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Integrin-Linked Kinase Is Involved in Cocaine Sensitization by Regulating PSD-95 and Synapsin I Expression and GluR1 Ser^{845} Phosphorylation

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Abstract

Our recent studies have demonstrated that integrin-linked kinase (ILK) is involved in the induction and maintenance of cocaine behavioral sensitization and chronic cocaine-induced neural plasticity in the nucleus accumbens (NAc) core. In the present study, we used ILK silencing to investigate how ILK may influence cocaine-induced neural plasticity. Adeno-associated virus carrying a small interfering RNA-ILK cassette under the control of an inducible Tet-On system was injected into the NAc core of Sprague–Dawley rats. Induced silencing was established during repeated cocaine injections (sensitization induction period) or between withdrawal days 9 and 22 (sensitization maintenance period). Under both paradigms, established cocaine sensitization under non-silenced conditions was associated with enhanced PSD-95 and synapsin I protein expression as well as enhanced Ser^{845} phosphorylation of the GluR1 subunit on withdrawal day. Silencing ILK expression under both paradigms prevented or reversed these changes. Importantly, ILK appears to form a complex with PSD-95 and synapsin I because it co-immunoprecipitated with

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each of these proteins. Together, these data suggest that ILK exerts pleiotropic actions by regulating pre- and postsynaptic neural plasticities within the NAc core in response to repeated cocaine exposure.

Keywords

Integrin-linked kinase; Cocaine; Behavioral sensitization; PSD-95; SiRNA; GluR1; Synapsin I; Synaptic plasticity

Introduction

In laboratory animals, cocaine behavioral sensitization and self-administration have long been considered models of human cocaine abuse (Wolf 2002; Davidson et al. 2002). Repeated administration of cocaine results in progressive and persistent augmentation in locomotor activity (Carlezon and Nestler 2002; Wolf 2002; Chen et al. 2008a). Behavioral sensitization is accompanied by structural changes in dendrites in the nucleus accumbens (NAc) and prefrontal cortex (Kolb et al. 2003; Robinson and Kolb 1999; Robinson et al. 2001; Norrholm et al. 2003; Robinson and Kolb 2004). These structural changes in the NAc are observed primarily in the distal dendrites of medium-sized spiny neurons (Robinson and Kolb 1997, 1999, 2004; Li et al. 2003), the primary loci of synapses. Our recent study using integrin-linked kinase (ILK) silencing indicates that ILK plays a critical role in cocaine sensitization by modulating dendritic changes in the NAc core (Chen et al. 2008a). While ILK has been investigated primarily for its contributions to oncogenic transformation and cell survival (Dedhar 2000; Zhang et al. 2003), this kinase may also participate in the regulation of neurite outgrowth and axonal myelination (Ishii et al. 2003; Chun et al. 2003). Since neurite outgrowth requires cytoskeletal re-arrangements, ILK may participate in regulating neural plasticities associated with cocaine sensitization.

Recent studies have demonstrated that dendritic spine morphology is highly dependent upon the dynamic organization of the postsynaptic density (PSD; Sekino et al. 2007), an important structural element located just beneath the postsynaptic membrane of dendritic spines (Kennedy 1997). PSD-95 is a prominent scaffolding protein in the PSD, and this protein binds many other signaling molecules and scaffolding proteins (Kim and Sheng 2004). Importantly, PSD-95 can regulate neuronal synaptic plasticity (Mulholland and Chandler 2007). For example, its PDZ domains can bind to synaptic AMPA receptors (AMPARs) and this protein–protein interaction appears to be a critical event mediating long-term synaptic changes such as long-term potentiation and depression (Chen et al. 2000; Ehrlich and Malinow 2004). PSD-95 has been identified also as an important regulator of early neurite outgrowth and dendritic pattering (Vessey and Karra 2007). We have recently demonstrated that chronic cocaine administration increases Ser^{845} phosphorylation of the GluR1 subunit of AMPARs in the NAc core, and this alteration parallels changes in ILK expression (Zhang et al. 2007; Chen et al. 2008a). Thus, changes in the levels of PSD-95 and phospho-GluR1 may reflect the role that ILK plays in regulating these postsynaptic proteins as well as the consequent changes in behavior due to chronic cocaine exposure.

Compared to an understanding of postsynaptic events in psychostimulant addiction, considerably less is known about the presynaptic molecular mechanisms that contribute to this condition. At presynaptic terminals, synapsin I has been shown to bind actin filaments and regulate the organization of the actin network (Jovanovic et al. 2001; Yamagata 2003; Yamamoto et al. 2003; Johnson and Ouimet 2006). In the present study, we investigated the relationship of ILK to GluR1, PSD-95, and synapsin I. Our findings suggest that ILK may

be involved in cocaine sensitization by regulating both presynaptic and postsynaptic plasticities within the NAc core.

Methods and Materials

Experimental Animals

Male Sprague–Dawley rats (Charles River Laboratories, Raleigh, NC, USA), initially weighing 125 to 150 g, were housed in pairs in the vivarium with a 12-h light/dark cycle (0700 to 1900 hours) for 1 week prior to experiments. Animals were housed individually after surgery and were given free access to food and water in a humidity- and temperature-controlled room. All experiments were conducted with an approved protocol from the Duke University Institutional Animal Care and Use Committee and in accordance with NIH guidelines.

rAAV Vectors and Microinjection

To produce the ILK-silencing recombinant adeno-associated virus (rAAV), each of the four preselected ILK small interfering RNA (siRNA) sequences was placed under the control of a tetracycline inducible H1 polymerase III promoter; expression of the Tet-On cassette was under the control of rat *synI* gene promoter (Chen et al. 2008a, b). The specificity and efficacy of these four siRNAs have been verified previously (Cordes 2004; Kumar et al. 2004; Tan et al. 2004; Nho et al. 2005; Chen et al. 2008a, b). A rAAV expressing green fluorescent protein (rAAV-GFP) was used as a control. Four microliters of virus was injected bilaterally into the NAc core at +1.5 mm (anterior/posterior— AP), 6.8 mm (dorsal/ventral—DV), and ±1.5 mm (lateral/ medial—LM) as described (Chen et al. 2008b). Importantly, we have already shown that the rAAV is limited to expression to the NAc core (Chen et al. 2008a).

Treatments and Behavioral Assessments

The overall experimental design has been previously described (Chen et al. 2008a).

Experiment 1—This experiment was designed to determine whether silencing of ILK during the withdrawal period could affect subsequent neurobiological responses to cocaine challenge. Rats were randomly divided into two groups each (n=16 rats/group) that received bilateral microinjections of rAAV-GFP (1.2×10^{10} viral particles) or rAAV-siILK (1.2×10^{10} viral particles) into the NAc core (AP +1.5 mm, DV 6.8 mm, LM ±1.5 mm). Following 8 days of postoperative recovery, nine rats which had been previously injected with rAAV-GFP (C-GFP) or rAAV-siILK (C-siILK) received 40 mg/kg/day cocaine (s.c.) for seven consecutive days, whereas the remaining seven rats in each group (S-GFP or S-siILK) were given saline. All rats were subsequently withdrawn for 8 days (22 days total, see below) to establish long-term behavioral sensitization in cocaine-treated groups (see Davidson et al. 2002). Starting on day 9 of cocaine or saline withdrawal, all animals were administered doxycycline (Dox; 2 mg/ml in drinking water) for 14 days. On day 15 of Dox treatment (i.e., day 23 of withdrawal), all rats were challenged with 15mg/kg cocaine (i.p.) and locomotor activity was assessed; rats were euthanized 24 h later to determine effects of ILK silencing on neurobiological markers.

Experiment 2—This experiment was designed to determine whether ILK silencing during the period of cocaine injections could prevent neurobiological alterations associated with behavioral sensitization. Rats were randomly divided into three groups (n=6 rats/group). One group received bilateral microinjections of rAAV-siILK (1.0×10^{10} viral particles) into the NAc core (AP +1.5 mm, DV 6.8 mm, LM ±1.5 mm; Paxinos and Watson 1986), while the other two groups received control rAAV-GFP microinjections (1.0×10^{10} viral particles).

Ten days after surgery, all rats were given Dox (2 mg/ml in drinking water) for a total of 11 days. On day 5 of Dox treatment, the rAAV-siILK group (C-siILK) and one rAAV-GFP group (C-GFP) received 40 mg/kg cocaine (s.c.) for seven consecutive days, and the other rAAV-GFP group (S-GFP) was administered parallel saline injections. All rats were then withdrawn from Dox and cocaine/saline for 20 days before assessment of acute cocaine challenge (15 mg/kg, i.p.). Rats were euthanized 24 h later for neurobiological markers (see Chen et al. 2008a).

Protein Extraction and Western Blot—Twenty-four hours after behavioral analyses, rats were euthanized and the NAc core was dissected and immediately frozen on dry ice. Tissues were homogenized and subjected to Western blot as described (Zhang et al. 2007; Chen et al. 2008a). Briefly, membranes were incubated with primary antibodies: anti-ILK antibody (1: 1,000 dilution; Cell Signaling Technology, Danvers, MA, USA), anti-PSD-95 antibody (1:1,000; Millipore, Bedford, MA, USA), anti-synapsin I antibody (1: 2,000; Millipore), or anti-GluR1 Ser^{845} antibody (1:500; Millipore) for 2 h, and blots were washed and then incubated with peroxidase-labeled secondary antibody (1:2,000; Cell Signaling Technology) for 2 h at room temperature. The blots were developed with chemiluminescent substrate (Pierce Chemical Company, Rockford, IL, USA). To control for loading efficiency, blots were stripped and re-probed with α-tubulin (Sigma-Aldrich, St. Louis, MO, USA). Western blot data were quantified as described (Zhang et al. 2007; Chen et al. 2008a).

Production of Glutathione-*S*-transferase Fusion Proteins—The full-length rat synapsin I (Syn I) complementary DNA (cDNA) was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from a rat RNA library with the forward primer: 5′-CGCGTCGACTGCCAATCTGCC TAATGGGTATATGAC-′ (*Sal*I site is underlined) and reverse primer: 5′- GAATGCGGCCGCTCAGTCGGA GAAGAGGCTGGCG-3′ (*Not*I site is underlined). The full-length rat ILK cDNA was amplified by RT-PCR from a rat RNA library with the forward primer: 5′-CGGGATCC GACGACATTTTCACTCAGTGC-3′ (*Bam*HI site is underlined) and reverse primer: 5′-CGGCTCGAGCTTGTCCTG CATCTTCTCCAAG-3′ (*Eco*RI site is underlined). The full-length cDNA rat PSD-95 was amplified by RT-PCR from rat RNA library with the forward primer: 5′-CGGAATTC GACTGTCTCTGTATAGTGACAACC-3′ (*Eco*RI site is underlined) and reverse primer: 5′-CCGCTCGAGTCA GAGTCTCTCTCGGGCTGGGAC-3′ (*Xho*I site is underlined). The amplified fragments were each cloned into the pGEX-4T-1 plasmid at the appropriate restriction sites downstream of glutathione-*S*-transferase (GST), producing a GST fusion construct.

BL21 (DE3) *Escherichia coli* were transformed with each of the three separate plasmids. After the bacteria were grown in SOC medium (20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCI, 2.5 mmol/l KCI, 20 mmol/l glucose) at 37°C to 0.8 U optical density at 550 nm, protein expression was induced with 0.4 mM isopropyl thio-β-D-galactoside for 5 to 6 h at 22°C. Cells were sonicated in a 10× volume of icecold phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysate was centrifuged at 10,000×*g* for 30 min, and the supernatant was applied to a PBS pre-equilibrated glutathione Sepharose column (GE Healthcare, Piscataway, NJ, USA). The bound protein was eluted with elution buffer [50 mM Tris–HCl (pH 8.0), 10 mM reduced glutathione] and quantitated (Bradford 1976). The intracellular domain of the rat GluR1 subunit was cloned, expressed in bacteria BL21, purified by Sepharose column, and phosphorylated with protein kinase A as previously described (Liu et al. 2009).

ILK Activity Assay—ILK activity was examined as described (Lin et al. 2007) with modifications. Briefly, GST-ILK was incubated with GST-Syn1, GST-cGluR1, and GST-

PSD-95, respectively; GST protein itself served as a negative control. Kinase activity was determined bymixing 1 μg protein with 200 nMATP and 20 μCi of [-^{32}P]ATP (6,000 Ci/mmol; GE Healthcare) in a 50-μl reaction volume containing 1× kinase buffer [12.5 mM Tris–HCI (pH 7.5), 0.05 mM Na_3VO_4, 5 mM MgCl2, 1 mM $MnCl_2$, 100 mM NaF] for 30 min at 30°C. The sample was boiled in 6× sample buffer [480 ml/l glycerol, 60 g/l sodium dodecyl sulfate (SDS), 12 mmol/l ethylenediamine tetraacetic acid (EDTA), 300 mmol/l Tris–HCl (pH 6.8), 17.28 mmol/l β-mercaptoethanol, 0.1 g/l bromophenol blue] for 10 min and separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels. After washing with kinase reaction buffer containing 1% SDS, the gel was dried and the phosphorylated products were visualized by autoradiography at −80°C. As a positive control, GST-ILK was incubated with inactive GST-Akt (Millipore), and phosphorylation of Akt Ser^{473} was detected using a site-specific anti- Akt Ser^{473} antibody (1:1,000; Cell Signaling Technology).

Immunoprecipitation Studies—To co-immunoprecipitate Syn1, GluR1, or PSD-95 with ILK from NAc core homogenates, the anti-ILK antibody (IgG was a negative control) was incubated with 100 μg homogenate for 3 h at 4°C and then incubated with protein A/G PLUS agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 2 h. To examine interactions between ILK and these proteins, 1 μg of GST-ILK (GST protein as a negative control) was incubated with 1 μg of GST-Syn1, GST-cGluR1, phospho-GST-cGluR1 Ser^{845}, or GST-PSD- 95, respectively, in homogenization buffer for 2 h at 4°C followed by incubation with protein A/G PLUS agarose for 1 h. To identify the specificity of the co-IP reaction, 1 μg of GST-ILK, GST-PSD-95, GST-Syn1, GST-cGluR1, and phospho-GST-cGluR1 Ser^{845} (GST protein for control) was incubated with their corresponding antibody in the homogenization buffer, respectively, and then precipitated with protein A/G PLUS agarose. After washing the protein A/G agarose six times with 1 ml washing buffer [1% SDS, 5 mM Tris–HCl (pH 7.4), 2 mM EDTA, 10 μg/ml aprotinin, 0.5 mM PMSF, 50 mM sodium fluoride, 50 mM sodium pyrophosphate, 20 mM 2-glycerol phosphate, 1 mM *p*-nitrophenyl phosphate, 2 μM microcystin LR], samples were boiled in 2× SDS sample buffer [16% glycerol, 2% SDS, 5.76 mM β-mercaptoethanol, 4 mM EDTA, 100 mM Tris–HCl (pH 6.8), 0.01% bromophenol blue] for 8 min, and the supernatant (10 μl) was separated on 10% SDS-PAGE gel. Western blot was performed with antibodies as indicated in the figure legend.

Data Analysis—The data are presented as means and standard errors of the mean. Statistical analysis was conducted with one-way ANOVA and post hoc Bonferroni comparisons. A $p<0.05$ was considered significant.

Results

ILK Regulates PSD-95 Levels and Physically Interacts with PSD-95

Our recent study has demonstrated that cocaine sensitization increases spine numbers in the NAc core and that siRNA-mediated silencing of ILK expression normalized these changes (Chen et al. 2008a). By comparison, overexpression of PSD-95 leads to an increase in the size and numbers of dendritic spines on hippocampal neurons (El-Husseini et al. 2000; Prange and Murphy 2001). To determine whether the ILK- and PSD-95-dependent events underlying these morphological changes played an interactive role in long-term maintenance of cocaine sensitization, experiment 1 examined whether cocaine sensitization increased the levels of PSD-95 protein in the NAc core and whether ILK silencing could reverse and/or prevent these changes. Two groups of S-GFP and S-siILK rats were given saline for seven consecutive days, while another two groups of C-GFP and C-siILK animals received cocaine for the same time period. All animals were first withdrawn for 8 days to establish

sensitization in the cocaine-treated groups (see Davidson et al. 2002), followed by silencing of ILK expression for 15 days. On the last day of Dox treatment (withdrawal day 23), all rats were given an acute cocaine challenge and euthanized 24 h later for biochemistry. As expected, Western blot demonstrated that behavioral sensitization (C-GFP group) increased levels of PSD-95 protein within the NAc core (Fig. 1a). Importantly, this increase was completely blocked by ILK silencing (i.e., C-siILK group). A one-way ANOVA supported these findings where significant main effects of treatment [$F(3, 31)=13.135$, $p<0.001$] were observed. Bonferroni tests showed that C-GFP animals had significantly higher PSD-95 levels in the NAc core than rats in the S-GFP, S-siILK, and C-siILK groups (ps<0.001). These results suggest that ILK plays a major role in maintaining elevated PSD-95 levels in the NAc following establishment of cocaine sensitization by intermittent cocaine injections and withdrawal.

Conditional silencing of ILK expression during repeated cocaine exposure prevents induction of behavioral sensitization (Chen et al. 2008a). In experiment 2, we determined whether ILK silencing during the period of cocaine injections could also block the long-term increases in PSD-95 levels. Animals received Dox in their drinking water 4 days prior to and throughout the seven consecutive days of saline or cocaine treatments. Rats were removed from Dox and withdrawn from saline/cocaine for 20 days, and then challenged with cocaine. Silencing ILK expression during daily cocaine injections prevented the increase in the levels of PSD-95 in the NAc core (Fig. 1b). A one-way ANOVA revealed significant main effects of treatment [$F(2, 17)=9.398$, $p<0.002$]. The NAc core from C-GFP animals had significantly higher PSD-95 levels than tissues from rats in the S-GFP and C-siILK groups (ps<0.01) which were not different from each other. These results indicate that ILK-mediated control of PSD-95 levels in the NAc core plays an important role in the induction of behavioral sensitization by repeated cocaine administration.

To determine whether ILK can complex with PSD-95 directly, we performed co-IP experiments using homogenates from the NAc core. The ILK antibody was sufficient to immunoprecipitate the PSD-95 protein (Fig. 2a) and the PSD-95 antibody immunoprecipitated ILK (Fig. 2b). To examine this relationship further, co-IP studies were conducted by incubating a mixture of GST-ILK and GST-PSD-95 proteins in homogenization buffer with either the ILK or PSD-95 antibodies. Here, the ILK antibody immunoprecipitated GST-PSD-95 protein (Fig. 2c), whereas the PSD-95 antibody immunoprecipitated GSK-ILK (Fig. 2d). The immunoprecipitation of the GST-ILK protein by the ILK antibody (Fig. 2e) and the immunoprecipitation of the GST-PSD-95 protein by the PSD-95 antibody (Fig. 2f), as well as the absence of binding of anti-ILK antibody (Fig. 2g) and PSD-95 antibody (Fig. 2h) to GST flag protein, further supported that the co-IP between ILK and PSD-95 is specific. To determine whether ILK can directly phosphorylate PSD-95 protein, we performed an ILK activity assay using GST-PSD-95 and GST-ILK proteins. This assay showed that ILK was unable to phosphorylate GST-PSD-95 (data not shown). Together, these results suggest that ILK interacts with PSD-95, but does not phosphorylate it.

ILK Regulates GluR1 Ser^{845} Levels and Physically Interacts with the Subunit

Our previous studies have demonstrated that established cocaine sensitization increases GluR1 Ser^{845} phosphorylation in the NAc core (Zhang et al. 2007) and that reversal of cocaine sensitization normalizes this change (Zhang et al. 2007). Since conditional silencing of ILK expression in the NAc core also reverses established cocaine sensitization (Chen et al. 2008a), experiment 1 was designed to ascertain whether silencing ILK expression in the NAc core during the cocaine withdrawal period could also abolish the long-term enhancement in levels of phospho-GluR1 Ser^{845} protein as has been observed in our pergolide-ondansetron pharmacological model (Zhang et al. 2007). The Western blot

demonstrated that silencing ILK expression normalized the cocaine-induced increase in GluR1 Ser^{845} phosphorylation in the NAc core at the time of cocaine challenge (Fig. 3a). A one-way ANOVA revealed significant main effects of treatment [$F(3, 31)=5.319$, $p<0.005$], and Bonferroni tests showed that the NAc core of animals in the C-GFP group contained significantly higher levels of phospho-Ser^{845} GluR1 protein than rats in the S-GFP, S-siILK, and C-siILK groups (ps<0.048). These results suggest that ILK controls the levels of phospho-Ser^{845} GluR1 protein in the NAc core following repeated cocaine exposure and withdrawal.

To separate possible effects of ILK silencing during repeated cocaine administration (i.e., sensitization induction) from those following withdrawal (i.e., sensitization maintenance), experiment 2 was conducted where expression of ILK was silenced during the time of repeated cocaine injection. As expected, levels of phospho-Ser^{845} GluR1 were significantly increased in non-silenced NAc core following long-term withdrawal (Fig. 3b). Conditional silencing of ILK during sensitization induction prevented this increase. A oneway ANOVA revealed significant main effects of treatment [$F(2, 17)=8.04$, $p<0.004$]. Bonferroni tests demonstrated that the NAc core from C-GFP animals had significantly higher levels of phospho-Ser^{845} GluR1 protein than those in the SGFP and C-siILK groups (ps<0.015) which did not differ from each other. These results suggest that repeated cocaine administration activates ILK, which in turn leads to long-term upregulation of phospho-Ser^{845} GluR1 in the NAc core.

To explore how ILK might play a role in GluR1 Ser^{845} phosphorylation, a co-IP experiment was performed. As shown in Fig. 4a, the ILK antibody immunoprecipitated the GluR1 protein. Another co-IP investigation was conducted by incubating the anti-ILK antibody with GST-ILK/GSTcGluR1 or GST-ILK/phospho-Ser^{845} GST-cGluR1 mixtures in homogenization buffer. The results showed that the anti- ILK antibody immunoprecipitated both the GST-cGluR1 (Fig. 4b) and phospho-Ser^{845} cGluR1 (Fig. 4c). Although the antibodies that were used to detect GluR1 and phospho- Ser^{845} GluR1 on the Western blot were found to be unsuitable for immunoprecipitation studies, the co-IP of GluR1 in the NAc core homogenate and the co-IP of GST-cGluR1 or phospho-cGluR1 in homogenization buffer with the ILK antibody support the contention that ILK can interact with the GluR1 and/or phospho-GluR1 subunits.

To examine whether ILK could directly phosphorylate GluR1, an ILK activity assay was run with GST-ILK and GST-cGluR1. As a positive control, GST-ILK was incubated with GST-Akt. ILK phosphorylated GST-Akt (Fig. 4d); however, it was unable to phosphorylate the GluR1 subunit (data not shown). These findings indicate that ILK interacts with GluR1 and phospho-GluR1 proteins, but does not phosphorylate the GluR1 subunit directly.

ILK Regulates Synapsin I Levels and Physically Interacts with This Protein

Synapsin I is a major presynaptic protein and it has been reported to play an important role in tethering the synaptic vesicle to the presynaptic actin network (Jovanovic et al. 2001; Yamagata 2003; Johnson and Ouimet 2006; Hilfiker et al. 1999). Experiment 1 examined whether ILK can alter levels of synapsin I in the NAc core during repeated cocaine administration followed by withdrawal. This procedure was associated with increased levels of synapsin I protein and ILK silencing fully normalized this response (Fig. 5a). A one-way ANOVA revealed significant main effects of treatment [$F(3, 31)=13.706$, $p<0.001$]. The NAc core from C-GFP animals had significantly higher synapsin I levels than tissues from rats in the S-GFP, S-siILK, and C-siILK groups (ps<0.01).

To determine whether blocking induction of cocaine sensitization by ILK silencing (Chen et al. 2008a) also prevented long-term increase in synapsin I levels, rats were treated as

described in experiment 2. Western blot revealed that synapsin I levels were significantly increased in the NAc core in non-ILK knockdown samples, whereas siRNA-induced silencing of ILK expression prevented this increase (Fig. 5b). The one-way ANOVA revealed significant main effects of the treatment [$F(2, 17)=5.331$, $p< 0.018$], and Bonferroni tests demonstrated that the NAc core from C-GFP animals had significantly higher synapsin I levels than samples from rats in the S-GFP and C-siILK groups (ps<0.031) that were statistically indistinguishable from each other. These results suggest that ILK activation during repeated cocaine exposure plays an important role in the subsequent upregulation of synapsin I within theNAc core.

To investigate whether ILK interacts with synapsin I, we performed a co-IP experiment by incubating the ILK antibody with NAc core homogenates. Here, synapsin 1 was immunoprecipitated with the ILK antibody (Fig. 6a). Conversely, the anti-synapsin I antibody could co-immunoprecipitate ILK protein (Fig. 6b). To further investigate this interaction, the ILK antibody was incubated with a mixture of GST-ILK and GST-Syn I in homogenization buffer. This antibody successfully immunoprecipitated GST-Syn I (Fig. 6c). Conversely, the anti-synapsin I antibody immunoprecipitated the GST-ILK protein (Fig. 6d). Since the ILK antibody recognizes ILK (Fig. 2g), because the GST-Syn I protein is immunoprecipitated by the synapsin I antibody (Fig. 6e), and as the synapsin I antibody does not bind the GST flag protein, our results show that the co-IP between ILK and synapsin I is specific. To determine whether ILK directly phosphorylates synapsin I, an ILK activity assay was performed with GST-ILK and GST-Syn I. GST-ILK was unable to phosphorylate GST-Syn I protein (data not shown). These findings suggest that ILK directly interacts with synapsin I, but does not phosphorylate the protein.

Discussion

Our recent study has demonstrated that silencing ILK expression in the NAc core during the chronic cocaine withdrawal period reverses established cocaine sensitization and normalizes structural changes in the dendrites; similar silencing during cocaine treatment also prevents the induction of cocaine sensitization (Chen et al. 2008a). The present study extends these findings by showing that cocaine sensitization increases expression levels of PSD-95 and synapsin I proteins in the NAc core, and these enhancements are normalized or prevented by conditional ILK silencing. This study also confirms our previous finding that repeated cocaine exposure can lead to long-term augmentation of levels of phospho-GluR1 in the NAc core (Zhang et al. 2007), and it demonstrates that this increase can be abolished by silencing ILK expression. These effects of ILK silencing on changes in these synapse-associated molecules suggest that ILK may play a critical role in modulating changes in synaptic plasticity associated with the induction and maintenance of cocaine sensitization.

Repeated psychostimulant exposure has been reported to change expression of PSD-95, a major postsynaptic protein involved in glutamate signaling (Elias et al. 2008). For instance, cocaine behavioral sensitization is absent in PSD- 95 homozygous mutant mice (Yao et al. 2004). PSD-95 has also been reported to contain PDZ domains that promote the clustering of *N*-methyl-D-aspartate (Kornau et al. 1995) and AMPARs (Chen et al. 2000; Ehrlich and Malinow 2004), and this action helps to tether them to the synapses. These changes may be critical to the induction and/or maintenance phases of glutamate-dependent synaptic plasticity (Mulholland and Chandler 2007). Since behavioral sensitization to cocaine or other psychostimulants may reflect neuroplastic changes associated with chronic treatment (Wolf 2002), it is intriguing that siRNA-mediated ILK silencing normalizes not only the enhancement in PSD-95 protein levels, but it also abrogates alterations in phospho- GluR1 levels. Although unclear at this time, the function of ILK in binding to PSD-95 could serve a number of different roles in synaptic plasticity from changing trafficking of phospho-GluR1

subunits within neurons, blocking the GluR1 subunit from binding to other AMPAR subunits, inhibiting proteins from binding to the PSD-95 scaffolding complex, or inhibiting its own activity. Future experiments will address these points.

ILK is a serine/threonine kinase consisting of a C-terminal kinase domain and an N-terminal ankyrin repeat domain (Brakebusch and Fässler 2003). Parenthetically, ILK has been reported to interact with many different proteins in cells (Dobreva et al. 2008). The present study suggests that ILK can form a complex with PSD-95, GluR1, phospho-GluR1, and synapsin 1, but it does not directly phosphorylate these proteins. Relative to its function as a kinase, the role of ILK as a scaffolding protein has not been fully investigated. On the other hand, ILK-PSD-95 protein–protein interaction is not unexpected because PSD-95 has been shown to bind proteins that have ankyrin repeats within the PSD (Kim and Sheng 2004) and because ILK has a N-terminal ankyrin repeat domain (Brakebusch and Fässler 2003). The ILK–GluR1 interaction is a novel finding. Here, ILK may act as a scaffolding protein, complexing with PSD-95 and/or GluR1-containing AMPA receptors in the PSD. If so, increased expression of ILK in established sensitization (Chen et al. 2008a) may provide a means to support the parallel increases in PSD-95 and AMPA receptors at the active synaptic site (i.e., PSD). Future experiments will examine these points.

The synapsins, including synapsin I, participate in modulating synaptic function (e.g., Jovanovic et al. 2001) and in trafficking mature vesicles to the synapse (Ferreira and Rapoport 2002; Hilfiker et al. 1999). Synapsin I binds to actin filaments and can regulate the organization of the presynaptic actin network (Jovanovic et al. 2001; Yamagata 2003; Johnson and Ouimet 2006). In a similar manner, the scaffolding protein ILK can couple integrin signaling to the actin cytoskeleton (Wu 2004; Hannigan et al. 2005). In the present study, it is interesting to find that the ILK antibody can co-IP purified GST-Syn1 protein as well as native synapsin I from NAc core homogenates. These data suggest that ILK may form a complex with synapsin I and physically interact with this protein. In the present experiment, levels of synapsin I are elevated in the NAc core from animals with established cocaine sensitization. Importantly, these changes can be reversed or prevented by siRNA-mediated ILK silencing during the respective maintenance or induction phases of behavioral sensitization. Together, the present results suggest that ILK may be able to modulate not only presynaptic, but also postsynaptic neuroplasticity events that occur as a result of repeated cocaine exposure.

One issue that arises from the present study is how alterations in expression of ILK protein can affect cocaine behavioral sensitization and associated changes in the levels of PSD-95 and synapsin I in the NAc core. Currently, there is no evidence available indicating that ILK can directly regulate the transcription of these genes. However, because levels of PSD-95 and synapsin I are enhanced in the NAc in the C-GFP group but not in the ILK-silenced group (i.e., C-siILK), ILK may play a secondary role where it influences transcription and/or translation of the messenger RNAs for these proteins through downstream signaling molecules. For instance, translation effects of ILK may be mediated through Ser^{473} phosphorylation of Akt, which in turn stimulates raptor/mTOR-mediated phosphorylation of S6 kinase (see Hay 2005; Kaeberlein and Kennedy 2007; McDonald et al. 2008). Parenthetically, ILK can phosphorylate Akt at the Ser^{473} residue either directly (present study) or through its interaction with rictor (McDonald et al. 2008). With respect to mediating phosphorylation events, the in vitro ILK activity assays show that ILK cannot directly phosphorylate PSD-95, synapsin I, or the GluR1 subunit. Since the Ser^{845} residue to the GluR1 subunit is a known substrate of protein kinase A (Roche et al. 1996), it may be the case that ILK can exert some indirect actions on this kinase through its interactions with rictor (see Mavrakis et al. 2007). ILK-mediated effects of protein kinase A could also influence transcription of PSD-95 and synapsin I. Although the precise mechanisms

underlying the actions of ILK on PSD-95, synapsin I, and GluR1 are unknown, our findings clearly show that this kinase plays a pivotal role in blocking behavioral sensitization and the appearance of neurobiological markers associated with this condition. These findings suggest that ILK may serve as an important therapeutic target for cocaine abuse.

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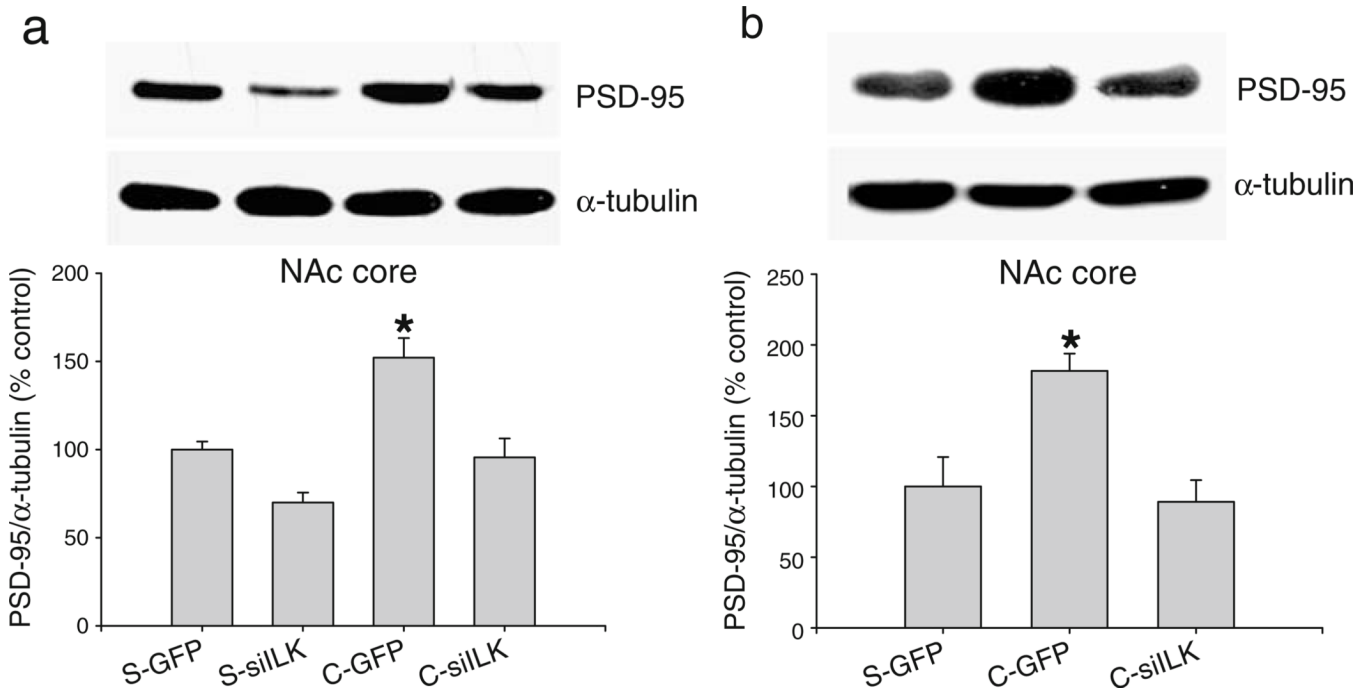

Figure 1.

ILK regulates PSD-95 expression. *Top,* Representative Western blots for PSD-95 and α-tubulin. *Bottom,* densitometric readings (PSD-95/α-tubulin) of the Western blot expressed as percent control (S-GFP set to 100%). **a** siRNA-induced silencing of ILK in the NAc core following cocaine sensitization and withdrawal reverses the increase in PSD-95 expression upon cocaine re-exposure. *$p<0.05$ for C-GFP versus all other groups. $n=7–9$ rats/group. **b** Silencing of ILK expression during repeated cocaine exposure blocks the cocaineinduced increase in PSD-95 expression at challenge. *$p<0.05$ for C-GFP versus all other groups. $n=6$ rats/group. *S-GFP* rAAV-GFP-infected rats given saline, *S-silLK* rAAV-silLK-infected rats given saline, *C-GFP* rAAV-GFP-infected rats given cocaine, *C-silLK* rAAV-silLK- infected rats given cocaine. The data are presented as means and SEM

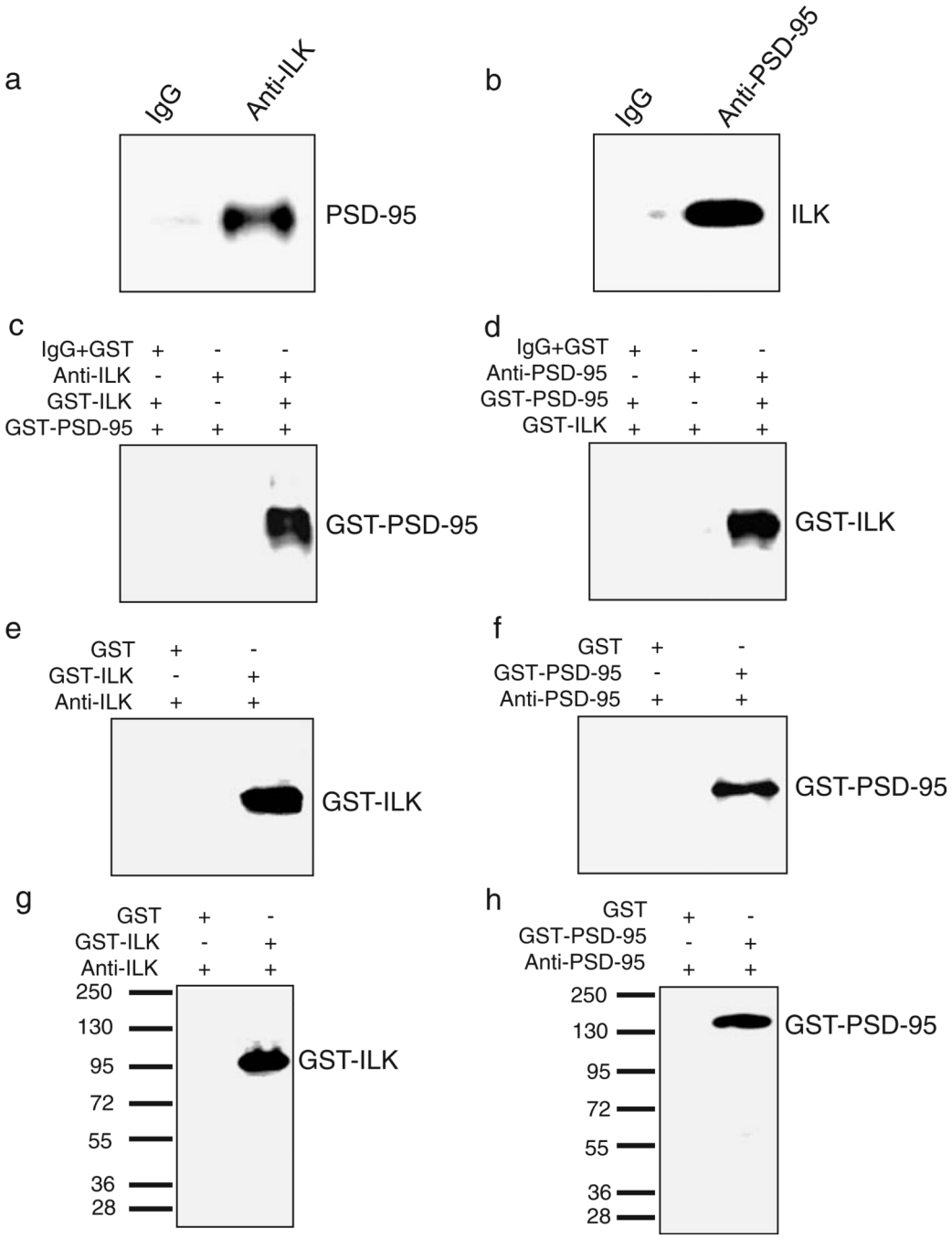

Figure 2.

ILK binds to PSD-95. **a** Immunoprecipitation of PSD- 95 from NAc core homogenates with the anti-ILK antibody and subsequent detection with the anti-PSD-95 antibody. **b** Immunoprecipitation of ILK from NAc core homogenates with the anti-PSD-95 antibody and subsequent detection by anti- ILK antibody. The IgG antibody serves as a negative control. **c** Co-IP of GST-PSD-95 using the anti-ILK antibody with detection by the anti-PSD-95 antibody and **d** co-IP of GST-ILK using the anti-PSD-95 antibody with detection by the anti-ILK antibody. GST serves as a negative control protein. **e** Immunoprecipitation of GST-ILK using the anti-ILK antibody with detection by the anti-ILK and **g** the anti-GST

antibodies. **f** Immunoprecipitation of GST-PSD- 95 using the PSD-95 antibody with detection by the anti-PSD-95 and **h** the anti-GST antibodies. The results show that ILK and PSD-95 can be co-immunoprecipitated. n=2–4 replicates

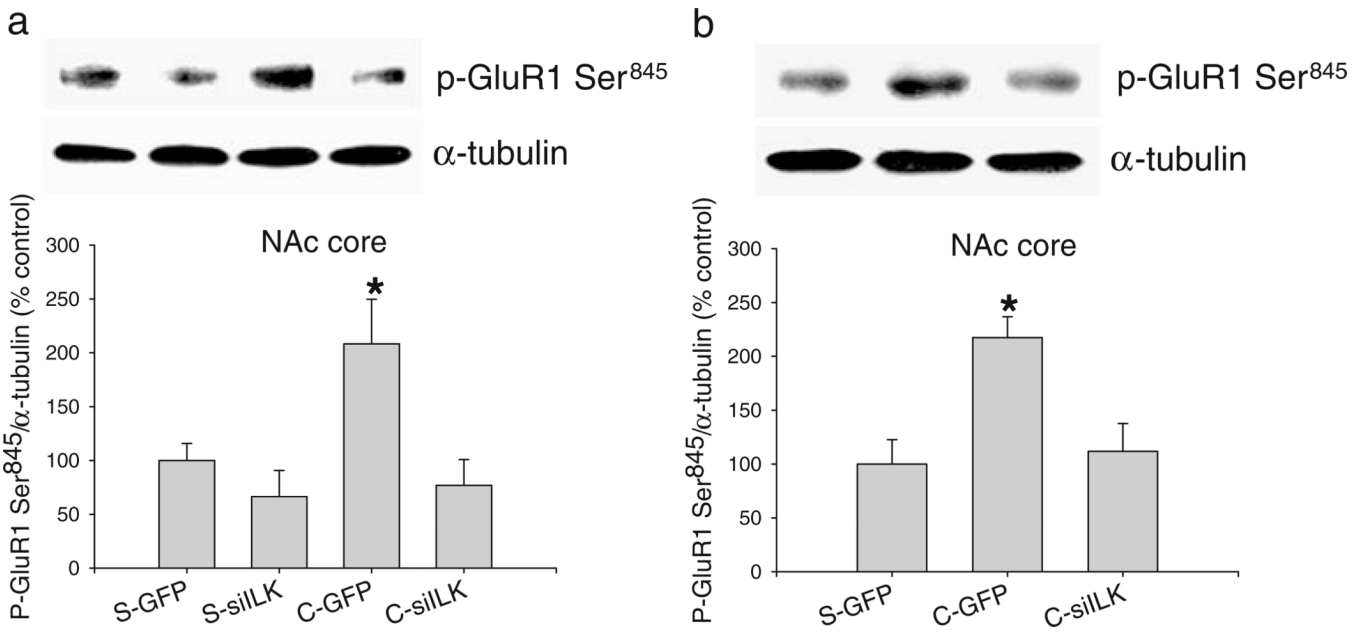

Figure 3.

ILK regulates GluR1 Ser^{845} phosphorylation. *Top,* Representative Western blots for phospho-Ser^{845} GluR1 and α-tubulin. *Bottom,* densitometric readings (phospho-GluR1/α-tubulin) of the Western blot expressed as percent control (S-GFP set to 100%). **a** Silencing ILK in the NAc core during the cocaine sensitization/ withdrawal period reverses the increase in the levels of phospho- Ser^{845} GluR1 protein at the time of cocaine challenge. *p<0.05 for CGFP versus all other groups. n=7–9 rats/group. **b** Silencing of ILK expression during repeated cocaine exposure blocks the cocaine-induced increase in Ser^{845} phosphorylation of GluR1. *p<0.05 for C-GFP versus all other groups. n=6 rats/group. See Fig. 1 for group designations

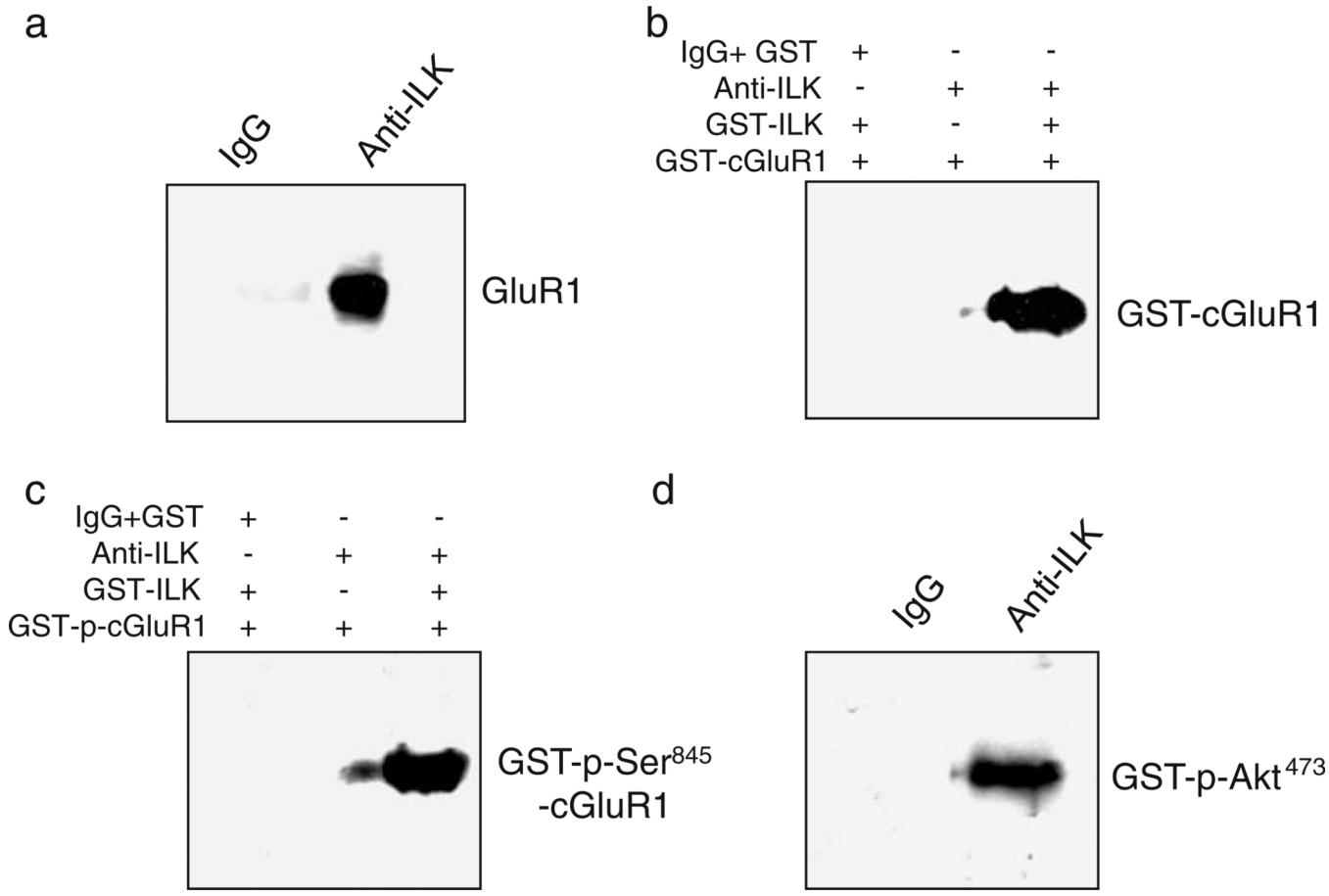

Figure 4.

ILK immunoprecipitates with GluR1. **a** Co-IP of GluR1 protein from NAc core homogenates using the anti-ILK antibody (*anti-ILK*) and detection by the anti-GluR1 antibody. The IgG antibody serves as a negative control. **b** Co-IP of GST-cGluR1 using the anti-ILK antibody. The ILK antibody co-immunoprecipitates the GST-cGluR1 protein. **c** Co-IP of phospho-Ser^{845} cGluR1 protein using the anti-ILK antibody. The ILK antibody co-immunoprecipitates the phospho-cGluR1 protein. GST is the negative control protein. **d** GST-ILK phosphorylates GST-Akt at the Ser^{473} residue. n=2–4 replicates

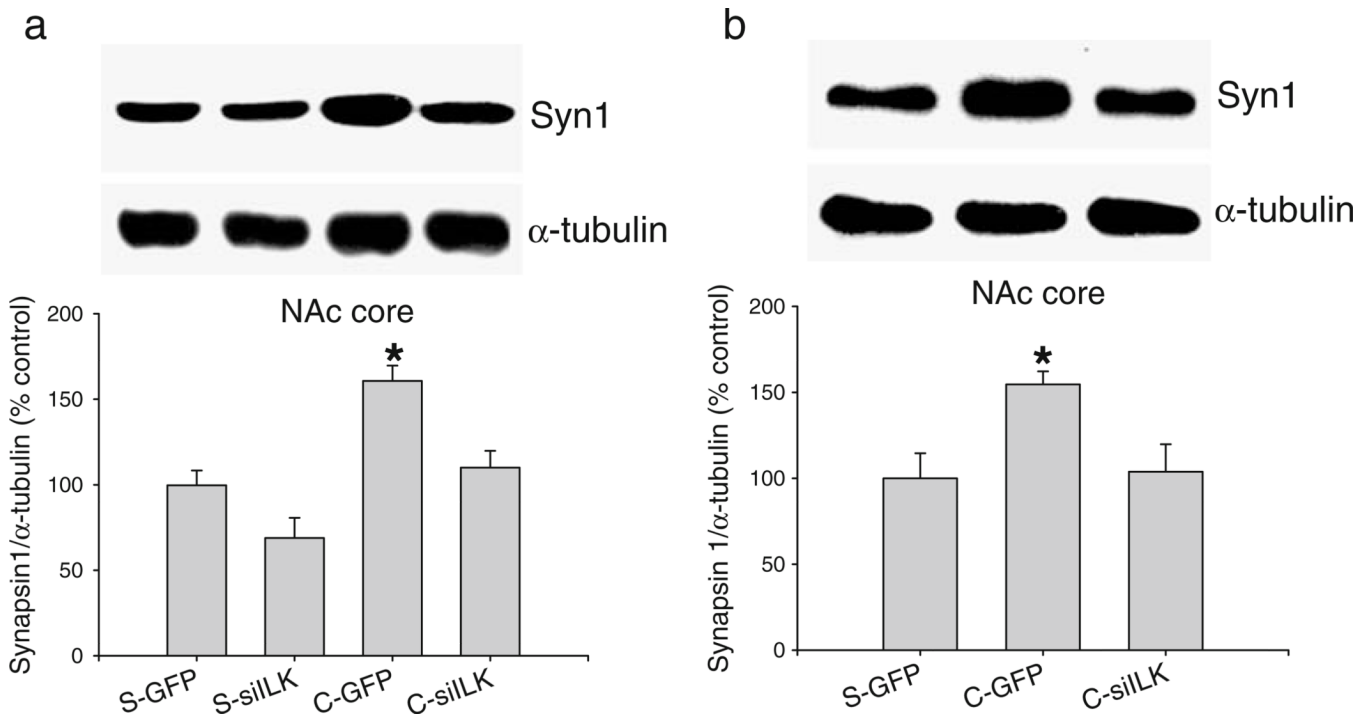

Figure 5.

ILK regulates synapsin 1 expression. *Top,* Representative Western blots for synapsin 1 and α-tubulin. *Bottom,* Densitometric readings (synapsin 1/α-tubulin) of the Western blot expressed as percent control (S-GFP set to 100%). **a** Silencing ILK in the NAc core during repeated cocaine exposure and the subsequent withdrawal period reversed the cocaine-induced increase in synapsin 1 expression. *p<0.05 for C-GFP versus all other groups. n=7–9 rats/group. **b** Silencing of ILK expression during repeated cocaine administration blocked the cocaine-induced increase in synapsin 1 protein. *p<0.05 for C-GFP versus all other groups. n=6 rats/group. See Fig. 1 for group designations

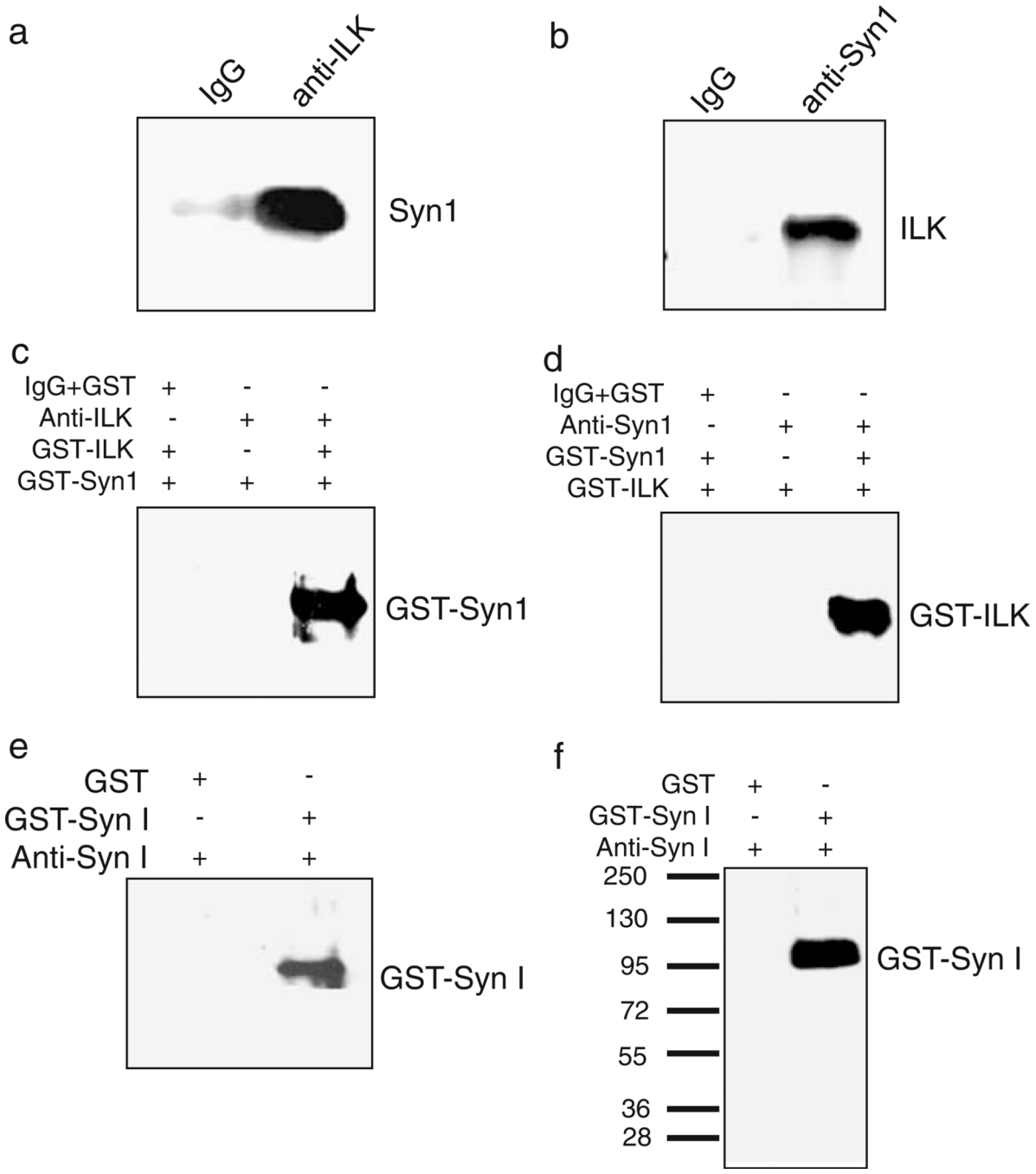

Figure 6.

ILK co-immunoprecipitates with synapsin 1. **a** Co-IP of synapsin 1 from NAc core homogenates using the anti-ILK antibody as detected with the anti-ILK antibody and **b** co-IP of ILK from NAc core homogenates using the anti-synapsin 1 antibody with detection by the antisynapsin I antibody. The IgG antibody serves as a negative control. **c** Co-IP of GST-Syn I using the anti-ILK antibody with detection by the anti-synapsin I antibody and **d** co-IP of GSTILK using the anti-synapsin 1 antibody and detection with the anti-ILK antibody. GST is a negative control protein. ILK and synapsin 1 can be coimmunoprecipitated with each other. **e** Immunoprecipitation of GST-Syn I using Syn I antibody as detected with the

anti-Syn I and **f** with the anti-GST antibody. Non-specific binding was not observed. n=2–4 replicates