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In Vivo Regulation of Homer1a Expression in the Striatum by Cocaine

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ABSTRACT

The glutamate receptor adaptor protein Homer is concentrated in the postsynaptic density of excitatory synapses and is critical for normal operation of synaptic transmission. In this study, we investigated the responsiveness of Homer family proteins to dopamine stimulation with the psychostimulant cocaine in rat striatal neurons both in vivo and in vitro. We found that a single dose of cocaine specifically induced a rapid and transient increase in protein levels of the Homer1a, but not Homer1b/c and Homer2a/b, isoforms in the striatum. This selective Homer1a induction was mediated primarily through activation of dopamine D1, but not D2, receptors. Both protein kinase A and Ca^{2+}/calmodulin-dependent protein kinases are important for mediating the cocaine stimulation of Homer1a expression. At the transcriptional level, cAMP response element-binding protein serves as a prime transcription factor transmitting the signals derived from D1 receptors and associative pathways to the CaCRE sites within the Homer1a promoter. From a functional perspective, non–cross-linking Homer1a, once induced, competed with the cross-linking isoforms of Homer proteins (Homer1b/c and Homer2a/b) to uncouple the connection of group I metabotropic glutamate receptors (mGluRs) with inositol-1,4,5-triphosphate receptors. These results indicate that cocaine possesses the ability to stimulate Homer1a expression in striatal neurons through a specific synapse-to-nucleus pathway. Moreover, inducible Homer1a expression may represent a transcription-dependent mechanism underlying the dynamic regulation of submembranous macromolecular complex formation between group I mGluRs and their anchoring proteins.

Synaptic Homer proteins are important for synaptic construction and function (Xiao et al., 2000; Sheng and Kim, 2002). Long-form Homer proteins (Homer1b/c, Homer2a/b, and Homer3) contain the N-terminal EVH1 (Enabled/VASP homology 1) domain, which binds the C terminus of group I metabotropic glutamate receptors (mGluRs), whereas the C-terminal coiled-coil structure and leucine zipper motifs render a capability for self-assembly (Brakeman et al., 1997; Xiao et al., 1998; Xiao et al., 2000). Thus, as a prominent scaffolding molecule concentrated in the postsynaptic density of excitatory synapses, Homer cross-links group I mGluRs to other targets in a specific subcellular microdomain to regulate a specific signaling activity. Emerging evidence indicates that Homer proteins play an essential role in the membrane trafficking of mGluR1α/5 (Ango et al., 2000); the coupling of mGluR1/5 to inositol-1,4,5-triphosphate (IP_3) receptors (Tu et al., 1998) and the cation (Ca^{2+} or K^+) channel (Yuan et al., 2003); the development of spines, axons, and synapses (Shiraishi et al., 2003); and drug addiction (Swanson et al., 2001; Szumlinski et al., 2004, 2005; also see below).

One distinctive member of Homer family is Homer1a (Brakeman et al., 1997; Xiao et al., 1998, 2000). Unlike the long-form of Homer proteins, this short-form of Homer lacks the C-terminal coiled-coil structure and leucine zipper motifs, rendering it incapable of self-assembly. Moreover, Homer1a, as one of immediate early genes, is constitutively

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; IP_3, inositol-1,4,5-triphosphate; PKC, protein kinase C; PKA, protein kinase A; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; CREB, cAMP response element-binding protein; pCREB, phosphorylated CREB; PP1γ1, protein phosphatase 1C; Gö6983, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide; SKF82958, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; KN-62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; SCH23390, (*R*)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride; Ro-31-8220, 3-[1–3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; CPu, caudate putamen; NAc, nucleus accumbens; CaMK, Ca^{2+}/calmodulin-dependent protein kinases.

expressed at a low level, but highly inducible in response to cellular stimulation. To date, Homer1a expression was found to be up-regulated by glutamate receptor ligands, KCl, protein kinase C (PKC) activators, and other experimental manipulations in vivo or in cultured neurons (Brakeman et al., 1997; Ango et al., 2000; Kato et al., 2001, 2003; Sato et al., 2001).

Psychostimulants are among the agents that readily increase *Homer1a* transcripts in the forebrain. Acute injection of a psychostimulant methylphenidate (Ritalin) increased *Homer1a* mRNA levels in the rat cortex and striatum (Yano and Steiner, 2005). Acute or "binge" administration of cocaine also produced the same effect (Brakeman et al., 1997; Yuferov et al., 2003). Thus, dopamine stimulation is thought to facilitate *Homer1a* transcription leading to enhanced transcript levels. However, whether increased *Homer1a* transcripts can translate to increased Homer1a protein expression after dopamine stimulation is unknown. More importantly, the signaling mechanism by which cocaine induces Homer1a expression and possible functional roles of induced Homer1a remain elusive.

In this study, we therefore examined the effect of acute cocaine administration on Homer1a protein expression in rat striatal neurons in vivo and in vitro. We found that cocaine strongly increased Homer1a protein levels in the striatum. This event was mediated through the D1 receptor and associated signaling pathways. Increased Homer1a proteins seemed to disrupt the binding of cross-linking Homer proteins to group I mGluRs and IP_3 receptors and thereby normalize stimulated group I mGluR signaling.

Materials and Methods

Animals and Drug Injections. Adult male Wistar rats weighting 200 to 225 g (Charles River, New York, NY) were individually housed in clear plastic cages in a controlled environment at a constant temperature of 23°C and humidity of 50 ± 10% with food and water available ad libitum. The animal room was on a 12-h/12-h light/dark cycle with lights on at 7:00 AM. Rats were allowed 6 to 7 days of habituation to the animal colony before any treatment began. All animal use procedures were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee. Saline or cocaine was injected i.p.

Primary Striatal Neuronal Cultures. Standardized procedures in this laboratory were employed to prepare primary striatal neuronal cultures from embryonic day-18 rat embryos (Charles River Laboratories, Inc., Wilmington, MA) (Mao et al., 2005a,b; Yang et al., 2006). Predominant GABAergic neurons were obtained using this procedure as evidenced by the fact that >90% of total cells were immunoreactive to glutamic acid decarboxylase-65/67, GABA, and the specific marker for neurons (microtubule-associated protein 2a + 2b), but not for glia (glial fibrillary acidic protein). Cells were cultured for 15 to 18 days before use.

Drug Treatments on Cultured Cells. Drug treatments were made according to the protocol described previously (Mao et al., 2005b; Yang et al., 2006). Cultures were washed with PBS and preincubated at 37°C for 60 min in the humidified atmosphere of 5% CO_2 in HEPES-buffered saline 154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 5.5 mM glucose, and 20 mM HEPES-KOH or HEPES-NaOH, pH 7.4. Cells were treated by adding drug to the HEPES-buffered saline. At the end of drug treatment, the cells were quickly washed with ice-cold PBS (pH 7.4; Ca^{2+}-free) and placed immediately on ice. The cell monolayer was rapidly scraped in ice-cold lysis buffer. All drugs were freshly made on the day of experiment. Drugs were dissolved in 1× PBS with or without an aid of dimethyl sulfoxide (DMSO). Whenever DMSO was used, PBS containing the same concentration of DMSO was used as the control vehicle.

Western Blot Analysis. Rats were anesthetized with pentobarbital/chloral hydrate (9 ml/kg, i.p.) and decapitated. Brains were removed, and the dorsal and ventral striata were separately removed into a 1.5-ml microtube containing ice-cold sample buffer (20 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 10 mM NaF, 2 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 5 μM microcystin-LR, and 0.5 mM phenylmethylsulfonyl fluoride). The sample was homogenized by sonication. The homogenate was centrifuged at 700*g* for 10 min at 4°C. The supernatant was again centrifuged at 10,000*g* at 4°C for 30 min to generate the pellet (P2), which was resuspended in ice-cold sample buffer. Protein concentrations were determined. The equal amount of protein (20 μg/20 μl/lane) was separated on SDS NuPAGE Novex 4 to 12% gels (Invitrogen, Carlsbad, CA). Proteins were transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA) and blocked in blocking buffer (5% nonfat dry milk in PBS and 0.1% Tween 20) for 1 h. The blots were incubated in a primary goat antibody against Homer1a (Santa Cruz Biotechnology, Santa Cruz, CA) or Homer2a/b (Santa Cruz Biotechnology); a rabbit antibody against pan Homer (Santa Cruz Biotechnology), c-Fos (Oncogene Research Products, San Diego, CA), cAMP response element-binding protein (CREB) (Santa Cruz Biotechnology), phosphorylated CREB (pCREB; Santa Cruz Biotechnology), type I IP_3 receptors (Chemicon, Temecula, CA), or β-actin (Santa Cruz Biotechnology); or a rat antibody against Homer1b/c (Chemicon), usually at 1:100 to 1:1000 overnight at 4°C. This was followed by 1-h incubation in a goat horseradish peroxidase-linked secondary antibody against goat, rabbit, or rat (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at 1:5000. Immunoblots were developed with the enhanced chemiluminescence reagents (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK), and captured on a Kodak Image Station 2000R (Eastman Kodak, Rochester, NY). Kaleidoscope-prestained standards (Bio-Rad Laboratories, Hercules, CA) and MagicMark XP Western protein standards (Invitrogen) were used for protein size determination. The density of immunoblots was measured using the Kodak 1D Image Analysis software, and all bands were normalized to percentages of control values. To enhance the faint Homer1a band, the blot was exposed for 20 to 40 min.

Coimmunoprecipitation. Coimmunoprecipitation was conducted as described previously (Yang et al., 2006). In brief, striatal tissue was homogenized in a microtube containing ice-cold sample buffer (10 mM Tris-HCl, pH 7.4, 5 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, and 1 mM EGTA). The homogenate was centrifuged at 800*g* (15 min) at 4°C. The supernatant was again centrifuged at 12,000*g* at 4°C for 30 min to obtain the P2 pellet. The P2 pellet was resuspended in sample buffer, and solubilized in 1% sodium deoxycholate. After incubation at 37°C for 30 min, Triton X-100 was added to a final concentration of 0.1%. Insoluble proteins were sedimented at 100,000*g* for 20 min at 4°C. The supernatants were used for coimmunoprecipitation. A rabbit antibody against mGluR1 (Upstate, Charlottesville, VA), mGluR5 (Upstate) or IP_3 receptors (Chemicon), or a rat antibody against Homer1b/c (Chemicon) was used to precipitate their targets with 50% protein A agarose/Sepharose bead slurry (GE Healthcare). Proteins were separated on Novex 4 to 12% gels and probed with the aforementioned antibodies or a rabbit antibody against protein phosphatase 1C (PP1γ1) from Chemicon. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence were used to detect proteins. Negative controls with antigen preabsorption were carried out for antibodies used in immunoprecipitation.

Oligonucleotides. The CREB antisense oligodeoxynucleotide (20-mer) has the sequence 5′-TGGTCATCTAGTCACCGGTG-3′. The sense oligonucleotide was used as a control: 5′-CACCGGTGACTAGATGACCA-3′. The sense and antisense oligonucleotides were synthesized with a 5′ thiol (SH). To increase the efficiency of

the oligonucleotide delivery into cultured neurons, the 5′ thiol of the oligonucleotide was linked to a vector peptide Penetratin1, a 16-amino acid peptide from the third helix of the *Antennapedia* homeodomain protein, by a disulfide bond, which is cleaved in the reducing environment of the cytoplasm, freeing the oligonucleotide (Troy et al., 1996). Three 3′ nucleotides were phosphorothioate-modified to minimize degradation and the cytotoxic effect. The delivery of the oligonucleotides into cells was aided with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Materials. Cocaine hydrochloride, Gö6983, phorbol 12-myristate 13-acetate, dopamine, SKF82958, eticlopride, 8-bromo-cAMP, and KN62 were purchased from Sigma (St. Louis, MO). Quinpirole and SCH23390 were purchased from Tocris Cookson Inc. (Ballwin, MO). H89 and Ro-31-8220 were purchased from Calbiochem (San Diego, CA).

Statistics. The results are presented as mean ± S.E.M. and were evaluated using a one- or two-way analysis of variance, as appropriate, followed by a Bonferroni (Dunn) comparison of groups using least-squares-adjusted means. Probability levels of <0.05 were considered statistically significant.

Results

Cocaine Specifically Increased Homer1a Expression. To evaluate the effect of cocaine on Homer1a expression, we measured changes in basal levels of Homer1a proteins in the striatum after a short-term cocaine injection in vivo. A low basal level of Homer1a was consistently seen in the caudate putamen (CPu; dorsal striatum) of saline-treated rats (Fig. 1A), indicating a low constitutive expression of this immediate early gene in striatal neurons. A single injection of cocaine (5, 10, or 20 mg/kg, i.p., 2 h before tissue collection) caused a dose-dependent stimulation of motor activities (increased levels of locomotion and stereotypical behaviors characterized by repetitive sniffing and biting) and elevation of Homer1a in the CPu (Fig. 1, A and B). Although cocaine at a low dose (5 mg/kg) did not alter behaviors and Homer1a expression, this drug at intermediate (10 mg/kg) and high (20 mg/kg) doses significantly increased motor activities and Homer1a levels. To determine the specificity of the cocaine effect among different isoforms of Homer proteins, experiments were expanded to the other two Homer family members (Homer1b/c and Homer2a/b) that are expressed by striatal neurons (Shiraishi et al., 2004). Unlike Homer1a, Homer1b/c and Homer2a/b showed a moderate level of constitutive expression in the CPu of saline-treated rats (Fig. 1A). However, these Homer proteins were not responsive to cocaine because their basal levels were not altered after cocaine injection at all doses surveyed (Fig. 1, A and B). These results indicate a specific effect of cocaine on Homer1a expression. To compare Homer1a to a well known inducible immediate early gene c-Fos in terms of responsiveness to cocaine, we found a similar dose-responsive increase in c-Fos protein levels in the same samples except that c-Fos seemed slightly more sensitive to a low dose (5 mg/kg) of cocaine (Fig. 1, A and B). The cocaine effect was not confined to the dorsal structure of the striatum because a similar dose-dependent increase in Homer1a levels was observed in the ventral [nucleus accumbens (NAc)] striatum (Fig. 1C). Furthermore, cocaine elevated Homer1a expression in the frontal cortex (Fig. 1D). In contrast, cocaine did not stimulate Homer1a

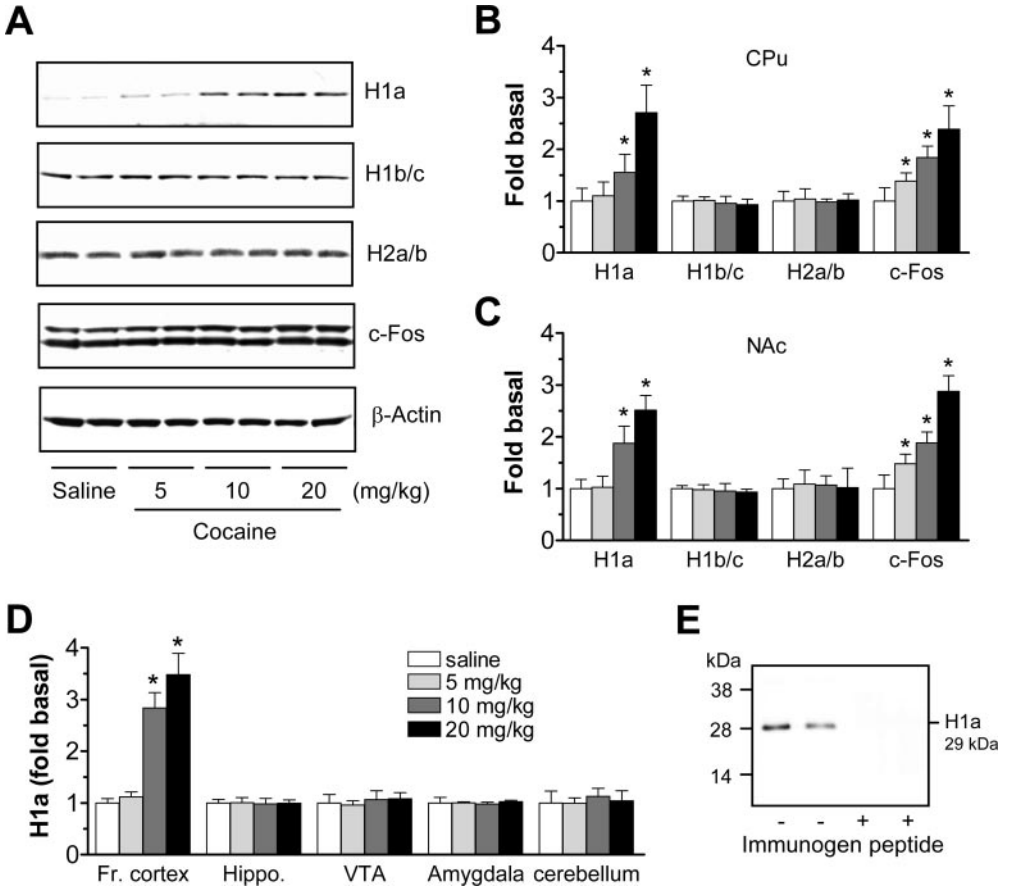

Fig. 1. Cocaine increases Homer1a expression in the rat striatum in vivo in a dose-dependent manner. A, immunoblots showing a dose-dependent increase in basal levels of Homer1a (H1a) and c-Fos, but not Homer1b/c (H1b/c), Homer2a/b (H2a/b), and β-actin, proteins in the CPu after cocaine injection. B, quantification of immunoblot results from A showing a dose-dependent increase in H1a and c-Fos, but not H1b/c and H2a/b, proteins in the CPu after cocaine injection. C, effects of cocaine on Homer and c-Fos protein expression in the NAc. D, effects of cocaine on H1a levels in the frontal cortex (Fr. cortex), the hippocampus (Hippo.), the ventral tegmental area (VTA), the amygdala, and the cerebellum. Note that cocaine increased H1a levels only in the frontal cortex, but not in other areas. E, evaluation of the selectivity of the anti-H1a antibody. A blot band (left two lanes) was produced in a molecular mass predicted for the size of H1a (29 kDa). Preabsorption of the antibody with an immunogen peptide [antibody/immunogen ratio, 1:3 (w/w)] prevented the detection of H1a band (right two lanes). A single dose of cocaine (5, 10, or 20 mg/kg) or saline was injected i.p. and rats were sacrificed 2 h after injection. The quantified data (B–D) are expressed as means ± S.E.M. ($n = 4–6$ per group). *, $p <$ 0.05 versus saline.

expression in several other brain areas, including the hippocampus, the ventral tegmental area, the amygdala, and the cerebellum. These results indicate a spatial specificity of the cocaine effect. The selectivity of antibody used in this study to detect Homer1a immunoreactivity was validated in a number of previous reports (Giuffrida et al., 2005; Stokely et al., 2006; Tappe et al., 2006) and also in a series of control experiments in this study. Omission of the anti-Homer1a antibody in an immunoblot produced no visible band. Addition of the antibody produced a band in a molecular mass (29 kDa) predicted for the size of Homer1a (Fig. 1E). Furthermore, no immunoblot band was displayed after preabsorption of the anti-Homer1a antibody with an immunogen peptide (Fig. 1E).

Increase in Homer1a Proteins: Temporal Characteristics. One key characteristic of inducible immediate early gene expression in response to acute psychostimulant administration is its rapid and transient nature of induction. To characterize the temporal property of Homer1a induction, alterations in Homer1a levels were investigated in a detailed time course study in rats treated with a short-term injection of saline or cocaine (20 mg/kg). Cocaine did not alter basal levels of Homer1a in either the CPu or the NAc at an early time point (0.5 h after injection; Fig. 2). A maximal increase in Homer1a levels was seen in the two striatal regions at either 2 or 3 h, whereas no significant change in a total amount of Homer1b/c proteins was seen at these two time points (Fig. 2). The cocaine-stimulated Homer1a expression showed a reversible nature of induction: the elevated levels of Homer1a declined at 6 h and returned to the normal level at 12 h (Fig. 2). Throughout the time course, Homer2a/b proteins exhibited no significant change in the striatum (data not shown). Together, the data here reveal a relatively rapid and reversible nature of Homer1a induction in response to acute cocaine administration, and this temporal pattern is parallel in the dorsal and ventral striatum.

Dopamine D1, but Not D2, Receptors Mediated the Cocaine Effect. Dopamine receptors are classified into two major groups: a D1 class (i.e., D1 and D5 subtypes) and a D2 class (i.e., D2, D3, and D4 subtypes). The D1 and D2 subtypes are enriched mostly in the mammalian striatum. It has been well documented that cocaine enhances synaptic dopamine availability by blocking the reuptake of released dopamine into the presynaptic terminal. Enhanced synaptic dopamine then activates D1 and D2 receptors to achieve its biological action. Because two dopamine receptors are heterogeneous in their pharmacology, cellular distribution, and linkage to second messenger systems, we set out to evaluate the relative importance of either subtype in mediating the cocaine effect on Homer1a expression. We first examined whether the two receptors are involved in determining the level of constitutive expression of Homer1a. We found that neither the D1 receptor antagonist SCH23390 (0.1 mg/kg, 2 h) nor the D2 receptor antagonist eticlopride (0.5 mg/kg, 2 h) had any effect on basal levels of Homer1a in the CPu and the NAc (Fig. 3A), although both the antagonists seemed to sedate animals with reduced motor activities. Thus, under normal conditions, endogenous dopaminergic tone on D1 or D2 receptors is not involved in determining basal levels of Homer1a expression. We then examined the role of D1 and D2 receptors in the Homer1a induction by cocaine. We found that cocaine (20 mg/kg, 2 h) produced a significantly less induction of Homer1a proteins in rats pretreated with SCH23390 (15 min before cocaine) in the CPu and the NAc compared with that seen in rats treated with cocaine alone (Fig. 3B). In contrast, in rats pretreated

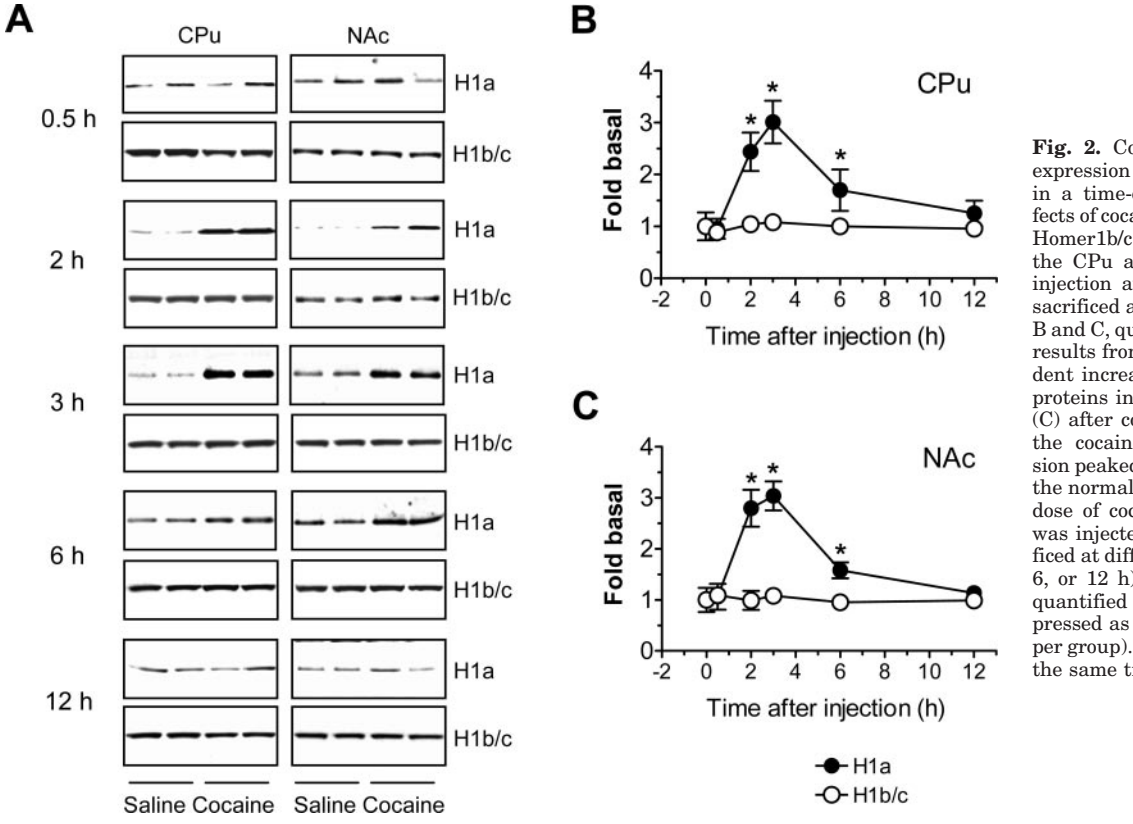

Fig. 2. Cocaine increases Homer1a expression in the rat striatum in vivo in a time-dependent manner. A, effects of cocaine on Homer1a (H1a) and Homer1b/c (H1b/c) protein levels in the CPu and the NAc after cocaine injection at a same dose. Rats were sacrificed at the different time points. B and C, quantification of immunoblot results from A showing a time-dependent increase in H1a, but not H1b/c, proteins in the CPu (B) and the NAc (C) after cocaine injection. Note that the cocaine-stimulated H1a expression peaked at 2 to 3 h and returned to the normal level within 12 h. A single dose of cocaine (20 mg/kg) or saline was injected i.p. and rats were sacrificed at different time points (0.5, 2, 3, 6, or 12 h) after drug injection. The quantified data (B and C) are expressed as means ± S.E.M. (n = 4–6 per group). *, $p < 0.05$ versus saline at the same time point.

with eticlopride (15 min before cocaine), cocaine increased Homer1a to an extent comparable with that induced by cocaine alone (Fig. 3B). To validate the selectivity and effectiveness of the two antagonists in blocking their targets, the effect of these antagonists on c-Fos induction was tested in the CPu. Cocaine has been shown to evoke c-Fos expression via a D1, but not D2, receptor-mediated mechanism (Moratalla et al., 1996). Consistent with this, the cocaine-stimulated c-Fos expression here was blocked by SCH23390, but not by eticlopride (Fig. 3C). In response to all drug treatments, Homer1b/c and β-actin did not display any changes (Fig. 3). From a behavioral perspective, both SCH23390 and eticlopride blocked cocaine-stimulated motor activities as a result of a well known D1/D2 synergy in processing the behavioral effect of cocaine. These results reveal a specific sensitivity of the cocaine effect to the D1, but not D2, receptor antagonist, indicating a specific role of D1 receptors in processing the cocaine effect.

We next tested whether selective D1 or D2 receptor activation with the selective agonist affects Homer1a expression. A single injection of the full D1 receptor agonist SKF82958 (2 mg/kg, i.p., 2 h) alone increased motor activities and basal levels of Homer1a, but not Homer1b/c, in the CPu (Fig. 4A) and the NAc (Fig. 4B). In contrast, the D2 receptor agonist quinpirole (2 mg/kg, i.p., 2 h) alone did not affect behaviors and Homer1a levels in the CPu (Fig. 4A) and the NAc (Fig. 4B). Coadministration of these two agonists produced an increase in Homer1a comparable to that observed after injection of SKF82958 alone (Fig. 4, A and B) except that the behavioral response to the two agonists seemed to be greater than that to SKF82958 alone. In addition, SKF82958 increased basal levels of c-Fos proteins in the CPu, whereas quinpirole had no effect (Fig. 4C). The SKF82958-stimulated Homer1a expression in the CPu was blocked by 0.1 mg/kg SCH23390 (Fig. 4D). These results provide further evidence in favor of the notion that the enhanced dopaminergic tone on D1 rather than D2 receptors triggers a stimulus-transcription coupling for Homer1a induction.

Identification of Protein Kinases Important for Homer1a Induction. After the clarification of a significant role of D1 receptors in the cocaine effect, we then set out to identify the protein kinases that transmit D1 receptor signals to Homer1a. The Gs-protein-coupled D1 receptor activates adenylate cyclase, followed by increased cAMP formation and activation of protein kinase A (PKA) in many cell lines and neurons (Neve et al., 2004). In striatal neurons, the cAMP/PKA system is among important pathways mediating inducible gene expression (Konradi et al., 2003). Thus, PKA was speculated to be a first candidate to play a significant role in dopamine-stimulated Homer1a expression. To clarify this speculation, a well characterized striatal neuronal culture model (Mao et al., 2005b; Yang et al., 2006) was used by taking advantage of this model that cell-permeable pharmacological tools for manipulating PKA activity can be readily and reliably applied to cultured neurons. Parallel with the in vivo finding, dopamine increased basal levels of Homer1a proteins in vitro in a concentration-dependent manner (Fig. 5A). This increase was blocked by the D1 receptor antagonist SCH23390 (5 μM), but not the D2 receptor antagonist eticlopride (10 μM), indicating a specific role of D1 receptors in mediating this event. In an attempt to evaluate the PKA involvement, we found that the PKA inhibitor H89 (10 μM) attenuated dopamine-stimulated Homer1a expression (Fig. 5B). Moreover, the PKA activator 8-bromo-cAMP (1 mM)

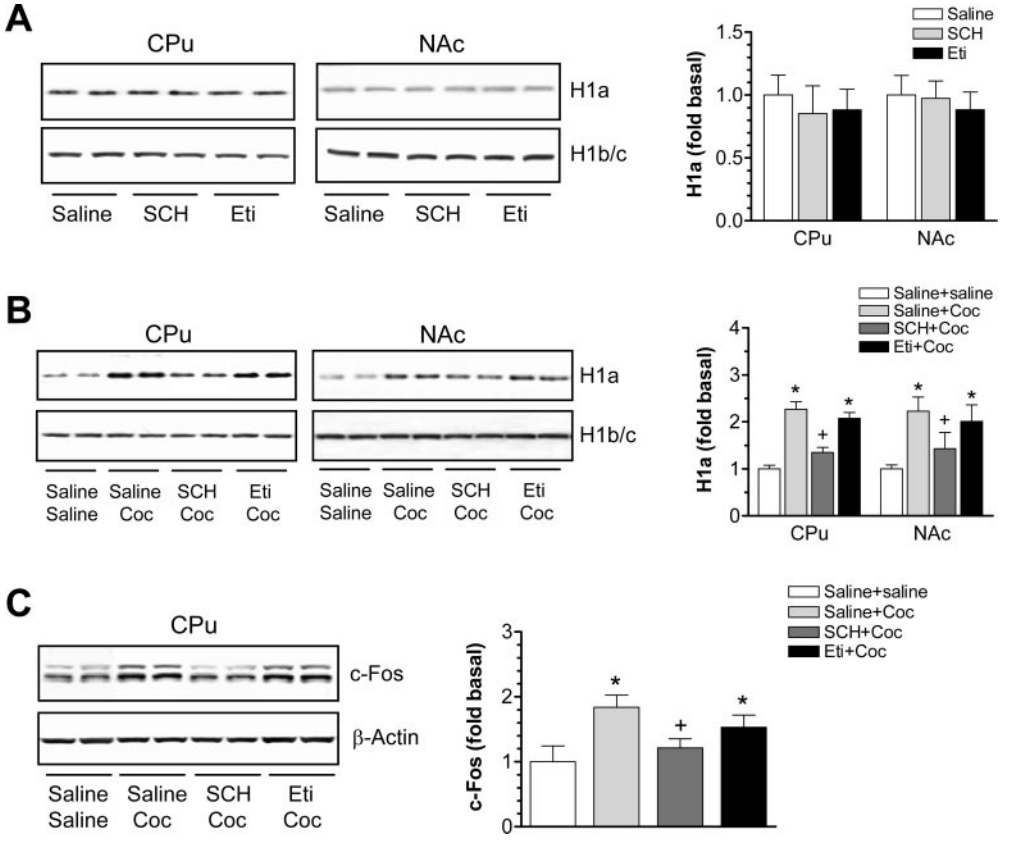

Fig. 3. Effects of a D1 or D2 receptor antagonist on basal and cocaine (Coc)-stimulated Homer1a (H1a) expression in the CPu and the NAc. A, effects of SCH23390 (SCH) or eticlopride (Eti) on basal levels of H1a and Homer1b/c (H1b/c). Neither SCH23390 nor eticlopride altered basal levels of the two proteins. Rats were given a single dose of SCH23390 (0.1 mg/kg, i.p.), eticlopride (0.5 mg/kg, i.p.), or saline and were sacrificed 2 h after drug injection. B and C, effects of SCH23390 or eticlopride on the cocaine-induced increases in H1a in the two striatal structures (B) and increases in c-Fos proteins in the CPu (C). SCH23390 (0.1 mg/kg, i.p.) or eticlopride (0.5 mg/kg, i.p.) was injected 15 min before an i.p. injection of saline or cocaine (20 mg/kg), and rats were sacrificed 2 h after cocaine injection. Note that SCH23390, but not eticlopride, reversed the effect of cocaine on H1a (B) and c-Fos (C). Representative immunoblots are shown left to the quantified data of H1a or c-Fos. Data are presented as means ± S.E.M. from four to six experiments. *, $p < 0.05$ versus saline; +, $p < 0.05$ versus saline + cocaine.

mimicked the effect of dopamine in stimulating Homer1a expression (Fig. 5B). Thus, PKA activation is required for the D1 receptor-dependent up-regulation of Homer1a expression. In contrast to PKA, PKC seems to be insignificant. The PKC inhibitor Ro-31-8220 (1 μM; Fig. 5C) or Gö6983 (1 μM) did not affect dopamine-stimulated Homer1a expression despite the fact that these inhibitors at 1 μM blocked the phosphorylation of extracellular signal-regulated protein kinases induced by a PKC activator phorbol 12-myristate 13-acetate in a same culture model (Yang et al., 2004). Finally, we tested whether Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) participate in this event. We found that the CaMK inhibitor KN62 (20 μM) reduced the level of dopamine-stimulated Homer1a expression (Fig. 5D). Thus, the CaMK, like PKA, is among essential kinases important for the dopamine effect. In all drug treatments, Homer1b/c exhibited no significant changes in their protein levels (data not shown).

CREB Facilitated Homer1a Transcription. The transcription factor CREB is densely expressed in the nucleus of brain cells and is activated through phosphorylation at serine 133 by cAMP and Ca^{2+} signals. pCREB has been shown to facilitate expression of many inducible immediate early genes, including c-Fos (Lonze and Ginty, 2002; Carlezon et al., 2005). In this study, we then wanted to determine the role of active CREB in mediating Homer1a induction. Dopamine stimulation of D1 receptors has been well documented to induce a rapid and transient increase in CREB phosphorylation in striatal neurons both

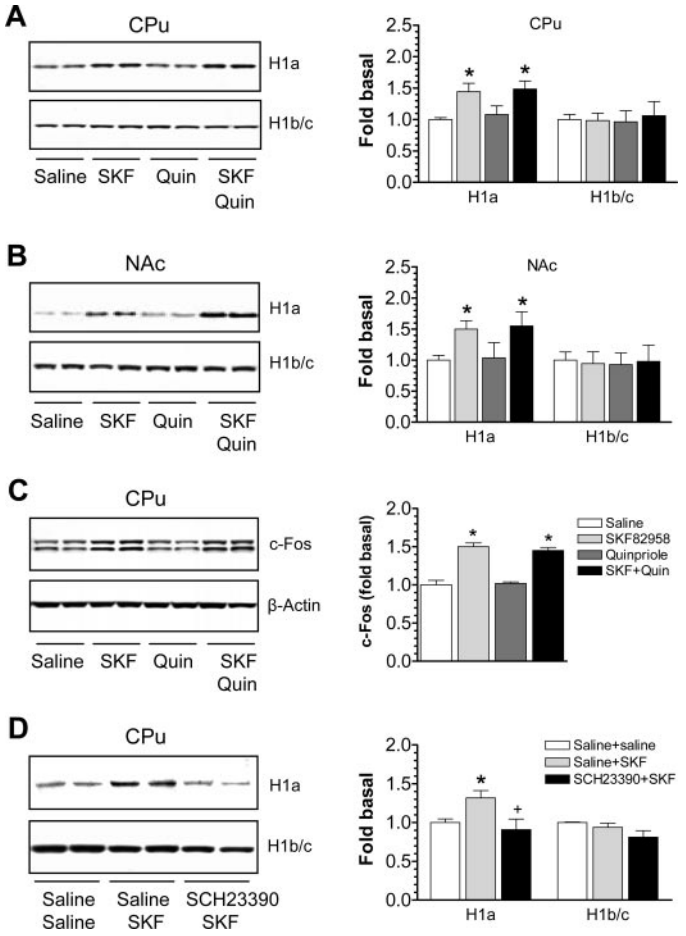

Fig. 4. Effects of a D1 or D2 receptor agonist on basal Homer1a (H1a) and Homer1b/c (H1b/c) expression in the CPu and the NAc. A and B, effects of SKF82958 (SKF) or quinpirole (Quin) on basal levels of H1a and Homer1b/c (H1b/c) in the CPu (A) and the NAc (B). Note that SKF82958 increased H1a expression in the two areas, whereas quinpirole did not affect it. C, effects of SKF82958 or quinpirole on basal levels of c-Fos and β-actin in the CPu. SKF82958 increased, whereas quinpirole unaffected, c-Fos expression in this region. D, effects of SCH23390 on SKF82958-stimulated H1a expression in the CPu. Representative immunoblots are shown left to the quantified data of H1a and H1b/c (A, B, and D) and c-Fos (C). Rats were given a single dose of SKF82958 (2 mg/kg, i.p.) or quinpirole (2 mg/kg, i.p.) or a combination of the two agonists and were sacrificed 2 h after drug injection (A–C). In experiments with SCH23390 (D), saline or SCH23390 (0.1 mg/kg, i.p.) was given 15 min before saline or SKF82958 (2 mg/kg, i.p.), and rats were sacrificed 2 h after final drug injection. Data are presented as means ± S.E.M. from three to five experiments. *, $p < 0.05$ versus saline.

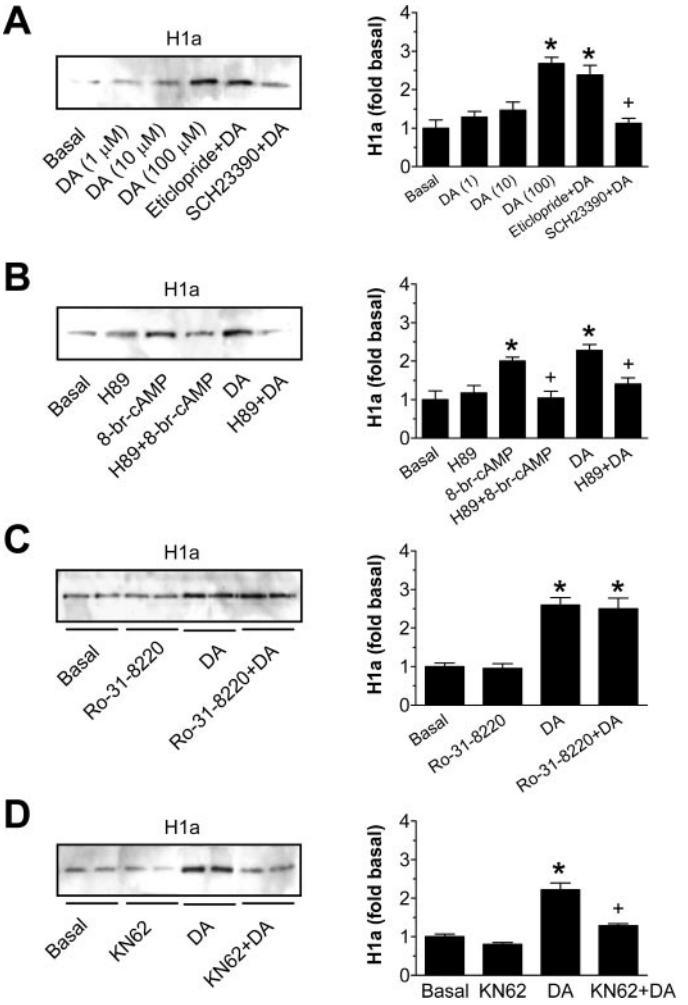

Fig. 5. Effects of dopamine (DA) receptor antagonists and protein kinase inhibitors on dopamine-stimulated Homer1a (H1a) expression in cultured rat striatal neurons. A, dopamine concentration-dependently increased basal levels of H1a proteins, which was blocked by the D1 receptor antagonist SCH23390 but not by the D2 receptor antagonist eticlopride. Dopamine (1, 10, or 100 μM) was incubated for 1.5 h. SCH23390 (5 μM) or eticlopride (10 μM) was incubated 15 min before and during a 1.5-h incubation of dopamine (100 μM). B, the PKA inhibitor H89 blocked H1a expression induced by either 8-bromo-cAMP or dopamine. C, the PKC inhibitor Ro-31-8220 did not affect dopamine-stimulated H1a expression. D, the CaMK inhibitor KN62 attenuated dopamine-stimulated H1a expression. H89 (10 μM), Ro-31-8220 (1 μM), or KN62 (20 μM) was incubated 15 min before and during a 1.5-h incubation of 8-bromo-cAMP (1 mM) or dopamine (100 μM). Representative immunoblots are shown left of the quantified data presented as means ± S.E.M. (n = 3–4). *, $p < 0.05$ versus basal levels; +, $p < 0.05$ versus 100 μM dopamine.

in vivo and in vitro (Liu and Graybiel, 1998). Likewise, dopamine (100 μM, 15 min) elevated levels of pCREB, but not CREB, in this striatal culture preparation (Fig. 6, B and C), which was sensitive only to 10 μM SCH23390 (data not shown). To determine the role of active CREB in facilitating Homer1a transcription, an antisense approach was used to selectively reduce nuclear levels of CREB. As shown in Fig. 6A, sense controls (0.5 μM) had no effect on, whereas antisense oligonucleotides (0.5 μM) significantly reduced, CREB levels. At the same time, dopamine (100 μM, 15 min) induced a significantly less increase in pCREB levels (Fig. 6B). In cultures treated with antisense but not sense oligonucleotides (0.5 μM), dopamine (100 μM, 1.5 h) induced an insignificant increase in Homer1a levels (Fig. 6D). These results suggest that active CREB is a critical transcription factor responsive to D1 receptor stimulation for the facilitation of Homer1a transcription.

Homer1a Disrupted the Formation of mGluR1/5-Homer1b/c-IP$_3$ Receptor Complexes. Once induced, non–cross-linking Homer1a was thought to compete with the cross-linking form of Homer for their N-terminal EVH1 binding partners, and thereby reduce the adhesion capacity of the cross-linking Homer (Xiao et al., 2000). In particular, Homer1b/c links surface membrane-bound group I mGluRs to cytoplasmic membrane-bound type I IP$_3$ receptors to form an mGluR1/5-Homer1b/c-IP$_3$ complex, rendering a rapid and effective transmission of mGluR1/5 signals to IP$_3$ receptors for evoking intracellular Ca^{2+} release (Tu et al., 1998; Fig. 7A). To determine whether induced Homer1a disrupts Homer1b/c's role in bridging mGluR1/5 with IP$_3$ receptors, a coimmunoprecipitation method was used to examine dynamic changes in the formation of mGluR1/5-Homer1b/c-IP$_3$ complexes in response to cocaine stimulation in the striatum in vivo. We found that an amount of Homer1a proteins was increased in mGluR5 precipitates in rats treated with cocaine (20 mg/kg, i.p., 2 h; Fig. 7, B and C). In contrast, an amount of cross-linking Homer proteins (Homer1b/c and Homer2a/b) and IP$_3$ receptors in mGluR5 precipitates was decreased after cocaine stimulation (Fig. 7, B and C). Similar changes in levels of Homer proteins and IP$_3$ receptors were observed in mGluR1 precipitates (Fig. 7D). In a reverse coimmunoprecipitation, mGluR5 and IP$_3$ receptors were found to be decreased in their protein contents in Homer1b/c precipitates after cocaine administration (Fig. 7E). Likewise, mGluR5 and Homer1b/c proteins were reduced, whereas Homer1a was increased, in IP$_3$ receptor precipitates (Fig. 7F). Because cocaine treatment did not alter a total level of mGluR5, Homer1b/c, or IP$_3$ receptor proteins (Fig. 7, B–F), the changes in these proteins in coimmunoprecipitation results reflect a dynamic change in their ratios in the complex. In other words, endogenously induced Homer1a proteins by cocaine replace Homer1b/c in forming complexes with group I mGluRs and disrupt the linkage of group I mGluRs to their downstream signaling molecules. Homer1a seems to indistinguishably disrupt both binding sites (Homer1b/c with mGluR1/5 and Homer1b/c with IP$_3$ receptors) because 1) increased Homer1a and reduced Homer1b/c proteins were observed in both mGluR5 and IP$_3$ receptor precipitates, and 2) in Homer1b/c precipitates, both mGluR5 and IP$_3$ receptors were reduced. To control the selectivity of Homer1a action, changes in the binding of mGluR5 with another interacting protein were examined in the same samples. Like Homer proteins, PP1γ1 directly binds the C terminus of group I mGluRs, but at a different site (Croci et al., 2003). However, PP1γ1 binding to mGluR5 was not altered by cocaine, as evidenced by a lack of change in PP1γ1 protein levels in mGluR5 precipitates after cocaine stimulation (Fig. 7B).

D1 Receptors Mediated the Disrupting Effect of Homer1a. D1 receptors specifically mediate cocaine stimulation of Homer1a expression (above). Thus, it is anticipated that blockade of D1 receptors could also prevent Homer1a's function in disrupting the association of group I mGluR with

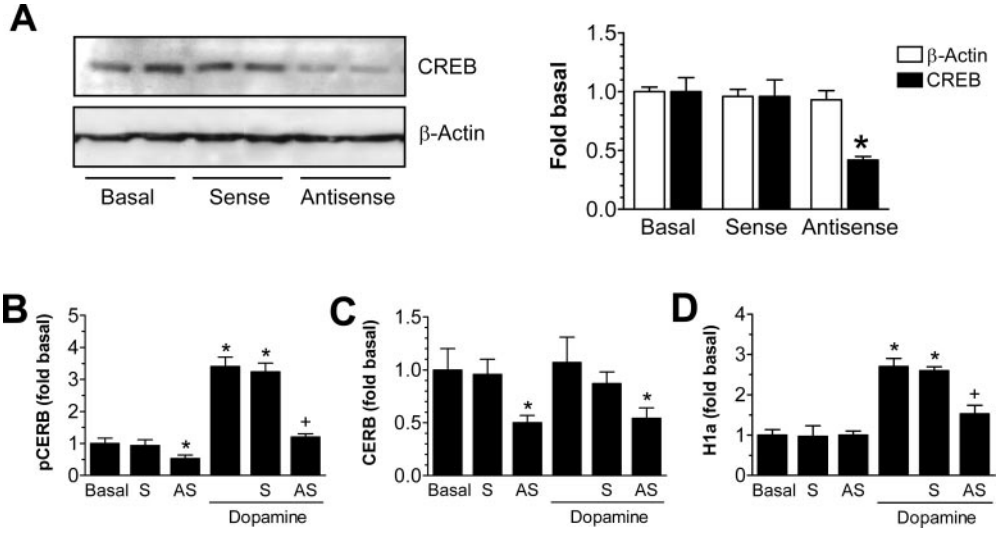

Fig. 6. Effects of CREB antisense oligonucleotides on basal levels of CREB and dopamine-stimulated CREB phosphorylation and Homer1a (H1a) expression in cultured rat striatal neurons. A, representative immunoblots illustrating that the CREB antisense oligonucleotide reduced cellular levels of CREB. Quantified data of β-actin and CREB proteins are shown right to immunoblots (means ± S.E.M., n = 3–5 per group). Antisense or sense oligonucleotides were incubated at 0.5 μM for 4 to 6 h and then washed off. Amounts of CREB and β-actin proteins were assayed 16 to 18 h after addition of oligonucleotides. B–D, effects of dopamine on pCERB (B), CREB (C), and H1a (D) protein levels in the presence of sense (S) or antisense (AS) CREB oligonucleotides. Sense or antisense oligonucleotides were incubated at 0.5 μM for 4 to 6 h, and the effect of dopamine (100 μM, 15 min for pCREB and CREB and 1.5 h for H1a) on pCREB, CREB, and H1a levels was assayed 16 to 18 h later. Data are expressed as means ± S.E.M. (n = 5–6). *, $p < 0.05$ versus basal levels; +, $p < 0.05$ versus sense.

their binding partners. Indeed, in this study, we observed that blockade of D1 or D2 receptors with SCH23390 (0.1 mg/kg, i.p., 2 h) or eticlopride (0.5 mg/kg, i.p., 2 h), respectively, did not alter basal levels of Homer1a and Homer1b/c in striatal mGluR5 precipitates (Fig. 8A). This indicates a minimal dopaminergic tone on either D1 or D2 receptors controlling the constitutive formation of mGluR5-Homer1b/c complexes. However, SCH23390 (15 min before cocaine) reversed an increase in an amount of Homer1a and a decrease in an amount of Homer1b/c in mGluR5 precipitates induced by cocaine (15 mg/kg, i.p., 2 h) (Fig. 8B). Eticlopride (15 min before cocaine), on the contrary, did not produce the same effect (Fig. 8B). These results suggest a D1 receptor-sensitive nature of the Homer1a action and support a model in which cocaine stimulates D1 receptors to increase Homer1a proteins, which in turn antagonize the adhesion capacity of cross-linking Homer proteins.

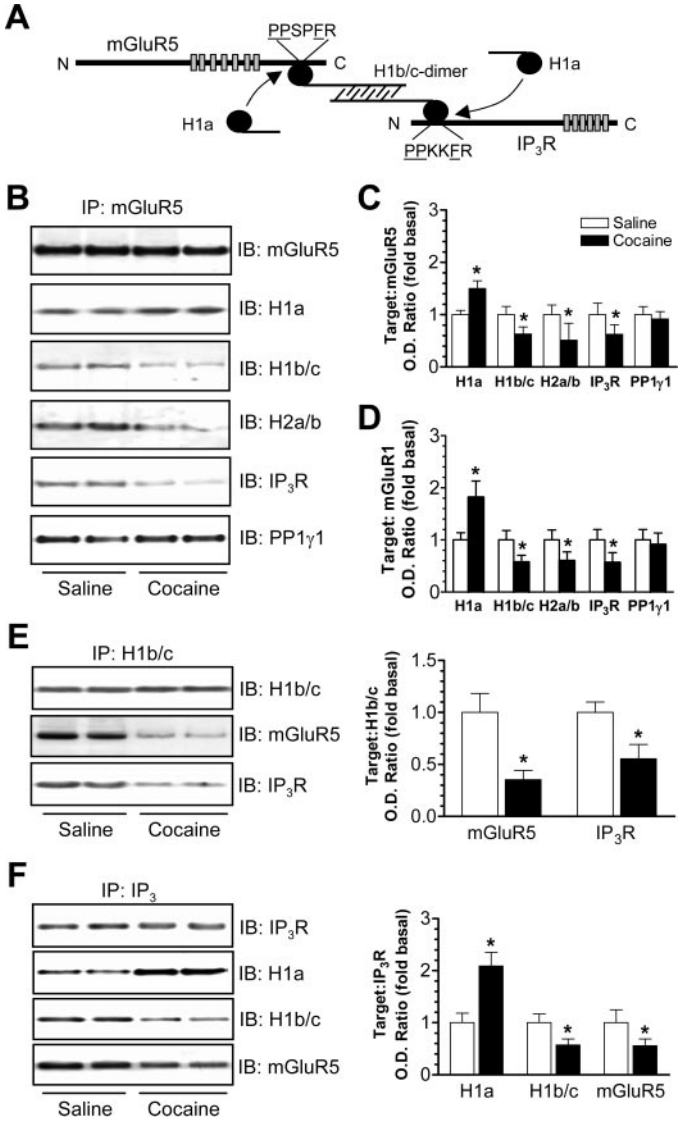

Fig. 7. Changes in the formation of mGluR5-Homer-IP_3 receptor complexes in the rat striatum after cocaine administration. A, a schematic diagram illustrating a Homer1b/c (H1b/c)-mediated linkage of mGluR1/5 and type I IP_3 receptors (IP_3R) through a motif of *PP*xx*F* (Tu et al., 1998). Due to the same structure of Homer1a (H1a) and H1b/c at their N-terminal regions, H1a is able to competitively antagonize H1b/c's binding to mGluR1/5 and IP_3 receptors. B, representative immunoblots (IB) illustrating increased H1a proteins, decreased H1b/c, Homer2a/b (H2a/b), and IP_3R proteins, and unaltered PP1γ1 proteins in mGluR5 immunoprecipitates (IP) in rats treated with cocaine. C, the quantified data from experiments in the (B). D, cocaine also increased H1a proteins and decreased H1b/c, H2a/b, and IP_3R proteins in mGluR1 immunoprecipitates. Data in C and D are expressed as means ± S.E.M. (n = 5–6). E, effects of cocaine on an amount of mGluR5 or IP_3R proteins in H1b/c immunoprecipitates. F, effects of cocaine on an amount of H1a, H1b/c, and mGluR5 in IP_3R immunoprecipitates. Representative immunoblots are shown left of the quantified data presented as means ± S.E.M. (n = 5–6). Rats were received a single i.p. injection of saline or cocaine (20 mg/kg) and were sacrificed 2 h after injection. ∗, $p < 0.05$ versus saline.

Discussion

This study investigated the regulation of Homer1a protein expression in striatal neurons in vivo and in vitro by dopamine stimulation with cocaine. We found that acute injection of cocaine consistently induced a rapid and transient increase in Homer1a expression in the dorsal and ventral striatum. The increase was blocked by a D1, but not a D2, receptor antagonist, indicating a specific role played by D1 receptors in mediating the effect of cocaine. Furthermore, in the effort to identify the protein kinases important for this event, we found that both PKA and CaMKs, although not PKC, were involved in transmitting D1 receptor signals to Homer1a induction. At the transcriptional level, CREB was found to be a key transcription factor in facilitating Homer1a transcription. Finally, induced non–cross-linking Homer1a showed the ability to disrupt the capacity of cross-linking Homer proteins and reduced the linkage of group I mGluRs with their downstream signaling molecules. These results reveal a positive regulation of Homer1a protein expression in the rat striatum by psychostimulant exposure.

Early studies have found that a single dose or "binge" administration of cocaine increased *Homer1a* transcripts in the striatum (Brakeman et al., 1997; Yuferov et al., 2003). This study confirms that the increase in *Homer1a* transcripts can be translated to a substantial increase in its protein content. The induction of Homer1a proteins was characterized by the following properties. First, low basal levels of Homer1a protein expression were present throughout the brain. Cocaine injection can therefore readily induce a dose-dependent increase in Homer1a expression. Second, the Homer1a induction was time-dependent. A rapid and transient increase in Homer1a levels was seen after cocaine administration, similar to a typical temporal pattern of many immediate early genes in their inducible expression in response to acute injection of cocaine or other dopamine stimulants (Simpson et al., 1995; Wang et al., 1995). Homer1b/c can be noticeably increased after a single dose of cocaine at a 24-h timepoint (Fourgeaud et al., 2004). Finally, Homer1a showed a region-specific response to cocaine stimulation. Among the brain regions surveyed, the two structures in the striatum (the CPu and the NAc) and the frontal cortex exhibited a profound increase in Homer1a expression after cocaine administration.

At the receptor level, the D1, but not D2, subtype seems to mediate the induction of Homer1a. This was supported by the data from a series of pharmacological studies: 1) the D1 receptor antagonist SCH23390, but not the D2 receptor antagonist eticlopride, blocked the Homer1a induction, 2) the full D1 receptor agonist SKF82958, but not the D2 receptor agonist quinpirole, mimicked the stimulative effect of cocaine

on Homer1a expression, and 3) coadministration of the D1 and D2 receptor agonists produced the same result as that induced by the D1 receptor agonist alone. D1 and D2 receptors are believed to be enriched in the different types of projection neurons in the striatum: D1 receptors in striatonigral neurons and D2 receptors in striatopallidal neurons (Gerfen, 2000). Thus, the Homer1a induction preferentially occurred in one specific subpopulation of output neurons (striatonigral neurons). This selective induction pattern in striatonigral neurons is consistent with other immediate early genes. For instance, the D1-dependent induction c-Fos was predominantly shown in striatonigral neurons (Moratalla et al., 1996).

At the protein kinase level, three kinases were evaluated for their contributions. PKA was the first to be considered as a prime kinase because it represents a major downstream transducer. In support of the role of PKA, the PKA activator triggers *Homer1a* mRNA expression in cultured sympathetic, cortical, and hippocampal neurons (Girard et al., 2004) and triggered Homer protein expression in striatal neurons (this study). Moreover, the PKA inhibitor blocked dopamine-stimulated Homer1a expression. Thus, PKA contributes to transmitting D1 receptor signals to Homer1a. Similar to PKA, PKC positively regulates Homer1a protein expression in cultured hippocampal neurons (Kato et al., 2001). However, PKC may not be an important kinase for mediating D1 receptor signals because the inhibition of this kinase did not effect dopamine-stimulated Homer1a expression. CaMKs are abundantly expressed at the postsynaptic density of glutamatergic synapses and have been documented to advance synaptic signals to the nucleus to facilitate immediate early gene expression (Lonze and Ginty, 2002; Carlezon et al., 2005). In this study, we found that the CaMK inhibitor KN62 attenuated dopamine-stimulated Homer1a expression. This supports the notion that CaMKs play an important role in transmitting D1 receptor signals to Homer1a.

The transcription factor(s) that possibly mediate inducible Homer1a expression at the transcriptional level have not been investigated experimentally until this study. Sequence analysis of the Homer1a promoter region has discovered multiple cAMP response element-binding sites specific for the transcription factor CREB (Bottai et al., 2002). Thus, CREB could be a transcription factor mediating the phasic Homer1a expression. Phosphorylation of CREB is a needed chemical process for activating this factor to facilitate D1 receptor-dependent gene expression in striatal neurons (Simpson et al., 1995; Konradi et al., 2003). Cytosolic second messengers (Ca^{2+} ions, PKA, and CaMKs) also phosphorylate CREB to facilitate target gene transcription in heterologous systems and cultured neurons (Lonze and Ginty, 2002; Carlezon et al., 2005). The results obtained from this study showed that selective knock-down of cellular CREB proteins through an antisense approach attenuated the induction of Homer1a. This supports the participation of CREB in facilitating Homer1a transcription after cocaine stimulation of D1 receptors and associated pathways.

Among all long forms of Homer proteins, Homer1b/c and Homer2a/b are constitutively expressed, whereas Homer3 is lacking in striatal neurons in vivo (Shiraishi et al., 2004) and in cultures (Ango et al., 2000). The C-terminal coiled-coil domain of these Homer proteins renders a cross-linking capability and promotes the formation of homomeric multivalent complexes in forms of dimers or tetramers. The highly conserved N-terminal EVH1 domain forms heteromeric binding to a proline-rich motif in the C terminus of group I mGluRs as well as to many other synaptic proteins. In the postsynaptic density of excitatory syn-

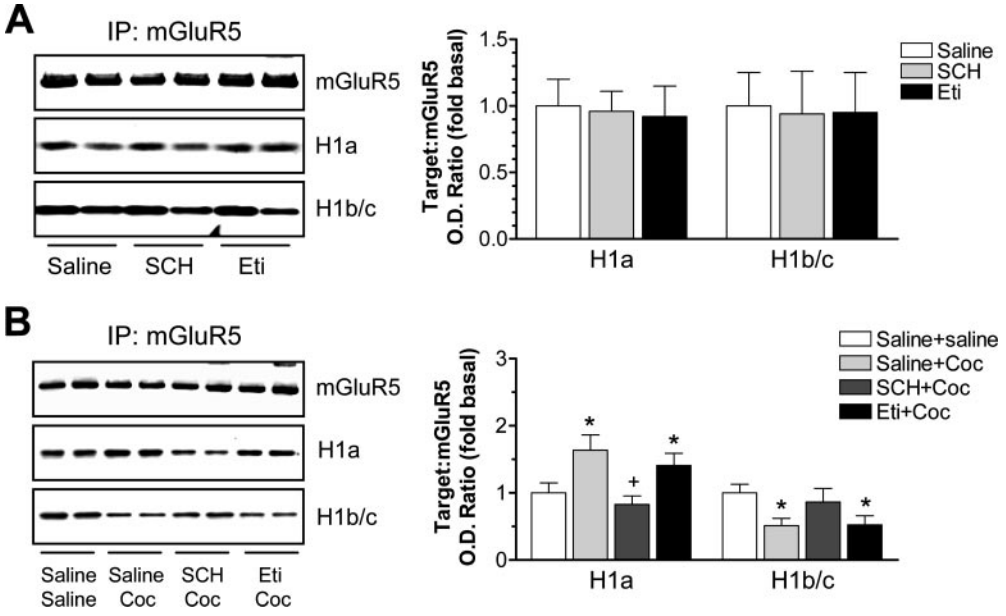

Fig. 8. Effects of a D1 or D2 receptor antagonist on the formation of mGluR5-Homer-IP_3 receptor complexes in the rat striatum. A, effects of SCH23390 (SCH) or eticlopride (Eti) on the constitutive formation of mGluR5-Homer complexes. Neither SCH23390 nor eticlopride altered Homer1a (H1a) and Homer1b/c (H1b/c) immunoblots (IB) performed from mGluR5 immunoprecipitates (IP). Rats were given a single dose of SCH23390 (0.1 mg/kg, i.p.) or eticlopride (0.5 mg/kg, i.p.) and were sacrificed 2 h after drug injection. B, effects of SCH23390 or eticlopride on the cocaine-induced changes in the formation of mGluR5-Homer complexes. SCH23390 (0.1 mg/kg, i.p.) or eticlopride (0.5 mg/kg, i.p.) was injected 15 min before an i.p. injection of saline or cocaine (20 mg/kg) and rats were sacrificed 2 h after cocaine injection. Representative immunoblots are shown left to the quantified data. Data are presented as means ± S.E.M. from four to six experiments. *, $p < 0.05$ versus saline; +, $p < 0.05$ versus saline + cocaine.

apses, Homer1b/c proteins are enriched in both the central and lateral region (Xiao et al., 1998), whereas mGluR1/5 subtypes are present densely at the lateral margin (Nusser et al., 1994; Lujan et al., 1997). Such an ultrastructural arrangement allows Homer1b/c to form a physical tether linking membrane mGluRs with other submembranous proteins. In fact, the cross-linking Homer proteins were found to bind the proline-rich motif in IP_3 receptors (Tu et al., 1998). Through this binding, Homer links the submembranous endoplasmic reticulum (i.e., Ca^{2+} stores bearing IP_3 receptors) in close proximity to mGluRs, rendering rapid transmission of mGluR signals to Ca^{2+} release. In contrast to cross-linking Homer, non–cross-linking Homer1a has been thought to be an intrinsic regulatory peptide that competes with cross-linking Homer proteins for the same binding partners and, as a result, disrupts the organizing capability of cross-linking Homer proteins (Xiao et al., 2000). The results from this study for the first time provide in vivo evidence for this notion. An elevated Homer1a binding to mGluRs or IP_3 receptors was displayed because an amount of Homer1a in mGluR5 or IP_3 receptor precipitates was increased after cocaine administration. At the same time, an amount of Homer1b/c in mGluR5 or IP_3 receptor precipitates was decreased. These correlated, opposite events indicate a possibility that Homer1a competes with Homer1b/c for the same N-terminal binding partners. Because this competition uncouples group I mGluRs from IP_3 receptors, increased Homer1a expression may seem to inhibit group I mGluR signaling. Glutamatergic tone on group I mGluRs is believed to be enhanced as a result of increased glutamate release in the striatum after cocaine or amphetamine stimulation (Reid et al., 1997; Del Arco et al., 1999). Homer1a is therefore involved in the establishment of a negative feedback mechanism to restrain the responsiveness of group I mGluRs to subsequent or sustained stimulation. It is currently unknown whether and how Homer1a could respond, and function in regulating behavioral sensitivity, to repeated cocaine administration. These issues remain as intriguing topics for future studies.

Acknowledgments

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