

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and a variety of open channel blocks, such as MK801, memantine, amantadine, ketamine, PCP, and DMI (Blanpied et al., 1997; Bresink et al., 1996; Chen et al., 1992; Huettner and Bean, 1988; MacDonald et al., 1991; Mayer et al., 1984; Sernagor et al., 1989). A common feature of these organic channel blockers is that they all possess positive charges which are provided by either one or several amine groups. The fact that dopamine and D1 ligands are monoamines raises the possibility that they may act as open channel blockers for NMDA receptors. Nevertheless, two recent studies suggested that NMDA receptors can be directly blocked by dopamine and by one of the D1 agonists (Castro et al., 1999; Masuko et al., 2004).

To accurately understand how NMDA receptors are modulated by dopamine receptors and to clarify properties of D1 ligands, we carried out electrophysiology studies to determine if there were direct channel-blocking effects of D1 ligands on NMDA receptors. Using the HEK cell recombinant system, hippocampal neurons, and D1 receptor knockout mice, we demonstrated that dopamine and several D1 ligands could directly block NMDA receptors independent of D1 receptors. They acted as voltage-dependent open channel blockers, regardless of whether they are agonists or antagonists for D1 receptors. Our result provides solid evidence for the mechanism underlying the direct channel blocking effect of D1 ligands. It suggests that D1 ligands can modulate NMDA receptors through not only the G-protein coupled pathway but also a channel pore blocking mechanism.

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Materials and Methods

Material. Glutamate, NMDA, glycine, and D1 ligands were from Tocris (Ellisville, MO) and Sigma (St. Louis, MO). 4-Ethylcatechol was from Alfa Aesar (Ward Hill, MA). All other chemicals were from Sigma or as specified.

Expression of NMDA receptors in HEK cells. Human embryonic kidney (HEK) 293 cells (CRL 1573, American Type Culture Collection, Manassas, VA) are maintained and transfected using conditions described previously (Cui and Mayer, 1999). To express NMDA receptors in HEK cells, cDNAs of NR1A and NR2A are transfected at a 1:3 ratio. cDNA of EGFP is co-transfected with NR1 and NR2A cDNAs to aid the identification of transfected cells. HEK cells are washed with PBS 12-18 hours after the transfection and maintained in the medium containing 1mM AP-5 and 2mM Mg^{2+} to protect the cells from glutamate induced toxicity until used for electrophysiological recordings.

***Acutely dissociated neurons*.** Acutely dissociated neurons are prepared from the dorsal striatum of 4 to 5 week old wild type and D1 receptor knock mice (Xu et al., 1994) using the enzyme aid dissociation method. Briefly, 400μm of brain slices are prepared in ice cold dissection solution containing (in mM): 138 NaCl, 5.3 KCl, 2 $MgCl_2$, 0.4 KH_2PO_4, 0.34 Na_2HPO_4, 10 HEPES, 16 glucose, and 20 sucrose, pH 7.4, and maintained in oxygenated EBBS (Invitrogen) prior to the dissociation. The brain tissue containing the nuclei of interest is dissected out and placed in the dissect solution (without Mg^{2+}) containing 100 units/ml papain and 0.1mg/ml cysteine (Sigma). The enzyme treatment is stopped after the incubation for 10 to 15 minutes at room temperature. The tissue is triturated gently in the dissociation solution containing 1mM $MgCl_2$, and the cell suspension is placed in 30mm dishes. Recordings are usually made within 0.5 to 2 hours following the dissociation.

Primary culture of hippocampal neurons. Hippocampal neurons were prepared according to a previously described procedure (Mayer and Westbrook, 1987). Briefly,

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hippocampi of E18 rat embryos were dissected, dissociated and plated on glial cell layers at low density (2.5 x 10^4 cells per 35mm dish). The neuronal culture was maintained in MEM with 5% of horse serum, 1% FBS, and a nutrient supplement (Mayer and Westbrook, 1987). One day after plating, 2-Deoxy-5-fluoro-uridine and Uridine was added to suppress the glial cell division. Electrophysiological recordings were done using 8 to 13 day old cultures.

Recording conditions. Electrophysiological recordings were performed using whole cell patch-clamping recording of HEK cells and dissociated neurons. Recordings were made using fire-polished thin-walled borosilicate glass pipettes (2-5 MΩ) with an Axopatch-200B amplifier (Axon Instruments, Foster City, CA). The intracellular recording solution for HEK cells contained (in mM): 120 CsMethansulphonate or CsCl, 10 CsF, 5 CsBAPTA, 0.5 $CaCl_2$, 3 Na_2ATP, and 10 HEPES, pH 7.3. For neurons, CsMethansulphonate was replaced with CsF. The extracellular recording solution contained (in mM): 145 NaCl, 5.4 KCl, 1 $CaCl_2$, 5 HEPES, and 10 Glucose, pH 7.3. Series resistance (3-10 MΩ) was routinely compensated by 70-80%. Records were acquired under the control of the data acquisition program pClamp9 (Axon Instrument, Union City, CA) and stored on a Pentium 4 PC computer with a 16 bit analog-to-digital converter (DIGIDATA 1322A; Axon Instruments, Union City, CA). 20μM glycine was included in glutamate or NMDA containing solutions. One minute recording intervals were allowed for the recovery from the desensitization between applications of glutamate or NMDA. A modified RSC-160 fast perfusion system (Biologic Scientific Instruments, France) was used for the solution exchange. Because of the light sensitivity of D1 ligands, solutions containing D1 ligands were prepared in the dark. The stock solution of dopamine was prepared in Na-ascorbate. Experiments were performed with dim ambient light and solution reservoirs were covered with aluminum foil during experiments.

Data Analysis. The procedure for the analysis of voltage-dependent block was similar to that described before (Cui and Mayer, 1999). Briefly, current-voltage (I-V) relationships with and without a D1 ligand were generated by applying voltage ramps from -120mV to +50mV

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(0.1mV/ms) at the steady state of currents. Procedures in the Igor program (WaveMetrics) and KaleidaGraph (Synergy Software) were used to generate and analyze voltage-dependent block using the Woodhull Model (Woodhull, 1973). The reversal potential for an I-V plot is estimated using the fifth order polynomial fits to leak subtracted responses. The conductance in the presence of D1 ligands (G_b) are normalized to the conductance of control responses (G_0) and fitted with the following equation:

$$\frac{G_b}{G_o} = \frac{1}{1 + \frac{[B]}{K_{d(0)} * \exp(\frac{z\delta * V * F}{RT})}} \quad (1)$$

Where, B is the concentration of D1 ligand, $K_{d(0)}$ is the dissociation constant at 0mV, z is the valence of D1 ligand, δ is the fraction of the electric field that D1 ligand experiences at the blocking site, V is the membrane potential, and F, R, and T have their usual meaning.

The dissociation constant at a particular voltage, e.g., -60mV, is determined using $K_{d(0)}$ and zδ value by:

$$K_d = K_{d(0)} * \exp(\frac{z\delta * V * F}{RT}) \quad (2)$$

To evaluate the interaction of the D1 ligand block and Mg^{2+} block, G_b/G_0 vs. V plots were fitted with Boltzman Functions. For the individual application of the D1 ligand and Mg^{2+}, G_b/G_0 vs. V plot was fitted with a Boltzman Function over the range of -120 to +50mV:

$$\frac{G_b}{G_0} = \frac{1}{(1 + \exp\frac{(V_b - V)}{k_b})} \quad (3)$$

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Where G_0 and G_b have the same meaning as defined in equation (1); V_b is the membrane potential for the half block; k_b is the voltage dependence of the block.

To determine the interaction of D1 ligand block and the Mg^{2+} block, we generated a $G_{(b1+b2)}/G_0$ vs V relationship used the following equation and compared it to the experimental data when the D1 ligand was co-applied with Mg^{2+}:

$$\frac{G_{(b1+b2)}}{G_0} = \frac{1}{(1+\exp\frac{(V_{b1}-V)}{k_{b1}})} \times \frac{1}{(1+\exp\frac{(V_{b2}-V)}{k_{b2}}} \qquad (4)$$

Where V_{b1} and k_{b1} are determined from the Boltzman fit of Mg^{2+} block and V_{b2} and k_{b2} are from the Boltzman fit of the SKF81297 block.

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Results

Direct block of NMDA receptors by the D1 agonist SKF81297

To reliably determine whether D1 agonists have direct blocking effects on NMDA receptor-mediated currents, we took advantage of the non-dopaminergic HEK cell recombinant system (Tiberi and Caron, 1994) and transfected the cells with NMDA receptor (NR1A and NR2A) cDNAs, but not a dopamine D1 receptor cDNA. As illustrated in Fig. 1A, B, 30μM D1 agonist SKF81297 significantly inhibited NMDA receptor-mediated currents at -60mV (32 ± 4% of peak amplitudes and 63 ± 5% of steady state amplitude, n=10, Fig. 1E). However, this inhibitory effect was not observed if SKF81297 was only applied immediately prior to the glutamate application (Fig. 1C, E). Moreover, the pre-application of SKF81297 (1s) did not change the 10-90% rise time of glutamate evoked currents ($P > 0.3$). In contrast, if SKF81297 was applied after receptor activation, when channels were open (Fig. 1D), a significant amount of block (62 ± 5%, n=12) was detected (Fig. 1E). This suggests the blocking effect of SKF81297 depends on the channel activation. Furthermore, we detected similar blocking effects when PKA or PKC inhibitors (100μM Rp-cAMPs or 2μM staurosporine) were included in the intracellular recording solution (data not shown). This indicates that the blocking effect is independent of the PKA or PKC second messenger cascade. The kinetics of onset or offset of the channel block was determined by fitting changes of current amplitudes upon the application or the removal of SKF81297 with single exponential functions. The mean time constants (τ) are 62 ± 9ms for the onset of the block and 173 ± 41ms for the offset of the block with 30μM SKF81297. We further examined the concentration dependence of the onset and offset of the block. The results shown in Fig. 1F indicate that the inverse of the onset time constant ($1/\tau_{on}$) significantly correlated with the concentration of SKF81297 ($P < 0.01$), while the inverse of the offset time constant ($1/\tau_{off}$) did not. This suggests that the rate of the block of NMDA receptors depends on the concentration of the D1 ligand applied while the recovery from the block does not depend on the

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ligand concentration. Taken together, these results demonstrate that the D1 agonist SKF81297 can directly block NMDA receptor-mediated currents even in the absence of dopamine D1 receptors. It blocks NMDA receptor-mediated currents when receptors are activated. In addition, the channel blocking action of SKF81297 is several magnitudes faster than the second messenger mediated effects (Krasel et al., 2004).

Voltage-dependent property of D1 ligand action

The D1 ligand SKF81297 could block NMDA receptors by interacting with several possible structural domains of the receptor, such as the N-terminal domain, the ligand binding domain, and the channel pore region. The blocking property of SKF81297 suggests that it may act as an open channel blocker and binds in the channel pore. One of the characteristic electrophysiological properties of channel pore block is the rectification of I-V relationship at negative membrane potentials. This is due to changing the binding affinity of channel blockers with the membrane potential. To investigate the mechanism underlying the blocking effect of D1 ligands, we first examined the action of SKF81297 at different holding membrane voltages.

As shown in Fig. 2A, B, 30μM SKF81297 caused more significant reductions of NMDA receptor-mediated currents at hyperpolarizing membrane potentials than at depolarizing membrane potentials. Consistent with this finding, I-V relationships obtained using voltage ramps in the presence of various concentrations of SKF81297 showed significant rectification at negative voltages (Fig. 2C). This voltage-dependent blocking effect could be detected at low μM concentrations. In particular, at 10μM (filled up triangles), SKF81297 blocked 35 ± 7% (n = 7) of NMDA receptor-mediated currents at -60mV.

To determine the voltage-dependence and binding affinity of the SKF81297 block, we analyzed the normalized conductance (G_b/G_0) vs. V plots using the Woodhull Model (Woodhull, 1973) (*see Data Analysis for detail*). The result shown in Fig. 2D indicated that SKF81297

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experienced 80% of the membrane electric field at the blocking site ($\delta = 0.80 \pm 0.06$). This suggests that SKF81297 may bind inside the channel pore. The voltage-dependent block of SKF81297 is manifested by changes in the binding affinity (K_d) with membrane potentials. At 30μM concentration, the K_d value of SKF81297 block is 124 ± 22μM at 0mV and 19μM at -60mV. Similarly, the IC_{50} value varies with membrane potentials and is 19 ± 2μM (n_H=1.05) at -60mV (Fig. 2E). These results provide direct biophysical evidence that SKF81297 binds in the membrane electric field, most likely the channel pore region, and blocks NMDA receptors in a voltage-dependent manner.

Not only D1 agonists but also antagonists directly block NMDA receptor mediated-currents

Regardless of whether they are agonists or antagonists, D1 ligands are monoamines, and many of them have structural similarities. It is highly possible that other D1 ligands may also directly block NMDA receptors. To test this possibility, we examined dopamine and several ligands which have high affinities for D1 receptors. These include SKF81297, SKF38393, Fenoldopam, and Apomorphine, which are agonists for D1 receptors, and SCH23390 and SKF83566, which are antagonists for D1 receptors. As shown in Fig. 3A, at 10μM concentration, SKF81297, SKF38393, Fenoldopam, SCH23390, and SKF83566 all blocked NMDA receptor-mediated currents in a voltage-dependent manner, regardless of whether they are agonists or antagonists for D1 receptors. The fact that both agonists and antagonists exhibited the channel blocking effect provides additional supporting evidence that the blockage is independent of D1 receptors.

To determine structural elements of the D1 ligands which are critical for the voltage-dependent block of NMDA receptors, we characterized actions of dopamine and several D1 ligands which have the core structure of phenyltetrahydrobenzazepine, including SKF38393, SKF81297, NM-SCH23390, and SCH23390. As illustrated in Fig. 3B, C, and Table 1, dopamine

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is the weakest blocker compared to other D1 ligands studied. Although SKF38393 has the closest structural similarity to dopamine, it has a significantly lower K_d value (Table 1). This is most likely due to the additional phenyl ring of SKF38393 that may contribute to the binding of the ligand to the channel pore. Comparing structures and effects of SKF38393 and NM–SCH23390 (Fig. 3B and Table 1), replacing the hydroxyl group by a chlorine atom at the position 8 significantly reduced the binding affinity. This suggests either the importance of the hydroxyl group at this position for the binding or that the chlorine atom causes structural hindrance at this position. However, if a chlorine atom is added at position 9 on the benzyl ring, comparing SKF81297 and SKF38393 (Fig. 3B and Table 1), it makes SKF81297 have the highest affinity among the ligands tested. These results suggest that hydrophobic interactions play important roles in binding of D1 ligands to NMDA receptor channels. In addition, the chlorine atom at the position 9 of phenyltetrahydrobenzazepine greatly increased the binding affinity.

Since the positively charged amine group is essential for the voltage-dependent block, we next investigated how modifying the chemistry of this moiety would affect the voltage-dependent channel block by D1 ligands. The amine group in NM-SCH23390 has a pKa value of 9.69 ± 0.40 (calculated by ACD/LogD Suite software, ACD/Labs, Toronto, CN). Changing this secondary amine to a tertiary amine by attaching a methyl group, as in SCH23390, decreases the pKa value to 8.24 ± 0.40. Although both ligands blocked NMDA receptors, they exhibited different voltage-dependent properties (Fig. 4A). The $z\delta$ value changed from 1.15 ± 0.08 of NM-SCH23390 to 0.75 ± 0.04 of SCH23390. This suggests that changing the pKa value of the amine group influences the voltage-dependence of the channel block. However, even with the reduced voltage-dependence, SCH23390 had a higher binding affinity with $K_{d(0)}$ value of 462 ± 73 μM, compared to 5860 ± 1840 μM of NM-SCH23390. It is possible that the methyl group may

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interact with other hydrophobic amino acid side chains in the channel pore and stabilize the binding of SCH23390.

We next examined the effect of changing the percentage of positively charged SKF81297 on the block. The amine group of SKF81297 has a pKa value of 9.36 ± 0.40. At pH 7.3, 99.1% of SKF81297 are protonated, while at pH 8.3 and 9.0 percentages of the protonated SKF81297 are decreased to 91.9% and 69.6%, respectively. Thus, changing the external pH from 7.3 to 9.0 will effectively alter the percentage of SKF81297 with the positively charged amine group. As shown in Fig. 4B, switching the external pH from 7.3 to 8.3 or 9.0 significantly altered the voltage-dependent channel block by 30μM SKF81297. Because the external pH change influences the intrinsic channel activity of NMDA receptors (Traynelis and Cull-Candy, 1990), we normalized the conductance in the presence of SKF81297 (G_b) to that of the control response (G_0) obtained at the same pH. The Woodhull analysis revealed $K_{d(0)}$ and zδ values of 152 ± 29 μM and 0.77 ± 0.05 at pH 7.3; 561 ± 54 μM and 0.75 ± 0.06 at pH 8.3; 1640 ± 323 μM and 0.64 ± 0.06 at pH 9.0. Thus, $K_{d(0)}$ values changed dramatically with the percentage of protonated SKF81297 (Fig. 4C). It suggests that the positively charged amine group plays a critical role for the channel block. The zδ value was not significantly changed when pH was switched from 7.3 to 8.3 ($P>0.1$). This is expected if pH only changes the percentage of positively charged SKF81297 and that is the only chemical form interacting with the channel. However, at pH 9.0, the zδ value was significantly reduced compared to that at pH 7.3 ($P<0.01$). This suggests that increasing the pH to 9.0 might not only alter the percentage of the positively charged SKF81297 but also influence other components involved in the channel block.

To further examine the importance of the amine group in the channel block and to complement the pKa and pH approaches, we compared channel blocking properties of dopamine and its analogue, 4-ethylcatechol. The only difference between these two chemicals is that 4-ethylcatechol lacks the amine moiety (Fig. 4D). At 100μM concentration, dopamine

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exhibited the voltage-dependent block of NMDA receptor-mediated currents (Fig. 4D), with $K_{d(0)}$ and zδ value of 2430 ± 209μM and 0.72 ± 0.02, respectively. However, at the same concentration, 4-ethylcatechol did not exhibit a significant voltage-dependent block (Fig. 4D). Thus, removing the amine group completely abolished the channel blocking property, which provides additional evidence that the amine moiety is essential for the voltage-dependent block of NMDA receptors.

Voltage-dependent block of NMDA receptor-mediated currents by D1 ligands in neurons

Because of the different cellular environments of HEK cells and neurons, results obtained in HEK cells may not necessarily be valid in neurons. To determine whether D1 ligands directly block NMDA receptor-mediated currents in neurons, we examined effects of D1 ligands using acutely dissociated medium spiny neurons from the striatum of D1 receptor knockout mice and primary cultured hippocampal neurons.

Given the fact that D1 agonists bind to both D1 and D5 receptors, D1 agonists can still activate dopamine D5 receptors and the coupled adenylate cyclase cascade in D1 receptor knockout mice. However, the distinct expression patterns of these two receptors in the brain allow us to use D1 receptor knockout mice to study the direct voltage-dependent effect in a background without the interference of D1 and D5 receptors. D1 receptors are highly expressed in the striatum (Missale et al., 1998). In contrast, D5 receptors have a significantly lower expression level in this brain region and only in the large sized cholinergic interneurons (Yan and Surmeier, 1997). In the striatum of D1 receptor knockout mice we used, there was no detectable binding of the high affinity D1/D5 receptor antagonist, [^{3}H]SCH23390 (Xu et al., 1994). This suggests no detectable functional expression of D1 and D5 receptors in this brain region. Therefore, medium spiny neurons in the striatum of D1 receptor knockout mice provide an ideal environment to examine the direct channel blocking effects of D1 agonists. As illustrated in Fig. 5A, 30μM of the D1 agonist SKF81297 significantly blocked NMDA receptor-

mediated currents from a medium spiny neuron of D1 receptor knockout mice. Furthermore, this blocking effect was similar to that of neurons isolated from wild type mice (Fig. 5D). These results indicate that the D1 agonist SKF81297 can directly block NMDA receptor-mediated currents independent of the significant expression of D1 receptors in medium spiny neurons. The comparable blocking effects in neurons isolated from D1 receptor knockout mice and wild type mice suggests that the short time (2 seconds) application of SKF81297 did not cause a significant activation of D1 receptor-dependent pathways.

Functional modulations of NMDA receptors by D1 receptors performed using dopamine and D1 ligands have been characterized in various brain regions, such as the striatum, nucleus accumbens, prefrontal cortex, and hippocampus. To determine whether dopamine and D1 agonists have direct channel blocking effects on NMDA receptors in these brain regions, we employed a pharmacological approach which allowed us to isolate and characterize this property of D1 ligands. Because D1/D5 receptors and NMDA receptors coexist in neurons, it is essential to prevent the activation of D1/D5 receptors by D1 agonists in order to accurately study the direct channel blocking effect. To achieve this, we used a D1/D5 receptor antagonist SCH23390 at low concentration (0.5μM) to block D1/D5 receptors. SCH39390 has high affinities for D1 and D5 receptors with K_i values of 0.2nM and 0.3nM, respectively (Seeman and Van Tol, 1994). At 0.5μM concentration, SCH23390 blocks 99.9% of D1 and D5 receptors while it has no significant blocking effect on NMDA receptor channels (data not shown). Therefore, by recording in the presence of 0.5μM SCH23390, NMDA receptor-mediated currents can be studied independent of modulations by D1 or D5 receptors. Employing this strategy we analyzed the direct blocking action of SKF81297 on NMDA receptors using hippocampal neurons. As shown in Fig. 5B, 30μM SKF81297 significantly blocked NMDA receptor-mediated currents with (black) or without (grey) the presence of 0.5μM SCH23390. At -60mV membrane potential, the percentages of current amplitude changes were 47.9 ± 2.7% and 48.5 ± 3.5% (n =

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9), respectively (Fig. 5D). I-V relationships of SKF81297 block exhibited voltage-dependence with a $K_{d(0)}$ value of 212 ± 43μM and a zδ value of 0.76 ± 0.03 (Fig. 5C). The difference between these values and those obtained using HEK cells expressing NR1A/NR2A receptors is most likely due to the distinct subunit composition of NMDA receptors in hippocampal neurons. We did not observe significant differences in the percentage of block, $K_{d(0)}$, and zδ values with or without the presence of SCH23390. This is consistent with the observation obtained using medium spiny neurons from D1 receptor knockout mice that a short application (2 seconds) of SKF81297 (black bar in Fig. 5B) did not cause a significant activation of D1 receptor-dependent pathways. These data indicate that the D1 agonist SKF81297 can directly block NMDA receptor-mediated currents in a voltage-dependent manner in hippocampal neurons, and this blocking effect is independent of dopamine D1 or D5 receptors.

Interaction with Mg^{2+} block in neurons

Mg^{2+} ions are the endogenous voltage-dependent channel blockers for NMDA receptors. Studies shown in Fig. 2D and 3B, C revealed that δ values of D1 ligands are very similar to that of Mg^{2+}. It is highly possible that the D1 ligand block will interact with Mg^{2+} block. To explore this possibility, we characterized the SKF81297 block with or without the presence of Mg^{2+} using hippocampal neurons. The I-V relationships illustrated in Fig. 6A indicate that 30μM SKF81297 (filled squares) had more blocking effect at low negative voltages compared to that of 1mM Mg^{2+} (filled triangles), and the two blocking actions cross at 32 ± 3mV (inset of Fig. 6A). As voltages proceeded to more hyperpolarization than this value, Mg^{2+} had a significantly more blocking effect. In addition, the co-application of SKF81297 and Mg^{2+} (filled circles) caused more channel block than that for Mg^{2+} applied alone, especially at depolarizing membrane potentials (inset of Fig. 6A, and Fig. 6B). To determine the relationship of Mg^{2+} block and SKF81297 block, we analyzed G_b/G_0 vs. V plots using Boltzman Functions (Fig. 6B) (*see Data Analysis for detail*).

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For the individual application of SKF81297 (filled squares) and Mg^{2+} (filled triangles), the membrane potential for half block (V_b) and the voltage dependence (k_b) are -76.4 ± 2.1mV and 36.3 ± 1.9 for SKF81297 block, and -44.4 ± 0.8mV and 14.9 ± 0.7 for Mg^{2+} block. If the binding of one blocker prevents the binding of the second one, we would expect the G_b/G_0 vs. V plot of the co-application to be fitted by a relationship defined in the equation (4) (*see Data Analysis*). Indeed, this relationship generated using the V_b and k_b values of the individual application of SKF81297 and Mg^{2+} (Fig. 6B, dash line) fits well with the experimental data of the co-application of SKF81297 and Mg^{2+} (filled circles). This suggests that these two blocking actions preclude each other. These results also provide additional evidence that SKF81297 binds in the channel pore region.

Results shown in Fig. 6B indicate that SKF81297 not only interacted with Mg^{2+} block at negative membrane potentials but also reduced the Mg^{2+} mediated potentiation at positive membrane potentials (comparing filled triangles and circles). Since the potentiation by Mg^{2+} is mediated by a binding site outside of the membrane electric field (Paoletti et al., 1995) SKF81297 may functionally interact with Mg^{2+} through multiple sites. To get a complete picture of functional interactions of SKF81297 and Mg^{2+} block, we determined how SKF81297 influenced Mg^{2+} block by comparing channel conductance in the presence of both Mg^{2+} and SKF81297 ($G_{Mg2++SKF}$) to that in the presence of Mg^{2+} alone (G_{Mg2+}). As shown in Fig. 6C, at negative membrane potentials, the conductance ratio of $G_{Mg2++SKF}/ G_{Mg2+}$ decreased in a voltage-dependent manner. In contrast, at positive membrane potentials the conductance ratio of $G_{Mg2++SKF}/ G_{Mg2+}$ was constant and independent of membrane potentials. $G_{Mg2++SKF}$ is 87 ± 2.8% of G_{Mg2+} at +20mV, which is similar to those at +40mV and +50mV (Fig. 6C). These data suggest that the SKF81297 block interacts with the Mg^{2+} block via both voltage-dependent and independent manners. Thus, they functionally interact both inside and outside of the membrane electric field.

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Discussion

Using a HEK cell recombinant system and dissociated neurons, we characterized the mechanism underlying the direct channel block by dopamine and D1 ligands and revealed structural moieties of D1 ligands which are important for the channel block. Our studies provide strong evidence that D1 ligands can directly block the NMDA receptor channel activity through a D1 receptor-independent pathway.

Dopamine D1 ligands as NMDA receptor channel blockers

It has been well established that NMDA receptors are voltage-dependently blocked by polyamines and a variety of structurally distinct organic molecules which have positive charge through amine group(s) (Blanpied et al., 1997; Chen et al., 1992; Huettner and Bean, 1988; MacDonald et al., 1991; Sernagor et al., 1989). From this perspective, it is not surprising that dopamine and D1 ligands can directly block NMDA receptors in a voltage-dependent manner, as they are monoamines with a primary, secondary, or a tertiary amine group. Analysis of the structural and functional relationships of D1 ligands (Fig. 3, 4) revealed that both hydrophobic and charge interactions are important for the binding of D1 ligands to the channel pore. This is consistent with the role of hydrophobic interactions in stabilizing the binding of polyamine, diamine, and MK-801 to the channels (Cui et al., 1998; Kashiwagi et al., 2002; Subramaniam et al., 1994). Interestingly, the additional chlorine atom at the position 9 made SKF81297 the most potent D1 ligand in blocking of NMDA receptors (Fig. 3, and Table 1). It is possible that the electronegative chlorine atom interacts with pore lining residues and forms an attraction for binding of the molecule in the channel vestibule, which leads to a higher binding affinity.

It is clear that D1 ligands can bind to dopamine D1 receptors and NMDA receptors through different structural elements. There are certain common and distinct features between

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these two types of ligand–receptor interactions. D1 ligands bind to the crevice of helical bundles formed by the transmembrane domains 3, 4, 5, and 6 of D1 receptors, and it is located two helical turns from the extracellular side (Floresca and Schetz, 2004). In comparison, D1 ligands bind in the channel pore region of NMDA receptors and sense 80% of the membrane electric field. Similar to binding to dopamine receptors, increasing the hydrophobicity or protonation of the amine moiety leads to higher binding affinities for NMDA receptors. For dopamine receptors, the amine group forms a reinforced ionic bond with an aspartic residue of dopamine receptors (Floresca and Schetz, 2004). For NMDA receptors, it is most likely that the amine group interacts with the asparagine residues located in the narrow constriction region of the NMDA receptor channel pore (Masuko et al., 2004; Wollmuth et al., 1998), which is also the main interaction site for Mg^{2+} and many organic channel blockers (Kashiwagi et al., 2002). The hydroxyl groups of D1 ligands play important roles in the orientation of a D1 ligand in the binding pocket of dopamine receptors by forming hydrogen bonds with serine residues (Floresca and Schetz, 2004). Changing the hydroxyl group at position 8 to a chlorine atom will completely change the ligand property from an agonist to an antagonist for D1 receptors. However, this moiety change does not reverse the channel blocking property of a D1 ligand towards NMDA receptors.

D1 ligands have δ values ranging from 0.76 to 1.15 (Table 1). These are similar to those of Mg^{2+} and other channel blockers (Blanpied et al., 1997; Sobolevsky and Yelshansky, 2000). The high δ values of SKF38393 and NM-SCH23390 (1.03 and 1.15, respectively) may suggest these molecules interact with multiple sites in the membrane electric field. Because of the possible interaction of permeable ions with channel pore blockers (Antonov and Johnson, 1999), the exact binding sites of D1 ligands to NMDA receptors need to be determined by taking the ion permeation into account. Analysis of the voltage-dependent block of SKF81297 and Mg^{2+} indicates that the functional interaction of these two molecules may occur both inside and

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outside of the membrane electric field (Fig. 6C). Binding of either blocker to the channel precludes the binding of the other. These results suggest that under physiological conditions D1 ligands could influence Mg^{2+} block and potentiation of NMDA receptors. Functional interaction of Mg^{2+} block and D1 ligand block implies that these two types of blockers may interact with similar amino acids residues of NMDA receptors. It is highly possible that, similar to Mg^{2+} block (Wollmuth et al., 1998), D1 ligand block of NMDA receptors is also NR2 subunit dependent.

Modulations of NMDA receptors by dopamine D1 ligands and receptors

Aside from the direct voltage-dependent block of NMDA receptors by D1 ligands presented in this and other studies (Castro et al., 1999; Masuko et al., 2004), there are several laboratories which have demonstrated modulations of NMDA receptors through D1 receptor-dependent pathways. Although multiple mechanisms have been proposed for the D1 receptor-dependent modulations, they fall into two types of pathways, through either second messenger cascades (Cepeda et al., 1998; Chen et al., 2004) or direct receptor-receptor interactions (Lee et al., 2002). To accurately understand modulations of NMDA receptors by dopamine D1 receptor/ligands, it would be necessary to dissect properties and relationships of these different types of modulations.

The direct channel block of NMDA receptors by D1 ligands has a time constant of 61.8 ± 8.9ms for the onset of the block at 30μM concentration. In contrast, the modulation through the G-protein-coupled second messenger cascade has much slower time scale. Although the precise onset time for G-protein coupled D1 receptor modulation of NMDA receptors is not known, it most likely has a similar time scale to other G-protein-coupled cascades, such as α2A-adrenergic receptors, and has $t_{1/2}$ of 19.6 seconds for the time-limiting step (Krasel et al., 2004). The different time scales of D1 ligand actions through these two different mechanisms create a time window within which the direct voltage-dependent block plays a significant role before the

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second messenger cascades become effective. Indeed, if the D1 agonist was applied for 1-2 seconds, no significant D1 receptor-dependent modulation was detected (Fig. 6). However, the different affinities of D1 ligands to D1 receptors (in the nM range) and NMDA receptors (in the μM range) generate a concentration window within which only the modulation through the second messenger cascade would play a significant role. Furthermore, the direct voltage-dependent block is affected by the membrane potential, while the modulation through the second messenger cascade can be independent of the membrane potential. In addition, the reduced Ca^{2+} influx through NMDA receptors due to the direct channel block by D1 ligands will influence the subsequent second messenger cascade. Thus, several factors will influence whether one or both mechanisms are involved in the modulations of NMDA receptors. Experiments performed using different time scales, concentrations of D1 ligands, or holding membrane potentials would likely lead to different or even contradicting results, depending on the direction of these two types of modulations.

The other mechanism which has been proposed for the modulation of NMDA receptors by dopamine D1 receptors is via the physical interaction between NMDA receptors and D1 receptors (Lee et al., 2002). However, the direct channel blocking property of SKF81297 revealed by our study suggests that it needs to be cautious in the interpretation of the functional consequence of the receptor-receptor interaction. We observed a similar amount of block by 10μM SKF81297 using HEK cells expressing NR1A/NR2A alone (Fig. 2B and Fig. 3A) to that obtained using HEK cells expressing NR1A/NR2A and D1 receptors (Lee et al., 2002). This suggests that the blocking effect originates from the direct channel bock of NMDA receptors by SKF81297 and is independent of D1 receptors. Furthermore, in our experiments using hippocampal neurons (Fig. 5), there was no significant difference in the amount of block by 30μM SKF81297 with and without the presence of 0.5μM SCH23390 (a D1 receptor antagonist). Thus, the effect of SKF81297 is from a D1 receptor-independent mechanism.

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However, we do not exclude the possibility that receptor-receptor interactions may lead to functional modulation. It is possible that, in the presence of 10μM SKF81297, the direct channel blocking effect may be dominant over the receptor-receptor interaction allosterically. Thus, it is necessary to use proper conditions to determine the functional consequence of the physical interaction between NMDA receptors and dopamine D1 receptors, such as at a low concentration of a D1 agonist, e.g., 0.1μM SKF81297.

In summary, our study reveals the mechanism underlying the direct channel block of NMDA receptors by dopamine and several D1 ligands. It suggests that D1 ligands can modulate NMDA receptors through both G-protein-coupled mechanism(s) and direct channel pore block. Results presented in this study also provide important information for potential targets of D1 ligands in the treatment of neurological disorders. Finally, our study reveals the structural possibility for designing a unique pharmacological approach to concurrently modulate NMDA receptors and dopamine D1 receptors.

Acknowledgments: We thank Drs. Steve Heinemann and Mark Mayer for the plasmids; Ms. Li Bai and Yufen Han for DNA preparations and neuronal cultures; Ms. Madelon Cook for helping in the manuscript preparation; Drs. Mark Mayer and Qi-Ying Liu for critical reading of the manuscript.

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Footnotes:

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Figure Legends

Figure 1. Effects of the D1 agonist SKF81297 on NMDA receptor (NR1A/NR2A) mediated currents. ***A,*** Control response to 100μM glutamate. ***B***, 30μM SKF81297 (black bar) significantly inhibited the NMDA receptor-mediated current. ***C***, No inhibition was detected when SKF81297 was only applied prior to the glutamate application. ***D***, Significant inhibition was observed when SKF81297 was applied 2 seconds after the receptor activation. ***E***, Summary of percentages of the current amplitude changes in ***B***, ***C***, and ***D***. Changes in peak and steady state (sst) current amplitudes were measured. ***F,*** The inverse of time constants of SKF81297 block, derived from single exponential fits of onset and recovery process, were plotted against the SKF81297 concentration. 20μM glycine was included in all glutamate containing solutions. Error bars represent standard errors.

Figure 2. NMDA receptors are voltage-dependently blocked by SKF81297. ***A***, Effects of 30μM SKF81297 tested at different voltages. ***B***, Percentage of steady state current amplitude changes by 30μM SKF81297 at different membrane potentials. ***C***, I-V relationships of NMDA receptor-mediated currents in the presence of various concentrations of SKF81297. Open circles: control; filled up squares: 3μM; filled triangles:10μM; filled circles: 30μM; filled downward triangles: 100μM; open squares: 300μM. ***D***, Analysis of voltage-dependent block of NMDA receptors by fitting normalized G_b/G_o vs. V plots with the Woodhull Model. See *Data Analysis* for detail. G_b and G_0 are conductance with and without SKF81297. ***E,*** Voltage-dependent variation of the dose response. IC_{50} values at -100mV, -60mV, and +40mV were determined using equation: $I_b/I_0 = 1/(1+([C]/IC_{50})^{n}{}_H)$. Data points represent mean ± SE, n = 3 - 6.

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Figure 3. Voltage-dependent block of NMDA receptor-mediated currents by various D1 Ligands. ***A***, Both D1 agonists and antagonists block NMDA receptors in a voltage-dependent manner. Mean values ± SE were presented (n = 4 - 7). The concentration tested was 10μM for each ligand. ***B,*** Structural and functional analysis of D1 ligand effects. Dopamine, SKF38393, SKF81297 are agonists, while SCH23390 and NM-SCH23380 are antagonists for D1 receptors. 100μM of each ligand was used to determine zδ and K_d values, and error bars represent mean ± SE (n = 4 - 6).

Figure 4. Importance of the positively charged amine group in D1 ligand-block of NMDA receptor-mediated currents. ***A*****.** Changing the pKa of the amine moiety in D1 ligands influenced the voltage-dependent channel block. 100μM SCH233890 and NM-SCH23390 were used. ***B, C***, Changing the external pH from 7.3 to 8.3, or to 9.0 significantly decreased the voltage-dependent block by 30μM SKF81297. To avoid the interference of the intrinsic channel property change upon increasing pH, G_b was normalized to G_0 obtained at the same pH. For the plot obtained at pH 9.0, the fitting was up to -100mV instead of -120mV. Mean value ± SE were presented (n = 4 - 6). ***D***, Comparing channel blocking properties of 100μM dopamine and 4-ethylcatechol. These experiments were performed at pH 7.3. Mean value ± SE were presented (n = 3 - 6).

Figure 5. Effects of SKF81297 on NMDA receptor-mediated currents in neurons. ***A***, 30μM SKF81297 significantly blocked NMDA receptor-mediated current from a medium spiny neuron acutely isolated from D1 receptor knockout mouse. ***B***, Using 0.5μM of D1 receptor antagonist, SCH23390, to detect the D1 receptor independent effect of SKF81297. The black trace is obtained in the presence of 0.5μM of SCH23390. ***C***, I-V relationship of effects of SKF81297 with and without the presence of 0.5μM SCH23390. The filled circles and open squares represent

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with and without 0.5μM SCH23390. ***D***, Comparing blocking effects of 30μM SKF81297 using medium spiny neurons isolated from wild type (+/+) and D1 receptor knockout (-/-) mice; and hippocampal neurons with or without 0.5μM SCH23390.

Figure 6. Functional interaction of D1 ligand block and Mg^{2+} block. ***A***, I-V relationships of NMDA receptor-mediated currents of hippocampal neurons in the presence of 1mM Mg^{2+}, 30μM SKF81297, or 30μM SKF81297+ 1mM Mg^{2+}. The section of the plots from 0 to -60mV was amplified and shown at the right of Fig. 6A. ***B***, G_b/G_0 vs. V plots of 1mM Mg^{2+} (filled triangles) and 30μM SKF81297 (filled squares) were fitted with the Boltzman Functions. Filled circles represent experimental data obtained with the co-application of 1mM Mg^{2+} and 30μM SKF81297. The dash line is generated using the relationship (4) defined in *Data Analysis* assuming these two blocking actions preclude each other. The error bars represent standard errors. ***C***, Changes of the conductance ratio with membrane potentials. G_{Mg2+} is the conductance in the presence of 1mM Mg^{2+}, and $G_{Mg2++SKF}$ is the conductance in the presence of 1mM Mg^{2+} and 30μM SKF81297.

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Table 1. Voltage-dependence and binding affinity of D1 ligand block of NMDA receptor mediated currents

D1 ligands	zδ	Kd(0) (μM)	Kd, -60mV (μM)
SKF38393 (n=6)	1.02 ± 0.04	612 ± 72	56.1
SKF81297 (n=6)	0.84 ± 0.05	120 ± 17	18.0
NM-SCH23390 (n=7)	1.15 ± 0.08	5860 ± 1840	382
SCH23390 (n=6)	0.75 ± 0.04	462 ± 73	93.6
Dopamine (n=4)	0.78 ± 0.02	2420 ± 209	380

$K_{d(0)}$ and zδ values were determined using the Woodhull Model by fitting normalized G_b/G_0 vs V plots. K_d at -60mV were determined by equation (2). See data analysis for the detail.

Figure 1

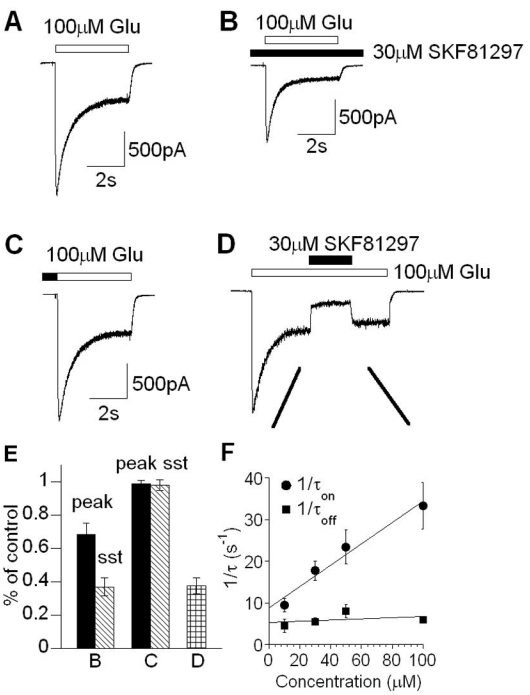
A
100μM Glu
500pA
2s
B
100μM Glu
30μM SKF81297
500pA
2s
C
100μM Glu
500pA
2s
D
30μM SKF81297
100μM Glu
E
peak sst
peak
sst
% of control
1
0.8
0.6
0.4
0.2
0
B
C
D
F
1/τon
1/τoff
1/τ (s-1)
40
30
20
10
0
0 20 40 60 80 100
Concentration (μM)

Figure 2

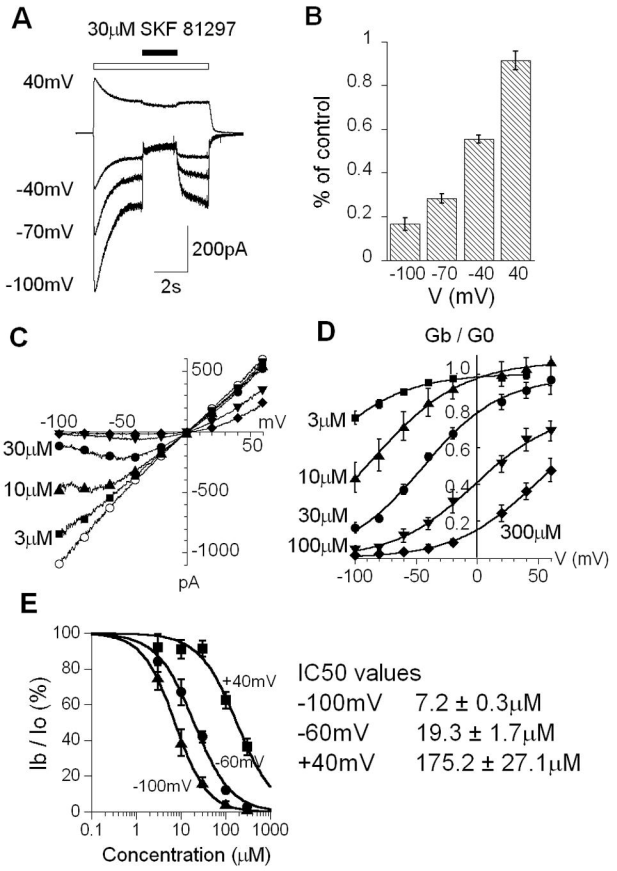
A
30μM SKF 81297
40mV
-40mV
-70mV
-100mV
200pA
2s
B
% of control
1
0.8
0.6
0.4
0.2
0
-100 -70 -40 40
V (mV)
C
500
-100
-50
mV
50
30μM
10μM
3μM
-500
-1000
pA
D
Gb / G0
1.0
0.8
0.6
0.4
0.2
3μM
10μM
30μM
100μM
300μM
-100 -50 0 50
V (mV)
E
100
80
60
40
20
0
Ib / Io (%)
+40mV
-60mV
-100mV
0.1 1 10 100 1000
Concentration (μM)
IC50 values
-100mV 7.2 ± 0.3μM
-60mV 19.3 ± 1.7μM
+40mV 175.2 ± 27.1μM

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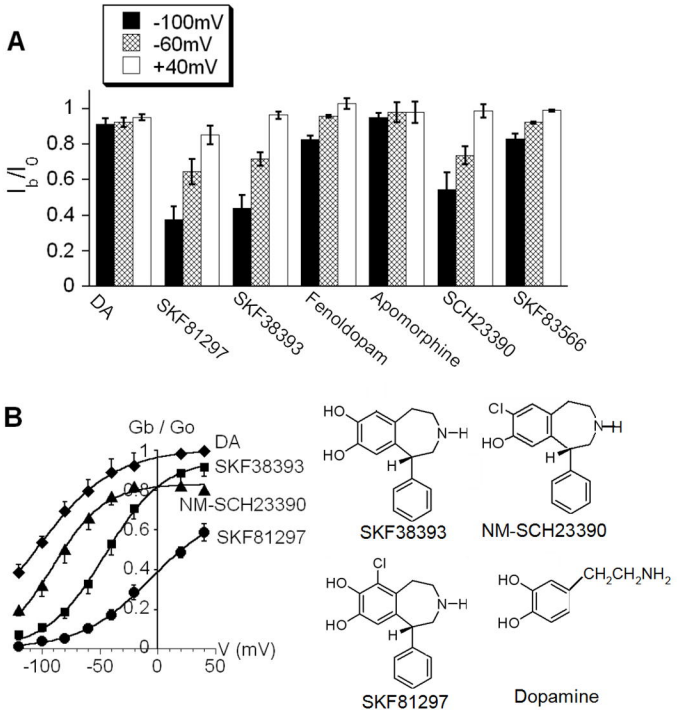
Figure 3



Molecular Pharmacology Fast Forward. Published on August 18, 2006 as DOI: 10.1124/mol.106.028332
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Figure 4

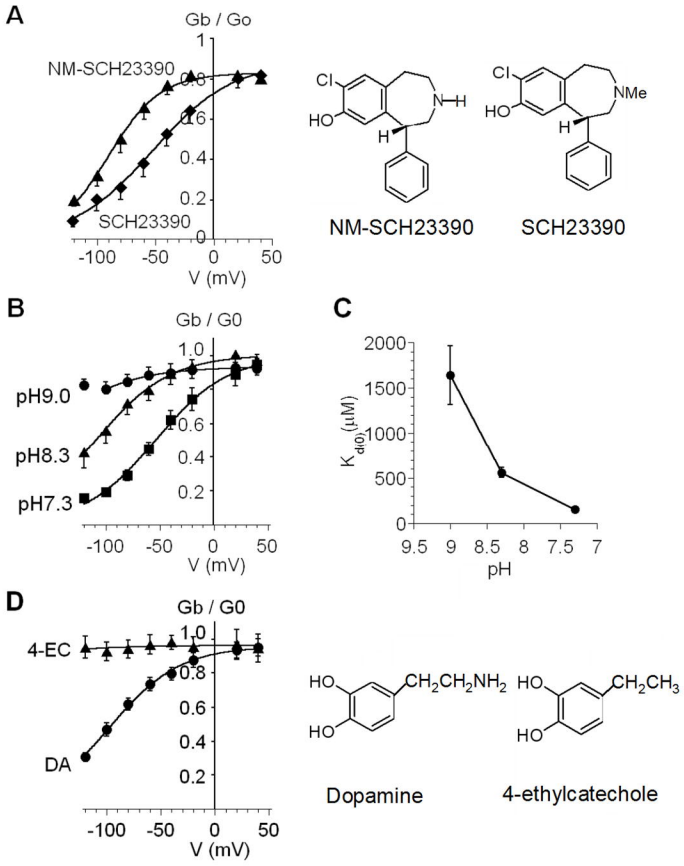
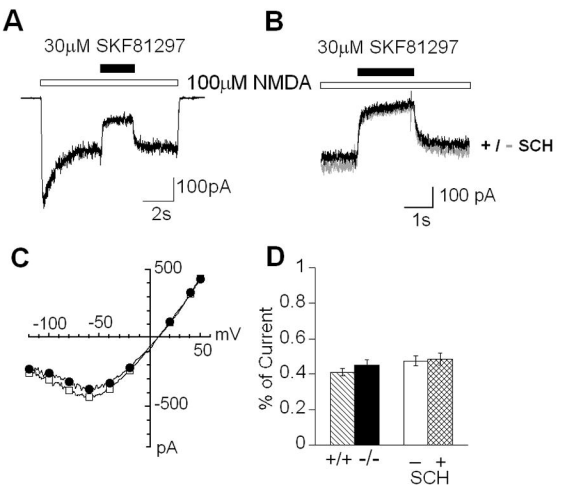


Figure 5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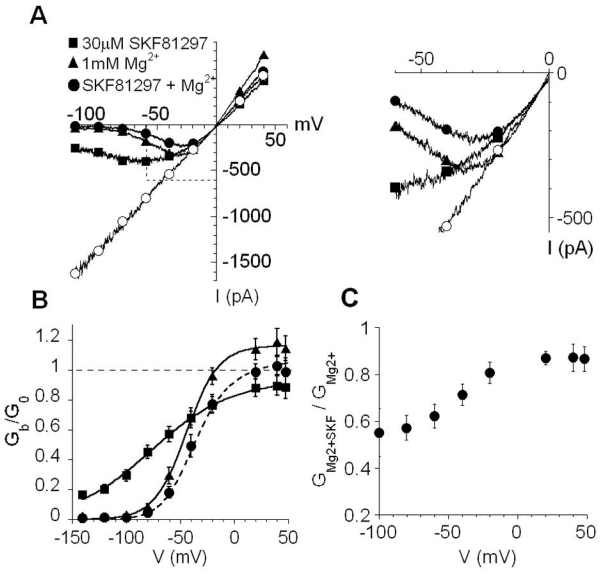
30μM SKF81297
100μM NMDA
100pA
2s
B
30μM SKF81297
+ / - SCH
100 pA
1s
C
500
-100
-50
mV
50
-500
pA
D
1
0.8
0.6
0.4
0.2
0
% of Current
+/+
-/-
–
+
SCH

Figure 6


A
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1mM Mg2+
SKF81297 + Mg2+
-100
-50
50
mV
-500
-1000
-1500
I (pA)
-50
0
0
-500
I (pA)
B
1.2
1
0.8
0.6
0.4
0.2
0
Gb/G0
-150
-100
-50
0
50
V (mV)
C
1
0.8
0.6
0.4
0.2
GMg2+SKF / GMg2+
-100
-50
0
50
V (mV)