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Proteasome phosphorylation regulates cocaine-induced sensitization

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ABSTRACT

Repeated exposure to cocaine produces structural and functional modifications at synapses from neurons in several brain regions including the nucleus accumbens. These changes are thought to underlie cocaine-induced sensitization. The ubiquitin proteasome system plays a crucial role in the remodeling of synapses and has recently been implicated in addiction-related behavior. The ATPase Rpt6 subunit of the 26S proteasome is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinases II alpha at ser120 which is thought to regulate proteasome activity and distribution in neurons. Here, we demonstrate that Rpt6 phosphorylation is involved in cocaine-induced locomotor sensitization. Cocaine concomitantly increases proteasome activity and Rpt6 S120 phosphorylation in cultured neurons and in various brain regions of wild type mice including the nucleus accumbens and prefrontal cortex. In contrast, cocaine does not increase proteasome activity in Rpt6 phosphomimetic (ser120Asp) mice. Strikingly, we found a complete absence of cocaine-induced locomotor sensitization in the Rpt6 ser120Asp mice. Together, these findings suggest a critical role for Rpt6 phosphorylation and proteasome function in the regulation cocaine-induced behavioral plasticity.

1. Introduction

The persistent neural and behavioral adaptations characteristic of addiction can render an addict permanently susceptible to relapse even years after cessation of drug use. The enduring nature of these modifications suggests the involvement of memory or memory-like neuronal remodeling (Robinson and Kolb, 1997; Russo et al., 2010; Howell et al., 2014). Behavioral sensitization is an increase in the sensitivity to a drug following repeated administration, characterized by enhanced locomotor activity, dopamine release, and the rewarding value of the drug (Robinson and Berridge, 2008). Sensitization is argued to mediate the transition from ordinary goal-seeking behavior to compulsive behavior as the drug progressively elicits a much stronger dopamine response than natural reinforcers. The involvement of protein synthesis is well demonstrated in traditional and addiction-related memories (Schafe and LeDoux, 2000; Hernandez et al., 2002), however the importance of protein degradation in memory and addiction-related plasticity and behavior has only recently been studied.

The ubiquitin proteasome system (UPS) plays a major role in the development, maintenance and remodeling of synaptic connections (Mabb and Ehlers, 2010; Hamilton and Zito, 2013). The 26S proteasome, which degrades ubiquitinated proteins, is a large multi-subunit energy-dependent protease formed by the co-assembly of a 20S proteasome catalytic core and 19S cap regulatory particle (RP) (Hershko and Ciechanover, 1998). We and others recently found a novel form of regulation for the 26S proteasome involving CaMKIIα, a key kinase involved in neuronal plasticity underlying learned behaviors. CaMKIIα phosphorylates the ATPase 19S (RP) subunit of the 26S proteasome, Rpt6, at serine 120 (S120) in an activity-dependent fashion to control the activity and distribution of proteasomes in neurons (Djakovic et al., 2009; Bingol et al., 2010; Djakovic et al., 2012). We have previously shown that Rpt6 is involved in the regulation of synaptic strength and activity-dependent generation of new spines (Djakovic et al., 2012; Hamilton et al., 2012). Here, we utilize recently generated Rpt6 S120D phospho-mimetic knock in (KI) mice (serine 120 to aspartic acid) to examine the importance of Rpt6 phosphorylation on cocaine-induced locomotor sensitization. We found that cocaine increases Rpt6 S120 phosphorylation in cultured neurons, the nucleus accumbens (NAc), and the prefrontal cortex (PFC) of wild type (WT) mice. Furthermore, repeated cocaine administration elevated proteasomal activity in both the NAc and PFC in WT mice but not in Rpt6 S120D KI mutant mice. Cocaine-induced locomotor sensitization was completely absent in the Rpt6 S120D KI mice. Together, these findings implicate a critical role for Rpt6 phosphorylation and proteasome function in the regulation of

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cocaine-induced behavioral sensitization.

2. Materials and methods

2.1. Generation of S120D Knockin mice

We generated Rpt6 phospho-mimetic (ser120 to aspartic acid; S120D) KI mice (iTL; www.genetargeting.com). The strategy for generating the KI mice is described in Fig. 2. The targeting vector was linearized and transfected by electroporation into BA1 (C57Bl/6 × 129/SvEv) (Hybrid) embryonic stem cells. Selected clones were expanded for southern blot analysis to identify recombinant ES clones (data not shown). The ES clones were microinjected into C57BL/6 blastocysts. After germline transmission, the Neo cassette was removed by mating to C57BL/6 FLP mice. Tail DNA was analyzed by PCR to identify heterozygous mice and verify deletion of the Neo cassette. Mutant heterozygous mice were backcrossed to C57BL/6. By visual inspection, Rpt6 S120D homozygous mutants (confirmed by PCR and sequencing) obtained by crossing heterozygous mutants, displayed normal body size, feeding, and mating behaviors. The intercross of heterozygotes resulted in production of wild-type, heterozygous, and homozygous offspring at the expected 1:2:1 Mendelian ratio. All procedures were approved by the UCSD IACUC and compliant with the NRC Guide.

2.2. Antibodies and reagents

Mouse mAb Rpt6 (Enzo mAb P45-110, BML-PW9265-0025), mAb 20S (Enzo mAb MCP231, BML-PW8195-0025), tubulin (Sigma T9026), mAb CaMKIIα (Abcam ab2725), tyrosine hydroxylase (Millipore AB152), MAP2 (Abcam AB5392) and custom rabbit pAb phospho-specific Rpt6 S120 (Djakovic et al., 2012) antibodies were used. *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC) substrate was used (BACHEM). Methamphetamine HCl (Sigma M8750), and atomoxetine HCl (TCI Chemicals A2357) were dissolved in water and citalopram HBr (Enzo BMC-NS112) was dissolved in ethanol. Cocaine HCl (Sigma) administered during behavioral assessment was dissolved in physiological saline to a dose of 15 mg/kg (salt weight) and administered i.p. (10 ml/kg).

2.3. Neuronal cultures

High Density Rat dissociated cortical neurons from postnatal day 1 pups of either sex were plated onto poly D-lysine-coated 6-well plastic dishes at ~500,000 cells per well (cortical cultures) and were maintained in B27 supplemented Neurobasal media (Invitrogen) until ≥ 14 d in vitro (DIV), as previously described (Djakovic et al., 2009).

2.4. Proteasome activity assays

Proteasome activity was measured as previously described (Kisselev and Goldberg, 2005) with slight modifications. Briefly, cultured neurons were incubated for 24 h in either plain media (control), or media containing Cocaine (1 μm, 5 μm), methamphetamine (500 μM), citalopram (25 μM) or atomoxetine (25 μM). Cultured neurons and brain tissues were then lysed or dounced, respectively, in Affinity Purification Buffer (APB) (25 mM Hepes–KOH, pH 7.4, 10% glycerol, 5 mM MgCl2, 1 mM ATP, and 1 mM DTT) (Besche and Goldberg, 2012), and lysates were cleared by centrifugation. Equal amounts of protein were used as inputs for the peptidase assay. Chymotrypsin-like activity was monitored over time using Suc-LLVY-AMC substrate. Assays were run in triplicate at 37 °C using a microplate fluorimeter (HTS7000 Plus, Perkin-Elmer) with excitation and emission filters of 360 nm and 465 nm, respectively. Kinetics data was taken every 60 s for 2 h using 96-well microplates (Costar). The data was averaged and plotted to find the kinetic rates of the chymotrypsin-like activity.

2.5. Western blot analysis

Equal or increasing amounts of protein lysates from dissociated cortical neurons as well as brain lysates were resolved by SDS-PAGE and probed with primary antibodies for total Rpt6, phospho-Rpt6 S120, CaMKIIα, and tyrosine hydroxylase. Resulting blots were digitized and band intensities quantitated using NIH ImageJ. For quantification of total phospho-Rpt6 S120 levels, band intensities in each condition were normalized to total Rpt6 band mean intensity from the same sample. Experimenters were blinded to condition during data collection and analysis.

2.6. qPCR

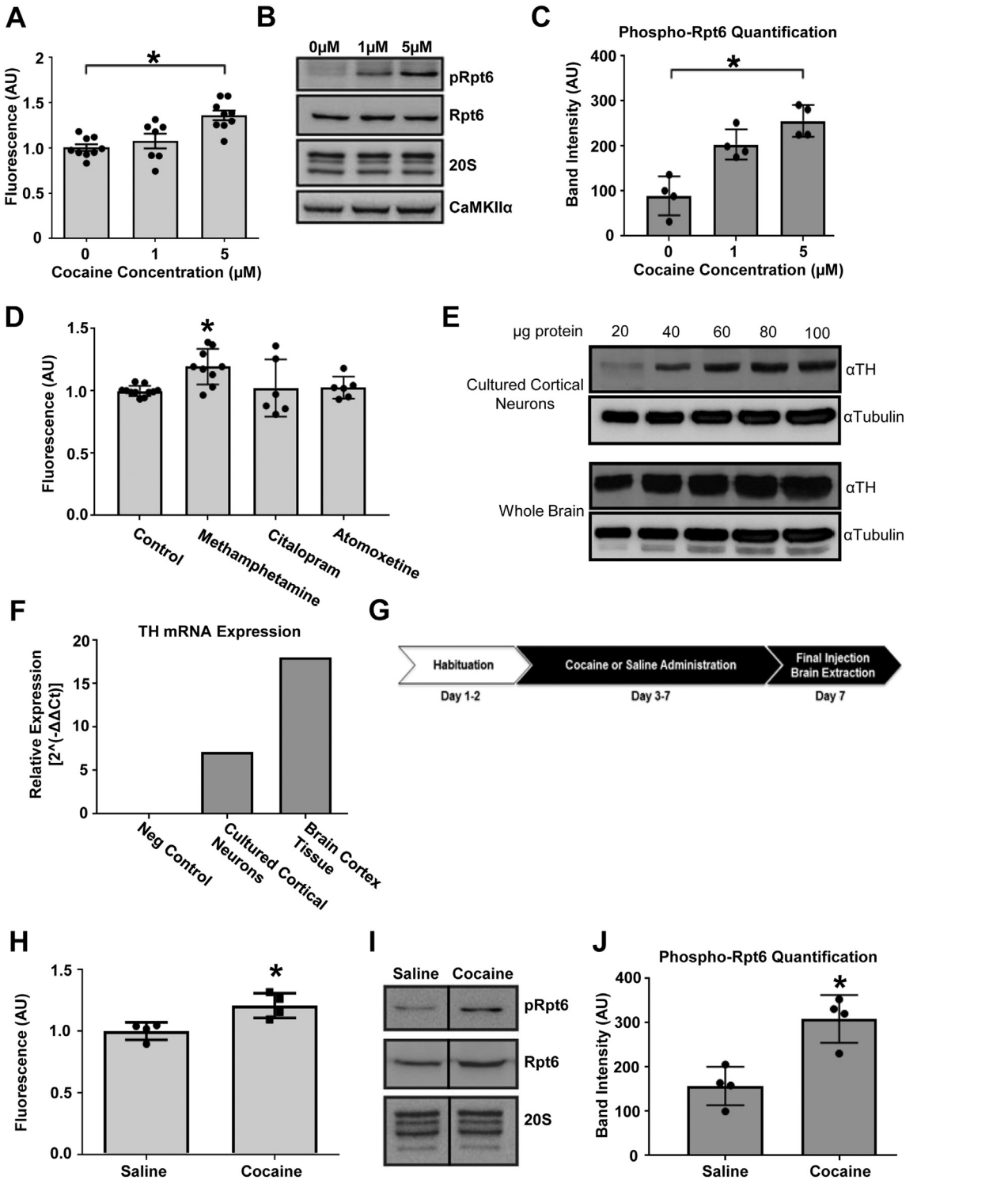
Total RNA was extracted from dissected samples or cultured neurons using Hybrid-R RNA purification kit (GeneAll Biotechnology). Purified RNA samples were reverse transcribed by using the SuperScript-IV First-strand cDNA synthesis kit (Invitrogen). qRT-PCR was performed by using TaqMan Gene Expression Assay Kit (Applied Biosystems). All TaqMan probes were purchased from Applied Biosystems and are as follows: TH (Mm00447557_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mm99999915_g1). Target amplification was performed by using ViiATM 7 Real-Time PCR System (Applied Biosystems). The relative mRNA expression levels were calculated via a comparative threshold cycle (Ct) method using GAPDH as an internal control: DCt = Ct (gene of interest) − Ct (GAPDH). The gene expression fold change was normalized to the control sample and then was calculated as 2-DDCt (Schmittgen and Livak, 2008).

2.7. Behavioral sensitization

40 adult Rpt6 S120D and WT mice were used for behavioral experiments. All animals were group housed. The vivarium was maintained on a 14:10 light:dark schedule. Mice had unrestricted access to food and water. All mice were handled for five days prior to behavioral assessment. Mice were then habituated to testing chambers during two, 1 h sessions (30 min per side). Testing was conducted during the light phase (Howell et al., 2014). Two-sided polycarbonate chambers, each side distinct in appearance and floor, bisected by an opaque acrylic wall were paired with saline or drug (counterbalanced). Mice were divided into four groups: S120D-Cocaine, WT-Cocaine, S120D-Saline, and WT-Saline. Sensitization was induced during four sessions of cocaine administration (as in Fig. 1C or 3A). All animals first received an injection of saline (10 ml/kg) and were placed in the saline-paired side of the chamber for 15 min. Mice were removed, given an injection of either cocaine (S120D-Cocaine, WT-Cocaine groups) or saline (S120D-Saline, WT-Saline groups) and immediately placed in the drug-paired side of the chamber for 15 min.

2.8. Histology

24 h post-sensitization, mice were given a final injection of cocaine (S120D-Cocaine, WT-Cocaine) or saline (S120D-Saline, WT-Saline) in their home cage. 30 min later, mice were anesthetized (isoflurane) and decapitated for fresh tissue collection. For whole brain experiments, brains were flash frozen in liquid nitrogen and stored at − 80 °C. For experiments targeting the NAc and PFC, brains were removed and frozen on dry ice. 1 mm coronal sections were obtained using an acrylic matrix (A/P: 2.10–1.10; Braintree Scientific). 1 mm punches (Miltex) were then taken from the NAc (M/L: ± 0.5, D/V: 2.0) and PFC (M/L: ± 1.25, D/V: 4.25), flash frozen in liquid nitrogen and stored at − 80 °C.



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2.9. Data analysis

For western blot experiments statistical significance was determined using unpaired Student *t*-tests (Prism). Behavioral data were entered into a multivariate ANOVA (SPSS). For proteasome activity assays, ANOVA was performed, or Mann-Whitney *U* test (for non-parametric data). If a significant omnibus comparison or group × time/session interaction was achieved, post-hoc comparisons were made using Fisher's protected least significant difference (PLSD). For all experiments, significance was set at a level of $p \leq 0.05$.

3. Results

3.1. Cocaine increases Rpt6 S120 phosphorylation and proteasome activity

It is known that cocaine activates CaMKIIα through T286 phosphorylation (Anderson et al., 2008). Since we discovered that CaMKIIα phosphorylates Rpt6 at ser120 which increases proteasome activity

Fig. 1. Cocaine increases Rpt6 S120 phosphorylation and proteasome activity via the dopaminergic pathway. *A*, Dissociated cortical neurons (DIV > 17) were treated with vehicle or cocaine (1 and 5 μM, 24 h) and chymotrypsin-like proteasome activity was measured in lysates with the fluorogenic substrate Suc-LLVY-AMC. Graph depicts rate substrate cleavage (mean ± S.E. fluorescence) normalized to control-treated neurons. Cocaine treatment (5 μM, 24 h) significantly increases proteasome activity (*, ANOVA: $F(2,22) = 11.81$, $p = 0.001$; unpaired Student's *t*-test: $p < 0.05$, n = 9). *B*, Representative western blot of lysates in (A) probed with phosphor-Rpt6 pS120, total Rpt6, phospho-CaMKIIα T286, total CaMKIIα, and 20S core antibodies. *C*, Quantification of phosphor-Rpt6 blot in (B). Cocaine increases Rpt6 S120 phosphorylation in a dose dependent-manner. *D*, Dissociated cortical neurons (DIV > 17) were treated with vehicle, methamphetamine (500 μM, 24 h), SERT inhibitor citalopram (25 μM, 24 h) or NET inhibitor atomoxetine (25 μM, 24 h) and chymotrypsin-like proteasome activity was measured in lysates with the fluorogenic substrate Suc-LLVY-AMC. Graph depicts rate substrate cleavage (mean ± S.E. fluorescence) normalized to control-treated neurons. Methamphetamine treatment significantly increases proteasome activity (*, ANOVA: $F(3,28) = 4.204$, $p = 0.014$; unpaired Student's *t*-test: $p < 0.001$, n = 9), while neither citalopram ($p = 0.73$, n = 6) nor atomoxetine ($p = 0.40$, n = 6) had any significant effect. *E*, Representative western blot of dissociated cortical neuron lysates (DIV = 21) compared to whole brain lysate and probed with tyrosine hydroxylase antibodies (Millipore AB152) to show relative expression levels of TH in dissociated cortical neuron cultures. *F*, qPCR of lysates from dissociated cortical neurons (DIV = 21) and whole brain cortex show expression of tyrosine hydroxylase in both lysates, indicating monaminergic systems in dissociated cortical neuronal cultures. *G*, Schematic of drug administration protocol. After a habituation period on day 1 and 2, cocaine (15 mg/kg, i.p) or saline was administered on days 3 thru 7 and a final injection was delivered on day 8 immediately prior to brain extraction. *H*, Whole brain lysates were prepared and proteasome activity and Rpt6 phosphorylation were examined as above. Proteasome activity increased in brains of cocaine-injected animals relative to saline (*, $p < 0.05$, Mann-Whitney *U* test; $U = 0$, $p = 0.029$, n = 4). *I*, Representative western blot of lysates in (H) probed with phospho Rpt6 pS120, total Rpt6 and 20S core antibodies shows that a concomitant increase in Rpt6 S120 phosphorylation occurs in cocaine-treated animals when compared to saline. *J*, Quantification of phosphor-Rpt6 blot in (I) shows a significant increase in Rpt6 phosphorylation in cocaine treated animals.

(Djakovic et al., 2009; Djakovic et al., 2012), we wondered whether cocaine could regulate Rpt6 S120 phosphorylation and proteasome activity. We examined Rpt6 S120 phosphorylation and proteasome activity in lysates from cultured neurons treated with increasing amounts of cocaine. Measuring proteasome peptidase activity, we found that treatment with 5 μM cocaine significantly increased proteasome activity (ANOVA $F(2,22) = 11.81$, $p = 0.001$; unpaired Student's *t*-test: $t(16) = 5.455$, $p < 0.001$) (Fig. 1A). While we observe an increase in Rpt6 phosphorylation after 1 μM cocaine treatment (Fig. 1B, C), we only observe a concomitant and significant increase in proteasome activity after 5 μM treatment (ANOVA $F(2,9) = 20.4$, $p < 0.001$; unpaired Student's *t*-test: $t(6) = 5.942$, $p = 0.001$). To determine if monaminergic systems were involved in this effect, we utilized a second dopamine re-uptake inhibitor, methamphetamine (which also inhibits serotonin and norepinephrine re-uptake, yet does not interact with sodium ion channels), a serotonin-specific re-uptake inhibitor (citalopram) as well as a norepinephrine re-uptake inhibitor (atomoxetine) to treat cultured neurons. We found that treatment with methamphetamine (500 μM) produced an increase in proteasome activity, similar to that of cocaine (ANOVA $F(3,28) = 4.204$, $p = 0.0142$; unpaired Student's *t*-test: $t(18) = 4.34$, $p < 0.001$), however neither citalopram (25 μM) ($t(16) = 0.3462$, $p = 0.734$) nor atomoxetine (25 μM) ($t(15) = 0.8767$, $p = 0.395$) had any significant effect (Fig. 1D). Additionally, we observe significant expression of tyrosine hydroxylase in our dissociated neuron cultures, both via western blot (Fig. 1E) as well as by qPCR (Fig. 1F), which suggest the presence of dopaminergic systems. We additionally evaluated whether cocaine would produce similar effects on Rpt6 S120 phosphorylation and proteasome activity when administered to mice (i.p.). We treated mice with either cocaine or saline for 6 days (Fig. 1G). Rpt6 S120 phosphorylation (Mann-Whitney *U* test: $U = 0$, $p = 0.029$) and proteasome activity (Mann-Whitney *U* test: $U = 0$, $p = 0.029$) was significantly increased in WT whole brains from mice treated with cocaine compared to saline (Fig. 1H, I, J). Taken together, we show that cocaine concomitantly increases Rpt6 phosphorylation and proteasome activity.

3.2. Generation of Rpt6 S120D Knockin (KI) mice

To begin to assess the biological relevance of *Rpt6 (PSMC5)* S120 phosphorylation, we generated genetically modified mice encoding a serine 120 to aspartic acid phospho-mimetic mutant of the Rpt6 protein (Fig. 2A), which can be distinguished from the wild type *Rpt6* gene by PCR-based genotyping (Fig. 2B and C). Rpt6 S120D homozygous KI mice expressed similar levels of Rpt6 protein as wild-type (+/+) mice. By visual inspection, Rpt6 S120D homozygous mutants were indistinguishable from wild type littermates and displayed normal body size, feeding, and mating behaviors. We found that our custom pS120 phospho-specific antibody, which does not recognize Rpt6 S120A mutant protein (Djakovic et al., 2012) and slightly cross reacts with the Rpt6 S120D which is likely due to the negative charge of the aspartic acid residue (Fig. 2D). Gross brain anatomy in Rpt6 S120D homozygous mice was comparable to wild-type (Fig. 2E).

3.3. Rpt6 S120 phosphorylation and peptidase activity is increased in NAc and PFC in cocaine treated wild type mouse brains, but not in S120D mutant mice

Major biochemical and structural changes in both the NAc as well as the PFC are associated with cocaine sensitization (Robinson and Kolb, 1997). To determine if cocaine increases Rpt6 phosphorylation and proteasome activity specifically in the NAc and PFC, mice were administered five cocaine (15 mg/kg, i.p.) or saline treatments across 6 days (Fig. 3A) and then lysates were extracted from tissue punched from the NAc and PFC of both WT and Rpt6 phospho-mimetic S120D mice (Fig. 3B). Western blot analysis and peptidase assays were performed. In WT mice, cocaine administration increased Rpt6 phosphorylation in the NAc in comparison to treatment with saline (Fig. 3E). This increase is also correlated with a significant enhancement in proteasome activity in the NAc ($U = 0$, $p = 0.029$) (Fig. 3C). Interestingly, however, cocaine-induced increases in proteasome activity was not observed in the NAc of S120D mice ($U = 7$, $p = 0.886$) (Fig. 3D). A similar trend was observed in PFC. Cocaine increased Rpt6 S120 phosphorylation in PFC in cocaine-treated WT mice (Fig. 3H), which correlated with increased peptidase activity ($U = 0$, $p = 0.029$) (Fig. 3F). As in the NAc, this increase was occluded in the PFC of S120D mutated mice, as no difference was observed between mice administered cocaine or saline ($U = 8$, $p > 0.999$) (Fig. 3F). Taken together, we show that cocaine concomitantly increases Rpt6 phosphorylation and proteasome activity specifically in the NAc and PFC and that the cocaine-induced increase in proteasome activity is occluded in the Rpt6 S120D mutant.

3.4. Behavioral sensitization is completely absent in Rpt6 S120D KI mutant mice

Locomotor sensitization to cocaine (15 mg/kg i.p.) was assessed in four groups of mice; S120D mice administered cocaine and saline (S120D-Cocaine (n = 11) and S120D-Saline (n = 5), respectively) and WT littermates administered cocaine and saline (WT-Cocaine (n = 18) and WT-Saline (n = 6), respectively). Baseline activity (prior to any injections; habituation (H), Fig. 4A) did not differ between groups [$F(3,36) = 0.037$, $p = 0.99$)]. Differences emerged during subsequent sessions [Fig. 4A, $F(3,36) = 5.79$, $p < 0.005$]. While locomotor activity increased in WT-Cocaine mice across repeated injections, activity levels of S120D-Cocaine mice remained constant (Fig. 4A, Fisher's PLSD, $p = 0.007$). Further, locomotor activity did not differ between S120D-Cocaine mice and groups administered saline (S120-Cocaine/S120D-Sal, $p = 0.59$; S120D-Cocaine/WT-Saline, $p = 0.36$). There were no between group differences during the first session of cocaine (or saline) administration [Fig. 4B, $F(3,36) = 0.778$, $p = 0.51$].

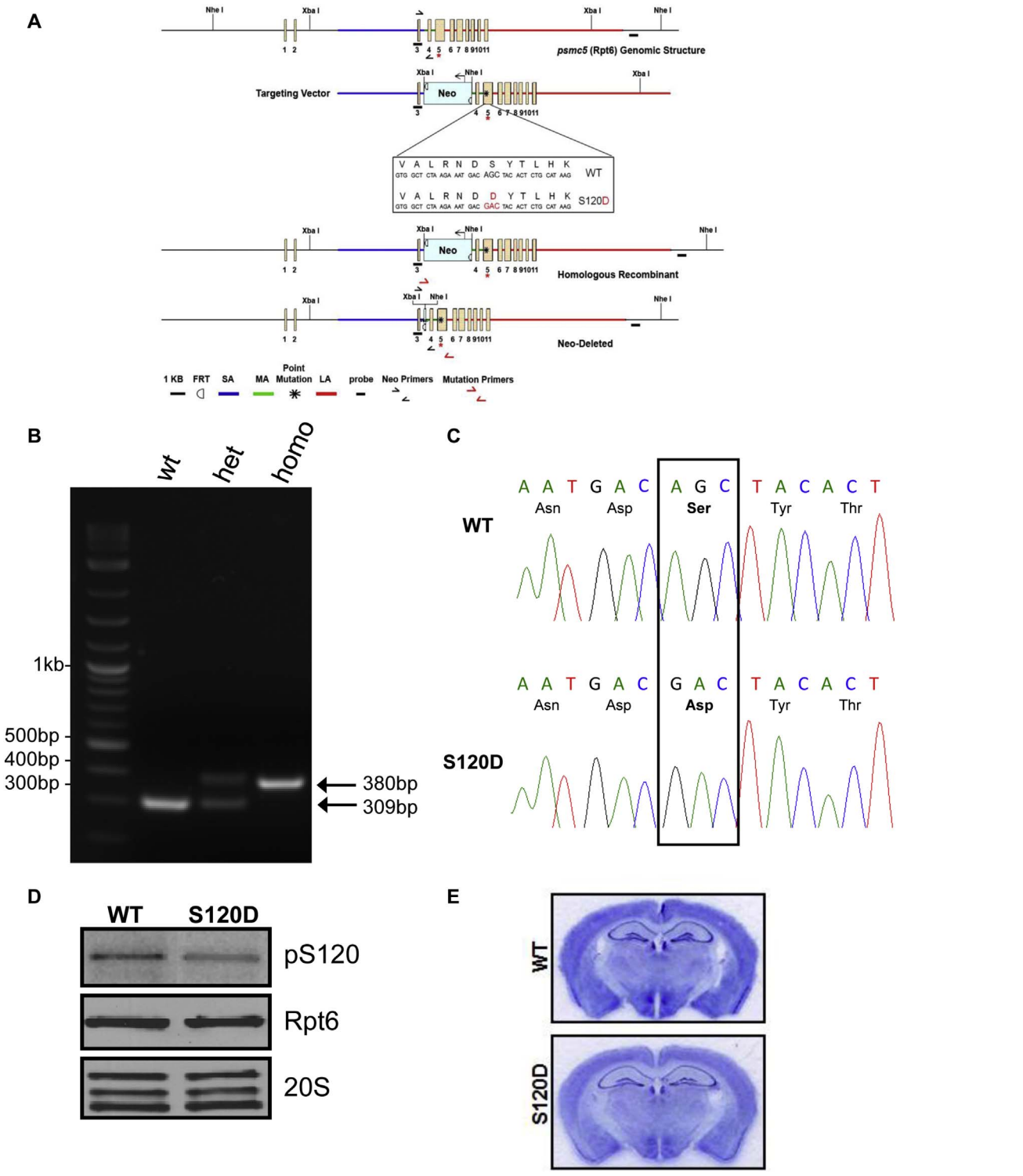

Fig. 2. Generation of *Rpt6* S120D Knockin (KI) mice. *A*, Schematic of targeting strategy. Genomic DNA structure of *psmc5 (Rpt6)* region is shown. The targeting vector was designed such that the long homology arm (LA) extends 5.98 kb 3′ to the first point mutation (asterisk; AG → GA, S120D) in exon 5. The FRT flanked Neo resistance cassette was inserted 463 bp 5′ to the point mutation. The short homology arm (SA) extends 2.79 kb 5′ to the FRT flanked Neo cassette. *B,* Tail genomic DNA was analyzed by PCR screening for genotyping and to verify deletion of the Neo cassette. *C*, Electropherograms confirming the presence of the mutation in homozygous Rpt6 S120D mutant male mice. *D*, Western blot analysis of affinity purified 26S proteasomes from Rpt6 S120D KI mice and wt littermates and probed with phospho Rpt6 pS120, total Rpt6 and 20S core antibodies. *E*, Representative images of Nissl stained fixed whole brain coronal sections of 60 day old mice.

Importantly, there was no difference in the acute (Day 1) response to cocaine between S120D and WT mice ($p = 0.76$). Session 4 locomotor activity was elevated in WT-Cocaine mice compared to all other groups [Fig. 4C, $F(3,36) = 4.57$, $p < 0.01$; S120D-Cocaine/WT-Cocaine $p = 0.01$]. Interestingly, locomotor activity did not differ between S120D-Cocaine mice and either saline group (S120D-Cocaine/S120D-Saline, $p = 0.64$; S120D-Cocaine/WT-Saline, $p = 0.67$). Sensitization was assessed as a difference between session 1 (acute) and session 4 (sensitized) activity (Fig. 4D). There were significant group differences [$F(3,36) = 3.70$, $p < 0.0.05$]. S120D-Cocaine mice did not sensitize,

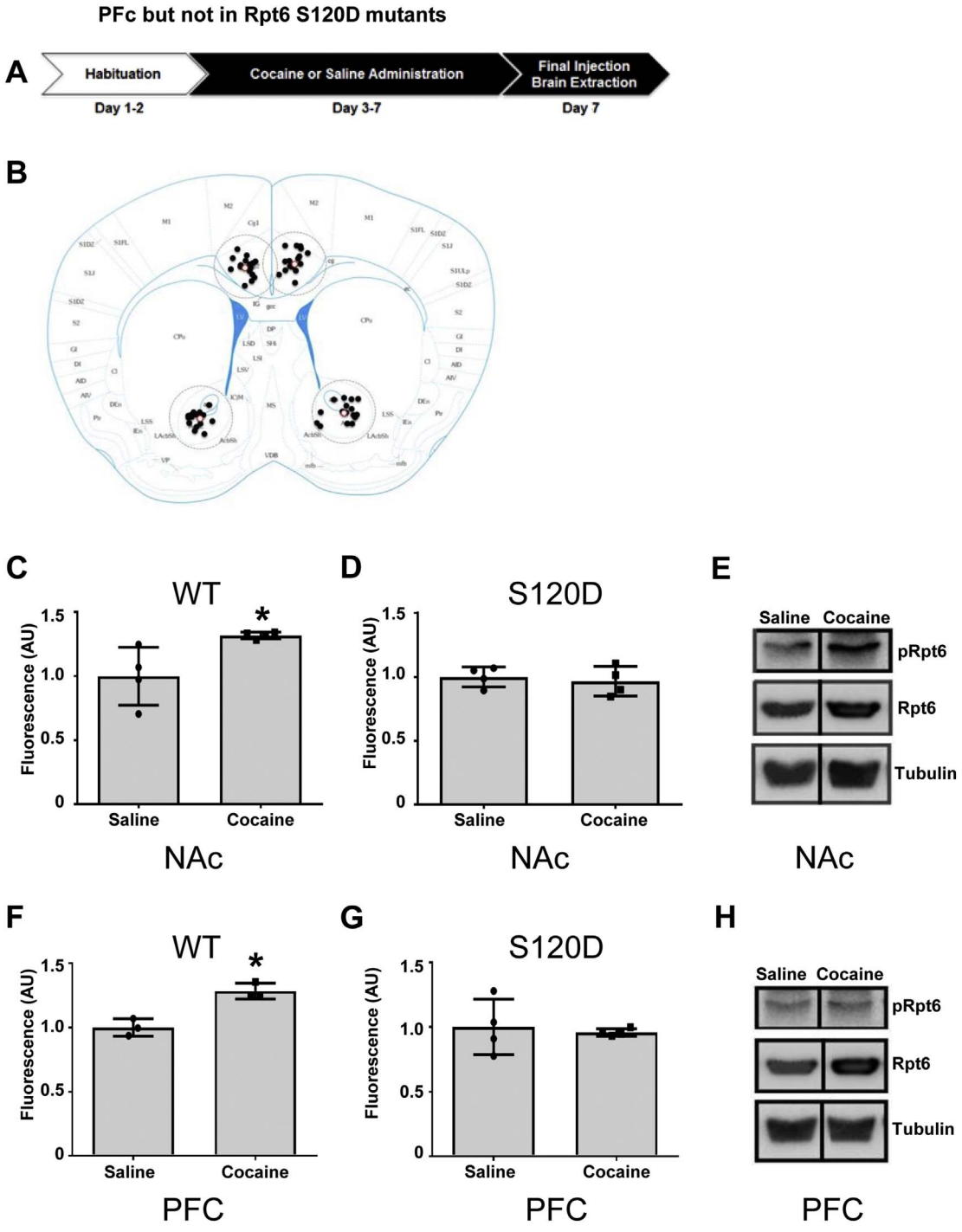

Fig. 3. Cocaine increases Rpt6 phosphorylation and proteasome activity in NAc and PFC in wildtype but not *Rpt6 S120D* KI mutant mice. *A*, Schematic of drug administration protocol. After a habituation period on days 1 and 2, cocaine (15 mg/kg, i.p) or saline was administered on days 3–7 and a final injection was delivered on day 8 immediately prior to brain extraction. *B*, 1 mm coronal sections were obtained using an acrylic matrix and tissue punches were taken from NAc and PFC. Depicted are the centers of individual punches (black dots), the arithmetic mean of the punch location (red dot), and average extent of all punches (dashed line). Lysates were prepared and chymotrypsin-like proteasome activity and Rpt6 phosphorylation was examined. *C thru E (NAc) and F thru H (PFC)*, Cocaine increases proteasome activity in NAc and PFC of wildtype (C, F) but not *Rpt6 S120D* KI mutant mice (D, G), measured by rate of substrate cleavage (mean ± S.E. fluorescence) normalized to saline-treated animals (*, $p < 0.05$, Mann-Whitney U test; n = 4: NAc: $U = 0$ and 7, $p = 0.029$ and 0.886 for wildtype and *Rpt6 S120D* cocaine to saline-treatments, respectively; and *, $p < 0.05$, Mann-Whitney *U* test; n = 3: $U = 0$ and 8, $p = 0.029$ and 0.999 for wildtype and *Rpt6 S120D* cocaine to saline-treatments, respectively). *E, H*, Representative western blot of lysates from wildtype NAc (E) and PFC (H) probed with phospho Rpt6 pS120, total Rpt6, and tubulin antibodies shows a dramatic increase in the NAc and slight increase in PFC for Rpt6 S120 phosphorylation in cocaine-treated animals compared to saline-treated controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

whereas WT-Cocaine mice demonstrated robust sensitization (Fig. 4D; $p = 0.008$). Again, there were no activity differences between S120D-Cocaine mice and saline control groups (S120D-Cocaine/S120D-Saline, $p = 0.87$; S120D-Cocaine/WT-Saline, $p = 0.79$).

4. Discussion

In order to assess the importance of proteasome-dependent protein degradation in addiction-related behavior we generated a novel line of mutant mice with a single point mutation on a single subunit of the 26S

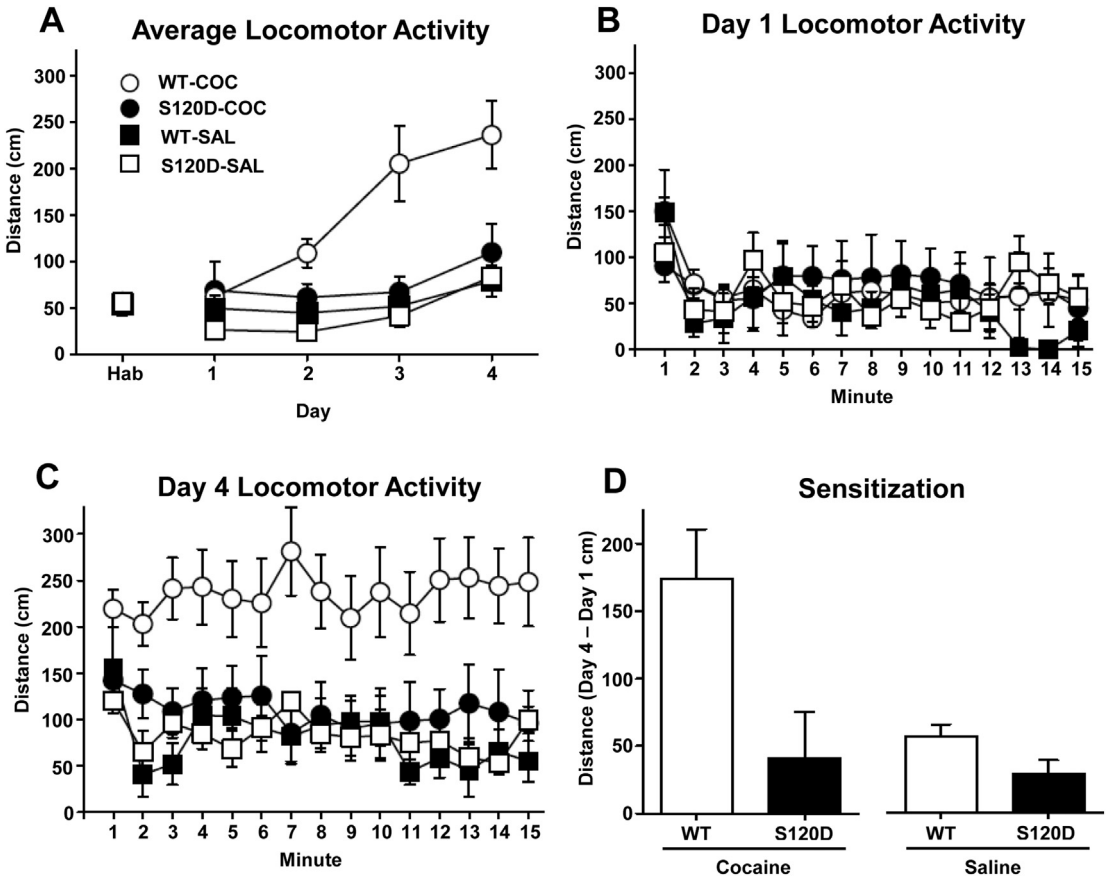

Fig. 4. Disruption of locomotor sensitization in S120D mice. *A,* S120D mice did not sensitize across 4 days of cocaine administration (n = 18 WT-Cocaine, n = 11 S120D-Cocaine, n = 6 WT-Saline, n = 5 S120D-Saline). The average distance traveled for each session (± SEM) is depicted. All mice showed a similar level of activity during habituation and day 1. However, during days 2–4, locomotor activity of WT-Cocaine mice was elevated compared to S120D-Cocaine mice and mice receiving saline (15 mg/kg, i.p; $p < 0.005$). *B,* Locomotor activity during day 1. The data represent the distance traveled per minute of the session (± SEM). There were no differences between the four groups ($p = 0.51$). *C,* Locomotor activity during day 4. Distance traveled per minute of the session (± SEM) is depicted. While the response to cocaine was elevated in WT-Cocaine mice, S120D-Cocaine mice did not exhibit locomotor activity different from saline groups (WT-Cocaine, S120D-Cocaine Fisher's PLSD $p = 0.01$; S120D-Cocaine, WT-Saline, S120D-Saline Fisher's PLSD $p > 0.5$). *D,* Sensitization was measured as the difference in locomotor activity between day 4 (sensitized response) and day 1 (acute response). The average difference in distance traveled (± SEM) per group is shown. S120D-Cocaine mice did not develop sensitization and did not differ from mice that received saline (S120D-Cocaine/S120D-Saline, S120D-Cocaine/WT-Saline, $p > 0.7$ WT-Cocaine mice displayed sensitized, elevated locomotor activity that differed from S120D-Cocaine mice ($p < 0.01$).

proteasome. Phosphorylation of the 19S ATPase subunit Rpt6 at S120 has been shown to be regulated by CaMKIIα in an activity-dependent manner, and increases in Rpt6 S120 phosphorylation correlated with increased proteasome activity (Figs. 1 and 3; Djakovic et al., 2012). Here, we demonstrate that Rpt6 phosphorylation at S120 is critical for cocaine-induced sensitization, a prominent addiction-related behavior. In cell culture, we observe a cocaine-induced significant increase in proteasome phosphorylation as well as chymotrypsin-like activity. Furthermore, we observed similar effects treating with methamphetamine, and not SERT-specific nor NET-specific inhibitors produced a similar effect. Together with TH immunoreactivity observed via western blot, as well as verification of mRNA expression via qPCR, we can conclude that this cocaine-induced response involves the dopaminergic system. In Rpt6 S120D mutant mice, Rpt6 S120 phosphorylation is locked in the phospho-mimetic state, behavioral sensitization to cocaine is completely absent, indicating the importance of dynamic regulation of Rpt6 S120 phosphorylation.

When treated with multiple administrations of cocaine, locomotor activity of S120D mice did not differ locomotor activity from mice receiving saline. It is important to note that while sensitization was blocked in these mice, the acute response to cocaine did not differ from WT animals (Fig. 4B). Thus, we are able to conclude that there is a disruption in nonassociative memory rather than simply an impaired response to cocaine.

Our results tend to support an interactionist view of memory and addiction (Volkow et al., 2002; Carmack et al., 2013). According to this model there are distinguishable associative and nonassociative components of addiction, and the molecular mechanisms and neural substrates underlying these processes may overlap with those involved in canonical forms of memory (Anagnostaras and Robinson, 1996; Anagnostaras et al., 2002; Russo et al., 2010). Here, we demonstrate that behavioral changes reflecting the nonassociative component of addiction, e.g. sensitization, that result from repeated drug administration are affected by changes to cellular plasticity mediated by the ubiquitin proteasome system.

Other recent studies have begun to investigate the importance of the ubiquitin proteasome system in addiction, specifically the effects of protein degradation inhibition during the development and expression of conditioned place preference (a task that models drug-seeking) and sensitization (Massaly et al., 2013; Ren et al., 2013). Development, but not expression of morphine-induced CPP was impaired by administration of a UPS inhibitor (Massaly et al., 2013). Similarly, treatment with a proteasome inhibitor during the induction of sensitization to morphine produced impairment (Massaly et al., 2013). Ren et al. (2013) investigated the effects of co-administration of a protein synthesis inhibitor and inhibitor of the UPS on cocaine-induced conditioned place preference. Co-treatment with a UPS inhibitor reversed the memory impairments typically produced by the administration of the protein synthesis inhibitor alone (Ren et al., 2013). Understanding the role of protein degradation during addiction is a young area of investigation

and many questions remain. The present study advances our current understanding by utilizing the first discrete mouse-model of altered proteasome function. This is important, as many of the previous studies have relied upon the use of inhibitors, which have been shown to be toxic to cells (Reaney et al., 2006).

We also found that the increase in proteasomal activity in the PFC and NAc typically induced by cocaine administration was absent in S120D KI mice, indicating that the effects of cocaine are occluded since we found an overall increase in proteasome activity from intact affinity purified 26S proteasomes from total brain homogenates in S120D KI mice (Gonzales and Patrick; data not shown). The PFC and NAc have previously been implicated in behavioral sensitization (Robinson and Kolb, 1997; Thomas et al., 2001; Steketee, 2003). Enduring morphological alterations were found in NAc and PFC neurons following amphetamine-induced sensitization including an increase in the length of dendrites, density of spines, and number of branched spines (Robinson and Kolb, 1997). These results highlight the long-lasting adaptations to synaptic connectivity that result from repeated experience with drugs of abuse. Specifically how these morphological changes contribute to addiction is unknown. Interestingly, the UPS has previously been shown to be implicated in spine stability and recently Rpt6 phosphorylation and proteasome function have been shown to regulate the formation of new dendritic spines (Hamilton et al., 2012). One intriguing possibility that may account for the present findings is that activity-dependent Rpt6 phosphorylation at serine 120 contributes to the structural changes that occur following repeated psychostimulant administration. Potentially, constitutively active phosphorylation at this site may interfere with the stimulant-induced morphological changes, thus preventing sensitization.

5. Conclusions

Surprisingly, we observed no locomotor sensitization of the Rpt6 S120D phospho-mimetic animals to cocaine, indicating this sensitization is occluded by the inability to modulate proteasome activity through its phosphorylation. Our results show that regulation of the proteasome itself to be important in cocaine-induced sensitization. However, future understanding of downstream UPS targets involved in these paradigms will be essential to better understand the molecular mechanisms underlying cocaine sensitization.

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