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## Cocaine effects on dopamine and NMDA receptors interactions in the striatum of Fischer rats

Wei-Lun Sun<sup>1,3,\*</sup>, Luyi Zhou<sup>2,4</sup>, Vanya Quinones-Jenab<sup>1,3,4</sup>, and Shirzad Jenab<sup>1,3</sup>

<sup>1</sup>Department of Psychology, Hunter College, CUNY, 695 Park Ave, New York, NY 10065

<sup>2</sup>Department of Biological Sciences, Hunter College, CUNY, 695 Park Ave, New York, NY 10065

<sup>3</sup>Biopsychology and Behavioral Neuroscience Doctoral Subprogram, Graduate School and University Center, CUNY, 365 Fifth Ave, New York, NY 10016, USA

<sup>4</sup>Biology Doctoral Program, Graduate School and University Center, CUNY, 365 Fifth Ave, New York, NY 10016, USA

### Abstract

Numerous studies have shown that biochemical and behavioral effects of cocaine are mediated by dopamine D1 receptor (D1R) and NMDA R1 receptor (NR1)-mediated transmissions. In this study, we investigated the physical interactions between D1R and NR1 in response to acute cocaine administration in a time course of 5 minutes to 60 minutes. In the caudate-putamen (CPu) of male Fischer rats, a single cocaine injection (30 mg/kg) reduced D1R-NR1 protein-protein interactions 30 minutes after treatment. In addition, activation or blockade of the NMDA receptor using NMDA (25 mg/kg) or MK-801 (0.25 mg/kg), respectively, also reduced the D1R-NR1 physical interactions. Acute cocaine administration did not alter total D1R or NR1 protein levels in our time course of study. These results indicate that D1R-NR1 physical interaction rather than total protein levels may regulate the intracellular signaling after acute cocaine administration.

### 1. Introduction

Cocaine, a psychostimulant, is one of the most widely abused drugs in Western countries. By acting as an indirect dopamine agonist, cocaine increases dopamine as well as glutamate release in the striatum [2,19,23,27,33,35,45]. At the receptor level, pharmacological or genetic manipulation of D1R and NMDA receptors attenuates acute cocaine-induced locomotor activity [18,20,32,41,48], reinforcing and rewarding effects of cocaine [1,17,30,32], and intracellular signaling alteration (e.g., the phosphorylation of extracellular signal-regulated kinase (ERK) and cyclic AMP response element-binding protein) in response to acute cocaine in the dorsal striatum/CPu [18,20,42,49]. Taken together, these findings indicate that both D1R and NMDA receptors are involved in cocaine-induced behavioral and biochemical changes. Although repeated cocaine exposure alters both D1R binding density in the striatum and NR1

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\*Corresponding author: Wei-Lun Sun; Department of Psychology; Hunter College, CUNY, 695 Park Ave, New York, NY 10065; wsun@hunter.cuny.edu; Tel: (212) 772-5625; Fax: (212) 772-4619

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Conflict of Interest

We declare that there is no conflict of interest.

mRNA/protein levels in the striatum and ventral tegmental area (VTA), acute cocaine administration does not change D1R binding or NR1 protein levels [4,12,14,21,22,39,40]. Therefore, how acute cocaine administration regulates D1R and NR1 protein levels remains ambiguous.

One mechanism may involve in D1R and NR1 communication via protein-protein interactions at their carboxyl terminal regions [10,24,25,31]. In the primary hippocampal neurons or dopamine D1 and NR1 co-transfected cell lines, the stimulation of D1R results in a dissociation of the receptors [25]. The activation of NMDA receptor enhances D1R cAMP accumulation and recruits D1R membrane surface insertion in a D1R/NR1 physical protein-protein interactions manner [31]. Taken together, it suggests that dopamine and glutamate levels modulate the physical interaction between D1R and NR1 receptors. The present study was designed to evaluate the effects of acute cocaine and NMDA receptor activation and blockade on NR1 and D1R interactions in the dorsal striatum.

## 2. Methods

### Animals

60-day-old male Fischer rats (Charles River, Raleigh, NC) were individually housed in Plexiglas chambers (20 × 20 × 41 cm) and maintained on a 12-hour light/dark cycle (lights on at 9:00 a.m.) with free access to food and water. Animal care and use was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, Bethesda, MD) and approved by the Institutional Animal Care and Use Committee of Hunter College.

### Drugs and antibodies

Cocaine hydrochloride, NMDA and MK-801 were purchased from Sigma Chemical Co. (St. Louis, MO). Antibody for D1R was purchased from Chemicon International (Temecula, CA). Mouse monoclonal antibody for NR1 was purchased from BD Pharmingen (San Diego, CA).  $\alpha$ -tubulin antibody and protein A/G agarose were from Santa Cruz Technologies (Santa Cruz, CA). Both horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Amersham Pharmacia (Piscataway, NJ).

### Drugs administration

For cocaine time course study, cocaine solutions were prepared by dissolution in physiological saline (0.9%) and injected intra-peritoneally (i.p.). Rats were injected with saline (1 ml/kg) or cocaine (30 mg/kg) and sacrificed 5, 15, 30 or 60 min later. A separate cohort of rats were injected with saline, NMDA (25 mg/kg, i.p.) or MK-801 (0.25 mg/kg, i.p) and sacrificed 30 min later.

### Protein preparation and measurement

After decapitation (following a brief 20 s exposure to CO<sub>2</sub>), rat brains were removed, flash frozen in 2-methylbutane (-40° C), and stored at -80° C until used. The coronal slices (1mm thick) were cut out in a matrix (ASI instruments, Warren, MI). CPU was dissected out on a cold glass plate, and then homogenized using a Polytron handheld homogenizer (Kinematica, Luzern, Switzerland) in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% Triton X-100, 1% Igepal CA-630, 1% sodium deoxycholic acid) containing phosphatase inhibitors mixture. After 30 min incubation, homogenates were centrifuged at 13,000 rpm for 15 min at 4° C. Supernatants were then collected and stored at -80° C until used. Total protein content was determined using a Bradford kit from Bio-Rad Laboratories (Hercules, CA).

### Western blot analysis

Protein samples were analyzed as previously described [18]. Briefly, 40 µg of proteins were boiled in Lammeli buffer containing 1% β-mercaptoethanol for 5 min and separated on 10% SDS-PAGE gels, then transferred to PVDF membranes. Membranes were blocked with 5% nonfat dry milk for 1hr at room temperature and incubated with antibodies for D1R (1:1000) or NR1 (1:1000) overnight at 4° C. After three washes with Tris-Tween-20 Buffer (TBST; pH = 7.4), membranes were incubated with their appropriate secondary antibodies (1:1000) for 1hr at room temperature followed by three more washes with TBST. Antibody binding was detected using an enhanced chemiluminescence kit (ECL; Amersham Pharmacia, Piscataway, NJ). Intensity of protein bands was quantified with a computer densitometer and Image Quant Program (Molecular Dynamics). For normalization of protein levels, all membranes were re-probed with α-tubulin antibody (1:1000).

### Immunoprecipitation analysis

For immunoprecipitation experiments, equal amount of protein extracts (500µg) were incubated in lysis buffer containing protease inhibitors mixture with anti-NR1 or anti-D1R primary antibody (1:50-100) overnight at 4° C, followed by the addition of protein A/G agarose (20 µl) for 4hrs. For control samples, protein extracts were incubated in absence of primary antibody. Beads were then washed four times in the lysis buffer. After final centrifugation, Lammeli buffer containing 1% β-mercaptoethanol (50-70 µl) was added and samples were boiled for 5 min. Equal amount of supernatants were loaded on 8% SDS-PAGE gels and the intensity of D1R or NR1 were analyzed via Western blot. Each sample for immunoprecipitation using anti-D1R antibody to pull down was performed three times to confirm the conclusion.

### Statistical analysis

Protein levels were expressed as a ratio to α-tubulin levels in Western blot analysis. For immunoprecipitation analysis, NR1 protein levels were expressed as a ratio over optic density of total NR1 levels obtained from Western blot experiments. Data was expressed as mean % ± SEM relative to respective saline controls, which were arbitrarily set at 100%. Student's t-tests were used to determine differences between cocaine- and saline-treated animals at each time point. To determine effects of NMDA and MK-801 administration on receptors physical protein-protein interactions, a one-way ANOVA was used followed by LSD post hoc analysis when appropriate. Differences were considered significant at 0.05 level.

## 3. Results

### Effects of acute cocaine on NR1 and D1R interactions

As shown in Figure 1, acute cocaine administration did not alter total D1R or NR1 protein levels in the CPu. Immunoprecipitation analysis however, indicated that 30 min after a single cocaine administration NR1 and D1R interactions were decreased as compared to the respective saline control [ $t(4) = 4.69, P < 0.01$ ; Fig. 2A]. This data was confirmed using a reciprocal immunoprecipitation approach: immunoprecipitation using NR1 antibody followed by probing with D1R antibody (Fig 2B).

### Effects of NMDA and MK-801 administration on NR1 and D1R interactions

Similar to acute cocaine, a single NMDA or MK-801 injection did not change the total protein levels of NR1 subunit or D1R [Fig. 3A and B, respectively]. However, immunoprecipitation experiment showed that there was a significant treatment effect on receptors interactions [ $F(2, 6) = 22.23, P < 0.05$ ; Fig. 4A]. Further, post hoc test revealed that NMDA and MK-801 treatments significantly reduced NR1 and D1R interactions as compared to saline-treated animals [ $P < 0.01$  and  $P < 0.01$ , respectively]. There were no differences between NMDA- and

MK-801-treated groups. This data was also confirmed using a reciprocal immunoprecipitation approach: immunoprecipitation using NR1 antibody followed by probing with D1R antibody (Fig 4B).

#### 4. Discussion

Results presented here demonstrated that acute cocaine administration time-dependently reduced NR1 and D1R protein-protein interactions in the CPu. In line with previous studies we also showed that acute cocaine did not change total protein levels of NR1 or D1R [12,39], suggesting the reduction of physical interactions between these receptors is not due to total proteins alteration. In addition, after NMDA or MK-801 treatment the association between NR1 and D1R was also decreased. Thus, although acute regulation of D1R or NR1 receptors does not change their total protein levels, it disrupts their physical interactions and thus may represent as an important step in initial cocaine-induced intracellular responses.

Several *in vitro* studies indicated a complex interaction between NR1 and D1R. For example, in hippocampal and striatal cultures, the stimulation of D1R and PKA resulted in the phosphorylation of NR1 subunits, increased in cytosolic calcium and phosphorylation of ERK proteins [3,5,26,38,43,47]. In the cultured striatal neurons or the VTA *in vivo*, D1R agonists or a single cocaine administration induced a rapid NR1 subunits postsynaptic membrane insertion [8,34]. The membrane insertion of NR1 subunits may contribute to the potentiation of NMDA receptor-mediated current by D1R. However, Lee et al [25] showed that the activation of D1R receptor by dopamine application disrupted the NR1/D1R physical interactions and reduced the NMDA receptor-mediated calcium influx via NMDA-NR2A/D1R carboxyl terminal interactions. Previously, we have demonstrated that, in the CPu, a single cocaine administration (30 mg/kg) transiently induced ERK phosphorylation, which gradually returned to basal levels 30 min after injection [37]. In present study, although the functional significance of cocaine-induced dissociation of NR1/D1R interactions is still unknown, it is tempting to postulate that the dissociation of receptors interactions may act as a time-limited gating mechanism on ERK activation in response to acute cocaine: (1) a rapid ERK phosphorylation due to potentiated NMDA-mediated calcium influx and/or NR1 redistribution through cocaine-elevated extracellular dopamine levels and D1R activation; followed by (2) a deactivation of ERK protein because of the decrease in intracellular calcium via the disruption of NR1 and D1R interactions.

Alternatively, the NR1/D1R physical associations may regulate the D1R surface expression. Recently, NR1/D1R oligomerization was also found in cells co-transfected with D1R and NMDA receptors including NR1/NR2B subunits as well as in the striatal post-synaptic density (PSD) [10,11]. In the presence of the NMDA-NR2B subunit, the NR1/D1R complex was delivered to membrane surface and then prevented the D1R agonist-induced D1R internalization, suggesting these receptors interactions may represent a mechanism to recruit D1R to the striatal PSD [10,29]. However, in the present study, we demonstrated a decreasing of NR1 and D1R receptor physical interactions after acute cocaine. We propose two plausible explanations. First, evidence has shown that D1R stimulation results in NMDA receptor membrane insertion in the striatum [7,8]. Thus, acute cocaine-induced D1R activation, may recruit NMDA receptor to the membrane region. The newly inserted NMDA receptor may not physically associate with D1R immediately but form receptors interactions at later time point. This temporal change in receptors interactions is consistent with present results in that transient reduction of NR1/D1R interactions was only observed 30 min after acute cocaine administration. Second, a previous *in vivo* study has shown that a single intrastriatal D1R agonist infusion or amphetamine injection (i.p.) rapidly induced D1R internalization in the striatum [6]. The modification of D1R distribution was found in cell bodies and dendrites but not in dendritic spines at the periphery of synaptic clefts, where PSD is located [6]. Thus, after

cocaine administration, the dissociation of NR1/D1R physical interactions may indicate D1R segregation and the reduction of receptor availability for extracellular ligands in a region-specific manner as suggested by Fiorentini and Missale [11]. However, further biochemical fractionation procedure and immunohistochemical labeling at the ultrastructural level should be conducted to examine cocaine-induced D1R internalization and the decrease of receptors interactions in different neuronal compartments.

In the CPu, we demonstrated that the NR1/D1R receptors physical interactions were decreased 30 min after MK-801 administration. Similarly, an *in vitro* study showed that NMDA receptor antagonist, AP-5, pre-application led to the dissociation between NR1 and D1R receptors in cells, suggesting that the NMDA receptor activation is necessary to maintain the protein-protein interactions between these receptors [31]. In the striatum, a recent study showed that MK-801 (0.1 mg/kg) did not modify NR1 and D1R interactions [11]. However, a higher dose of MK-801 (0.25 mg/kg) was used in the present study. Previous studies have shown that the higher MK-801 (0.2-0.5 mg/kg) doses elevated extracellular dopamine levels in the striatum [28,44,46]. Thus, by increasing synaptic dopamine levels, MK-801 itself may reduce NR1 and D1R interactions (similar to acute cocaine administration). Surprisingly, NMDA treatment also reduced NR1/D1R physical interactions, suggesting that the activation of NMDA receptor also disrupts the receptors interactions. In cortical neurons, a study has demonstrated that chronic neuronal activity promotes the degradation of NR1 through ubiquitin-proteasome system [9]. However, in the present study, total NR1 protein levels were not altered after NMDA administration, indicating that the reduction in NR1/D1R physical interactions is not due to the decrease in NR1 levels. In hippocampal neurons, PKC-induced rapid NMDA receptors dispersal and lateral mobility between the synaptic and extra-synaptic domains have been documented [13,15]. NR2B-containing NMDA receptors exhibit faster mobility than those containing NR2A subunit [16]. In addition, NR2B-containing receptors are prominently expressed in the striatum [36]. Thus, through NMDA receptors, the NMDA treatment (25 mg/kg i.p.) may induce PKC activation and lateral mobility of NMDA receptors and then contribute to the decrease of NR1/D1R physical interactions.

In summary, the present study demonstrated that acute cocaine reduced the NR1 and D1R physical protein-protein interactions in a time-dependent manner. We postulate that the dissociation may regulate cocaine- and/or dopamine-mediated intracellular signaling cascades and D1R distribution underlying the cocaine-induced behavioral changes. In addition, we have also showed that the homeostatic state of NMDA receptor is necessary for the NR1/D1R physical interactions, since both NMDA receptor activation and inhibition reduced receptors associations. However, the functional significance of their receptors interactions on NR1- and D1R-mediated intracellular signaling transduction and addictive behaviors needs further elucidation.

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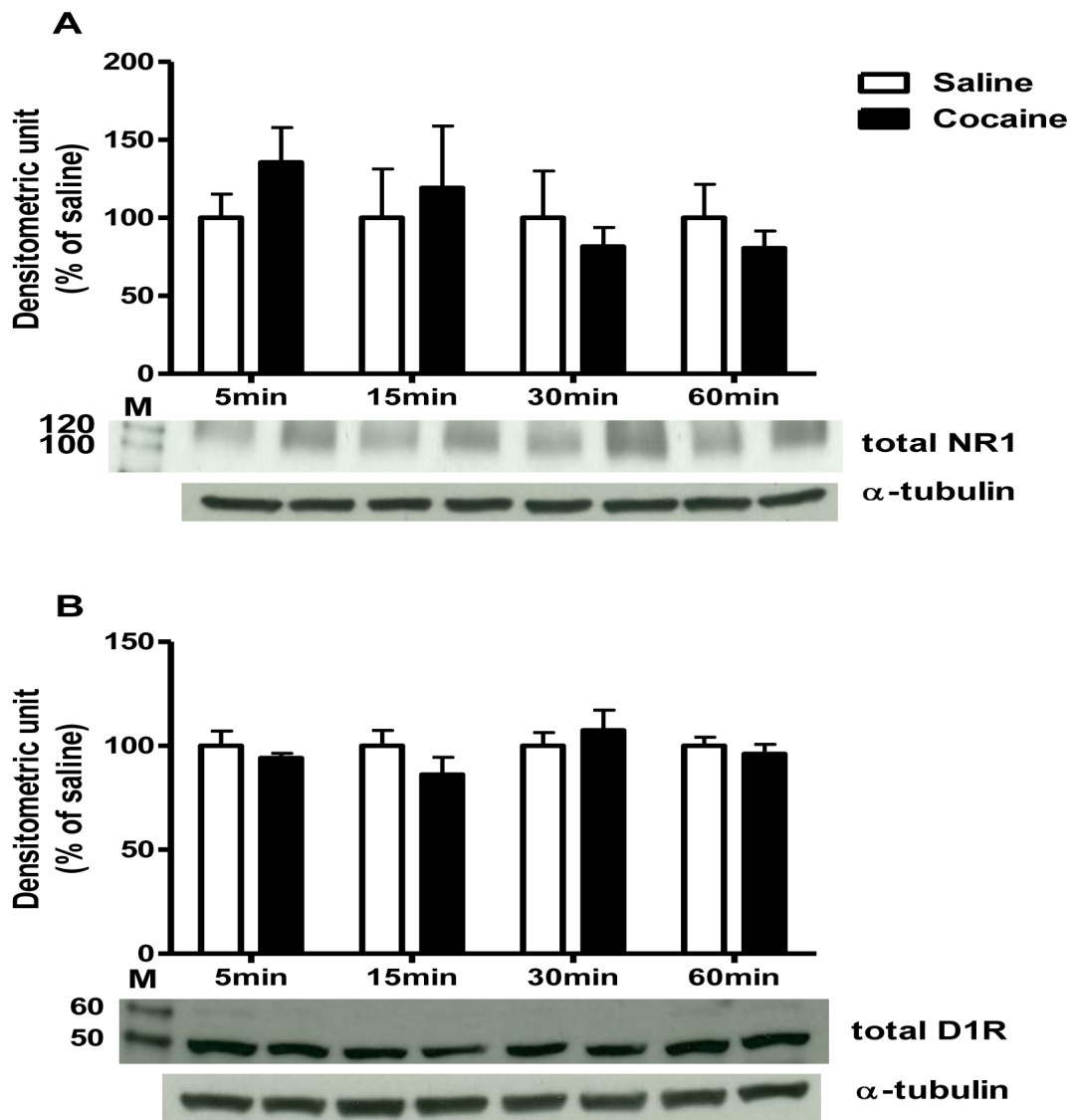


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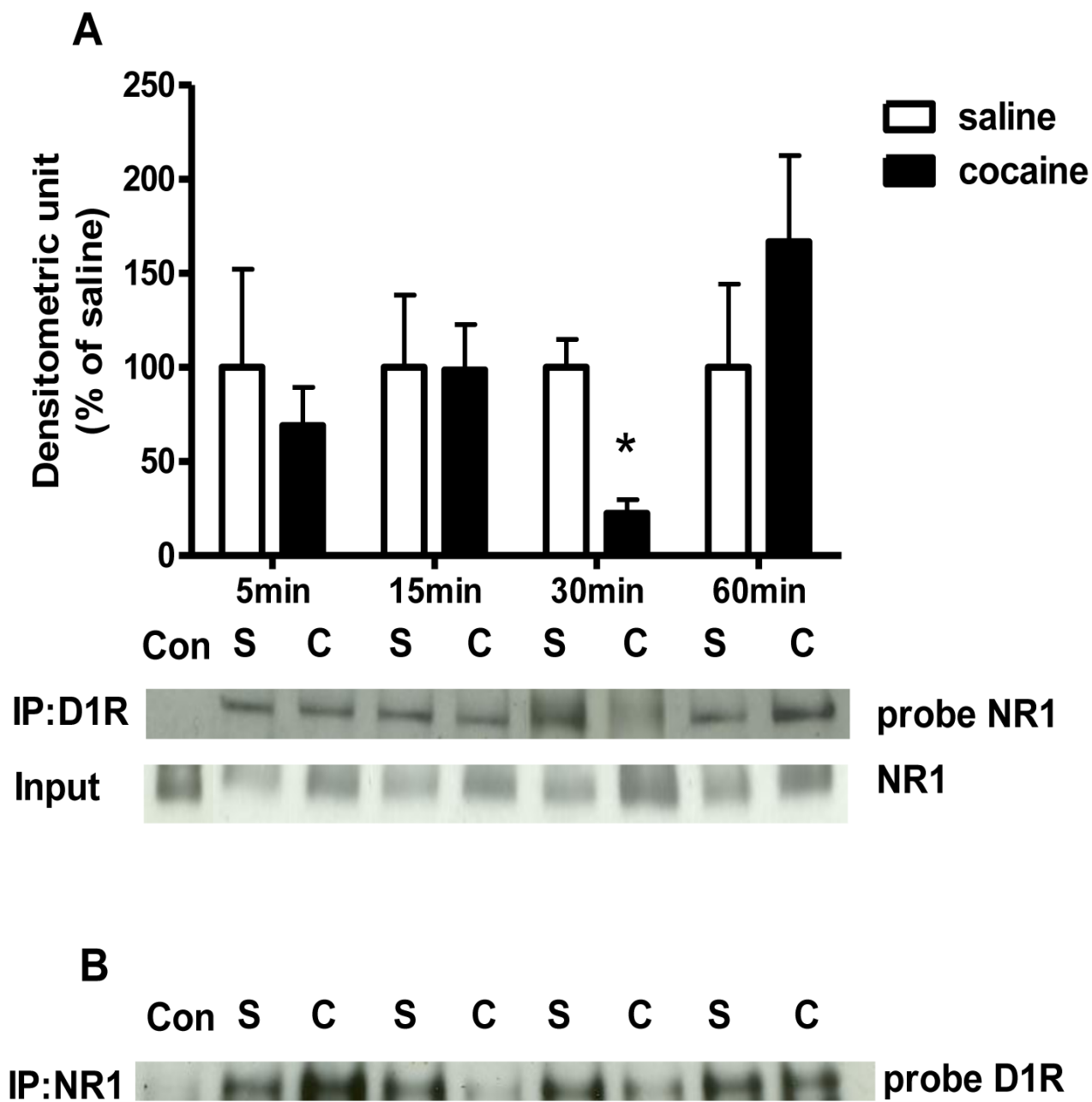
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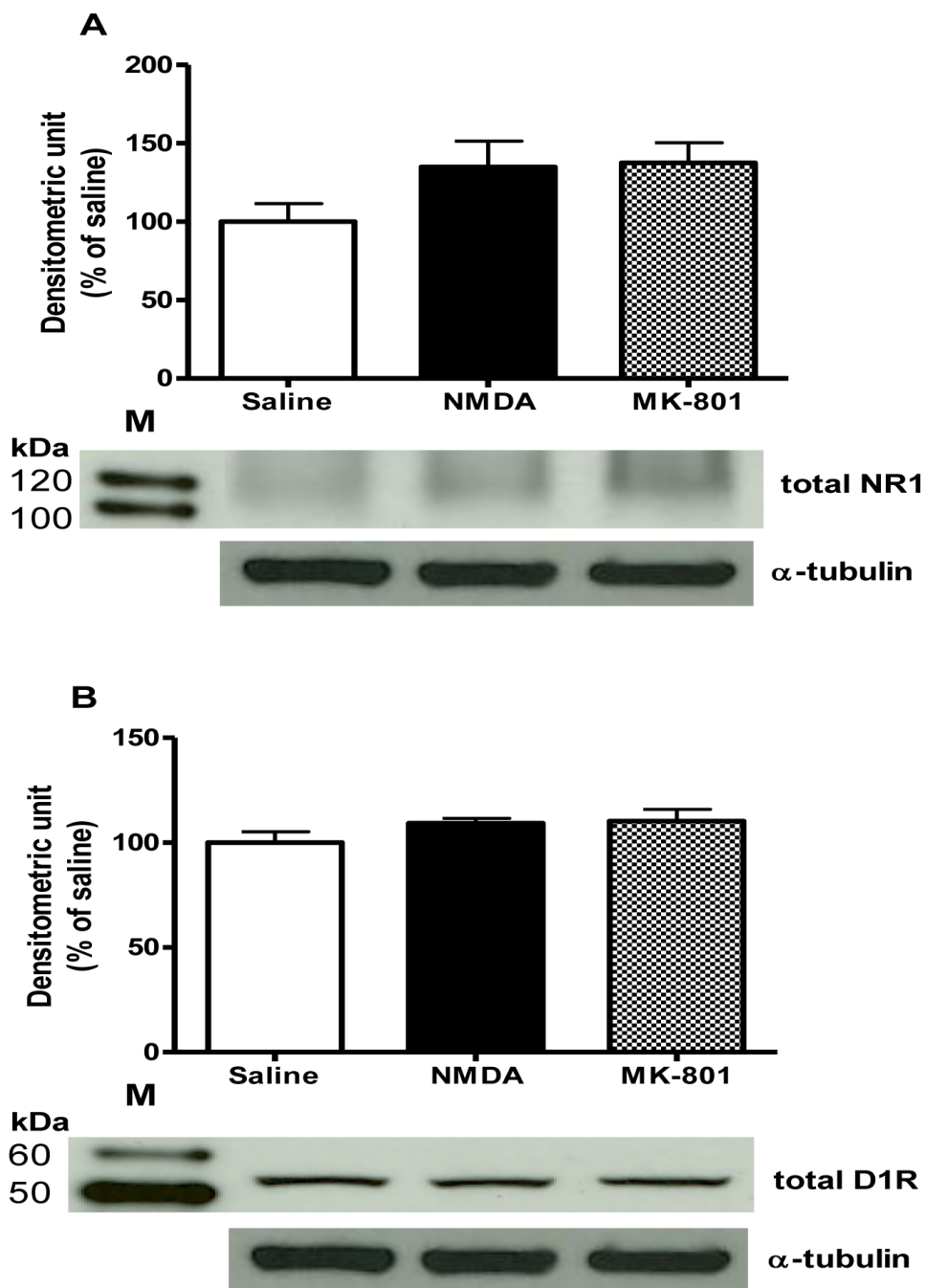




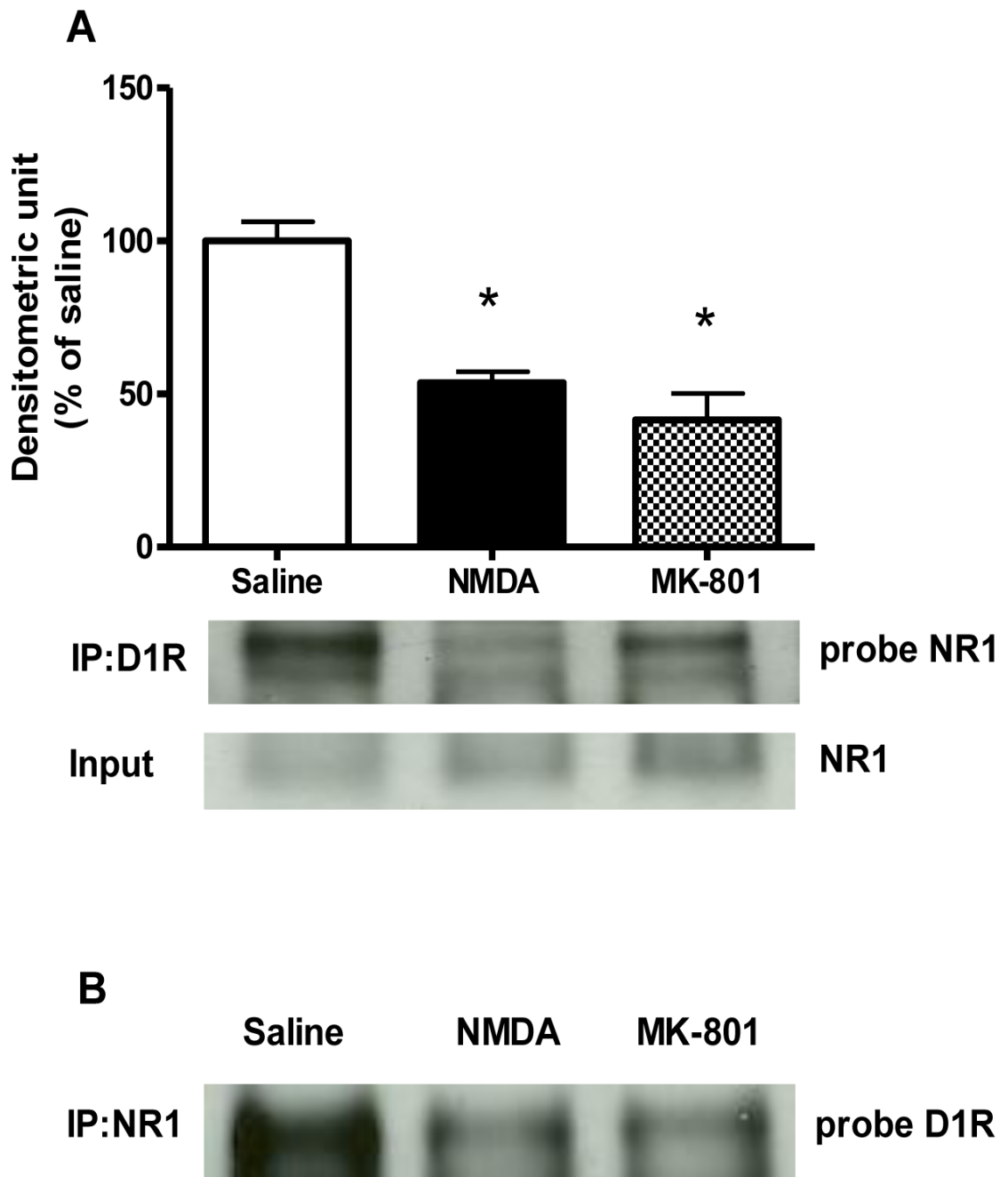
**Figure 1. Time course of cocaine effects on total NR1 and D1R in the CPu**  
 (A) NR1; (B) D1R. Total protein levels after 5, 15, 30, or 60 min of cocaine administration over  $\alpha$ -tubulin levels, expressed as percentage of saline control (4-6 animals per group). M is the molecular marker in kDa.



**Figure 2. Time course of cocaine effects on NR1 and D1R physical interactions in the CPu**  
 (A) NR1 protein levels after D1R antibody immunoprecipitation. Data shown is the protein level over total NR1 level, expressed as percentage of saline control after 5, 15, 30, or 60 min of cocaine injections (3-6 animals per group). \* $p < 0.05$  as compared with respective saline group. (B) A reciprocal immunoprecipitation showing D1R protein levels after NR1 antibody pull down (Con = Control; S = Saline; C = Cocaine).



**Figure 3.** Effects of NMDA and MK-801 administration on total NR1 and D1R in the CPu (A) NR1; (B) D1R. Results represent total protein levels over  $\alpha$ -tubulin levels, expressed as percentage of saline contro after 30 min of saline, NMDA or MK-801 injections (4-6 animals per group). M is the molecular marker in kDa.



**Figure 4. Effects of NMDA and MK-801 administration on NR1 and D1R physical interactions in the CPu**

(A) NR1 protein levels in D1R antibody pull down. Results represent protein levels over total NR1 levels, expressed as percentage of saline control, after 30 min of saline, NMDA or MK-801 injections (3 animals per group). \* $p < 0.05$  as compared with saline control. (B) A reciprocal immunoprecipitation showing D1a protein levels after NR1 antibody pull down.