

Supersensitivity to Amphetamine in Protein Kinase-C Interacting Protein/HINT1 Knockout Mice

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Protein kinase C interacting protein/histidine triad nucleotide binding protein 1 (PKCI/HINT1) is a member of the histidine triad protein family. Although this protein is widely expressed in the mammalian brain including mesocorticolimbic and mesostriatal regions, its physiological function in CNS remains unknown. Recent microarray studies reported decreased mRNA expression of PKCI/HINT1 in the frontal cortex of individuals with schizophrenia, suggesting the possible involvement of this protein in the pathophysiology of the disease. In view of the documented link between dopamine (DA) transmission and schizophrenia, the present study used behavioral and neurochemical approaches to examine the influence of constitutive PKCI/HINT1 deletion upon: (i) basal and amphetamine (AMPH)-evoked locomotor activity; (ii) DA dynamics in the dorsal striatum, and (iii) postsynaptic DA receptor function. PKCI/HINT1^{-/-} (KO) mice displayed lower spontaneous locomotion relative to wild-type (WT) controls. Acute AMPH administration significantly increased locomotor activity in WT mice; nonetheless, the effect was enhanced in KO mice. Quantitative microdialysis studies revealed no alteration in basal DA dynamics in the striatum or nucleus accumbens of KO mice. The ability of acute AMPH to increase DA levels was unaltered indicating that function in presynaptic DA neurotransmission in these regions do not underlie the behavioral phenotype of KO mice. In contrast to WT mice, systemic administration of the direct-acting DA receptor agonist apomorphine (10 mg/kg) significantly increased locomotor activity in KO mice suggesting that postsynaptic DA function is altered in these animals. These results demonstrate an important role of PKCI/HINT1 in modulating the behavioral response to AMPH. Furthermore, they indicate that the absence of this protein may be associated with dysregulation of postsynaptic DA transmission.

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INTRODUCTION

Protein kinase C interacting protein/histidine triad nucleotide binding protein 1 (PKCI/HINT1) is an ubiquitous member of the histidine triad (HIT) protein family characterized by the presence of a conserved HIT (HisX-HisXHis, X is a hydrophobic amino acid) sequence motif (Klein *et al*, 1998). The amino-acid sequences of the members of this family are conserved in a broad range of organisms including mycoplasma, plants, and mammals (Lima *et al*, 1996). Although murine PKCI/HINT1 is expressed in relatively high concentrations in brain, liver, and kidney, little is known about the physiological role of PKCI/HINT1 proteins. Studies on the function of PKCI/

HINT1 can only be found in a limited numbers of literatures. Bovine PKCI/HINT1 was originally identified as an inhibitor of PKC isoforms *in vitro* (McDonald and Walsh, 1985). However, the relevance of these *in vitro* effects to the physiological function of PKCI/HINT1 has been questioned (Klein *et al*, 1998). Studies in which X-ray crystallography and *in vitro* enzyme assays were used to elucidate the structure and function of PKCI/HINT1, suggested that it possesses nucleotidyl hydrolase or transferase activity (Lima *et al*, 1997). PKCI/HINT1 has also been shown to interact with the ataxia-telangiectasia group D protein and the *mi* transcription factor in the yeast two-hybrid system (Brzoska *et al*, 1995). PKCI/HINT1 KO mice display increased susceptibility to carcinogenicity, suggesting that PKCI/HINT1 may normally play a tumor suppressor role (Su *et al*, 2003). Later, the protein has been reported to interact with pontin and reptin and inhibit TCF- β -catenin-mediated transcription (Weiske and Huber, 2005).

To date, only one study has examined the CNS actions of PKCI/HINT1. Using yeast two-hybrid screening, we recently

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showed that PKCI/HINT1 specifically interacts with the C-terminus of the mu opioid receptor (MOR) (Guang *et al*, 2004). This interaction led to an attenuation of receptor desensitization and inhibition of PKC-induced MOR phosphorylation. Furthermore, a deficiency in the expression of PKCI/HINT1 in mice enhanced both basal and morphine-induced antinociception. Morphine tolerance was also increased.

Recently, PKCI/HINT1 was identified as one of the candidate molecules in the neuropathology of schizophrenia *via* microarray analysis (Vawter *et al*, 2001, 2002). Expression of this gene was significantly decreased in cortical samples from patients with schizophrenia compared with age- and gender-matched controls. This change was subsequently validated by real-time quantitative polymerase chain reaction (PCR) (Vawter *et al*, 2004). Schizophrenia is a complex human disorder with unknown etiology. Several lines of evidence, however, suggest that increased dopaminergic neurotransmission may contribute to the pathophysiology of this disease (Seeman, 1987, Lewis and Lieberman, 2000). PET studies have revealed increased D2 receptor occupancy in patients with schizophrenia (Abi-Dargham *et al*, 2000) and DA receptor antagonists are used for the treatment of this disorder (Baldessarini and Tarazi, 2006). Amphetamine-evoked hyperactivity and stereotypy are used to model the positive symptoms of schizophrenia in rodents and reversal of these effects is considered as a desired property of antipsychotic drugs (Segal *et al*, 1981). This premise led us to hypothesize that PKCI/HINT1 may play a role in dopaminergic function. Accordingly, in the present study, WT mice and mice lacking the gene encoding PKCI/HINT1 protein were used to assess the involvement of PKCI/HINT1 in: basal and amphetamine (AMPH)-evoked locomotor activity, DA dynamics in the dorsal striatum, and postsynaptic DA receptor function. We also examined the distribution of PKCI/HINT1 in specific brain regions, including both mesocorticolimbic and mesostriatal areas, which are enriched in dopaminergic neurons.

METHODS

PKCI/HINT1^{-/-} Mice

The generation of PKCI/HINT1^{-/-} mice was described previously and these mice have 96% of their genetic background from the 129 strain mice (Su *et al*, 2003). The breeding pairs of PKCI/HINT1^{-/-} mice and their wild-type (WT) littermates were received from Dr IB Weinstein at Columbia University where the original KO mice were generated. Drug naïve animals used for the present study were homozygous mutant mice and WTs derived from homozygous mutant breeding pairs or WT breeding pairs, respectively. In addition, KO and their littermate controls were also used in AMPH 2.5 mg/kg experiments. The genotype of all progeny was confirmed by using PCR analysis of DNA extracted from tail biopsies. Animals were housed 4–5/cage and maintained under standard laboratory condition with food and water provided *ad libitum*. Male animals were tested between 10 and 20 weeks of age. WT and PKCI/HINT1^{-/-} groups were matched for age in all experiments. All studies were conducted with an approved protocol of University of Maryland, School of Pharmacy

IACUC, or the IACUC of the NIH/NIDA IRP, and conformed to the NIH Guidelines for the Care and Use of Laboratory Animals.

Locomotor Activity Measurement

Basal and AMPH-evoked locomotor activity were monitored using a MED Associates' open-field activity monitoring system (Med Associates Inc., St Albans, VT). The chamber (27 × 27 × 20.3 cm) had sensor rings designed for use with mice. Each station has an on-board microprocessor to process beam breaks and prepare coordinate data for further analysis. Automated measurements of fine locomotion were previously validated (Sanberg *et al*, 1987). In our case, horizontal spontaneous locomotion activity, scored as ambulatory counts and stereotypic movements per 5 min, configured according to the manufacturer's manual, were recorded during the first 30 min of acclimation to the novel environment and (1) for the consecutive 60 min of spontaneous activity or (2) for the consecutive 120 min following drug treatment. Stereotypic behavior is defined as small and fast movements occurring when the animal breaks the same beam pattern repeatedly. This beam pattern is a square of the size of the animal, each episode is separated by 100 ms. Ambulation is defined as big and slow movements of the animal breaking a bigger beam pattern of two times the size of the animal. Data are expressed as total movement over the total recoded time. The animals were acclimated to a 12 h cycle light/dark cycle with the light on at 07:00 hours before the experiments. Dark phase experiments were conducted between 21:00 and 04:00 hours; all other tests were performed during the light phase of the cycle between 08:00 and 15:00 hours in the room where temperature was set at 23 ± 2°C. AMPH or other drugs were prepared freshly before each experiment by dissolving in saline (NaCl 0.9%) and administered *via* the intraperitoneal (i.p.) route in a 10 ml/kg volume of the indicated doses.

In Vivo Microdialysis

PKCI/HINT1^{-/-} mice and their WT controls were anesthetized and implanted unilaterally with a microdialysis guide cannula (CMA/11, CMA microdialysis) in the nucleus accumbens (AP: +1.5, L: -0.8, V: -3.8 mm from bregma) or the dorsal striatum (AP: +0.4, L: -2.1, V: -2.2 mm from bregma) using standard stereotaxic techniques, and allowed to recover for 5 days before the microdialysis experiment as described (Chefer and Shippenberg, 2006). Twelve hours before the start of experiments, a microdialysis probe (CMA/11, 1 or 2 mm membrane length for accumbens and dorsal striatum, respectively, CMA microdialysis, North Chelmsford, MA) was connected to the dialysis system and flushed with artificial cerebrospinal fluid (aCSF: 145 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 0.25 mM ascorbic acid, and 5.4 mM D-glucose, pH 7.2 adjusted with NaOH 0.5 M). The mouse was gently restrained and the probe was slowly inserted into the guide cannula. The dialysis system consisted of FEP tubing (CMA microdialysis) that connected the probe to a 1 ml gastight syringe (Hamilton Co., Reno, NV) mounted on a microdialysis pump (CMA/102) through a quartz-lined, low-resistance

swivel (375/D/22QM, Instech, Plymouth Meeting, PA). After probe insertion, the mouse was placed in the dialysis chamber with food and water freely available, and the probe perfused overnight with aCSF at a flow rate of 0.6 μ l/min. The next morning, the perfusion syringes were loaded with fresh aCSF and probes were allowed to equilibrate for an additional 1 h before the commencement of experiments. A flow rate of 0.6 μ l/min was used for all studies.

For no net flux experiments, five different concentrations of DA (0, 5, 10, 20, and 40 nM) in aCSF were perfused in random order through the dialysis probe. Each DA concentration was perfused for 30 min, and then 2×10 min samples were collected. Following completion of the no net flux experiments, normal aCSF was again perfused through the probe for 30 min, allowing for a period of equilibration. Consecutive 15 min samples were then collected. After three baseline samples, mice received a saline i.p. injection and three more samples were collected. Then mice received a 2.5 mg/kg D-AMPH i.p. injection and samples were collected for an additional 90 min. Samples were stored at -80°C until analysis. The DA content was determined by HPLC coupled to electrochemical detection with external standards for quantification. All the samples were analyzed within 48 h of collection. After experiments, mice were killed by a pentobarbital overdose and their brains were removed, frozen on dry ice, and 20 μ m sections were obtained on a cryostat for the histological verification of probe location. No net flux data were analyzed as described (Chefer and Shippenberg, 2006). The net flux of DA through the probe (DA_{in}–DA_{out}) was calculated and plotted against the concentration of DA perfused (DA_{in}). The following parameters were calculated from the resulting linear function. The *y* axis intercept, corresponding to zero DA perfused through the probe is the dialysate DA concentration (DA_{dial}) in a conventional microdialysis experiment. The *x* axis intercept corresponds to the point of equilibrium where there is no net flux of DA through the probe and provides an unbiased estimate of extracellular DA concentration (DA_{ext}). The slope of the regression line corresponds to the extraction fraction (Ed) which has been shown to be an indirect measure of DA uptake (Smith and Justice, 1994; Chefer and Shippenberg, 2006). In the conventional microdialysis experiments investigating the effects of AMPH, the average of the three baseline samples was calculated, and all the DA concentrations were expressed as % of baseline. Differences between WT and KO animals in the appropriate variables were assessed by comparing both groups using a Student's *t*-test.

Immunohistochemistry

Animals and preparation of tissue: PKCI/HINT1^{-/-} and WT control adult mice were anesthetized with 7.5 mg ketamine hydrochloride (Pfizer AB, Sweden) and 2.5 mg xylazine (Veterinaria AG, Switzerland) per 100 g body weight i.p. Animals were perfused transcardially with saline followed with 4% paraformaldehyde (PFA) in phosphate buffer (0.1 M, pH 7.4) for 10 min. The whole brain was removed, postfixed in 4% PFA at 4°C overnight, and equilibrated with 30% sucrose in phosphate buffer at 4°C for 48 h. Embedded whole brain (OCT, Tissue-Tek, Sakura Finetek

USA Inc.) was cut coronally into 25 μ m sections using a cryostat (OTF5000, Bright, Jencons, UK). All sections were kept with long-term protecting solution at 4°C until used.

Floating sections were incubated with 1% hydrogen peroxide in 70% methanol-tris buffered saline (TBST; 0.1 M Tris, pH 7.4, 0.9% saline, and 0.3% Triton X-100) for 30 min at room temperature (all incubations were performed at 22–25°C) to inhibit endogenous peroxidase, then washed three times with TBST and incubated for 1 h in 1% bovine serum albumin (BSA)—TBST. For staining, the sections were incubated with hPKCI antiserum (1:10 000 dilution in TBST, 1% BSA, 24 h), washed in TBST (three times of 5 min duration each), incubated for 1 h in biotinylated donkey anti-rabbit serum (1:1000 dilution; Biotin-SP-Conjugated Donkey Anti-Rabbit IgG (H+L), Jackson ImmunoResearch Lab Inc., West Grove, PA, USA), rinsed, and finally visualized with ABC reagent (ABC Kit PK-6100, Vector, USA) according to manufacturer's manual and mounted onto gelatin-coated slides in DPX mounting medium.

Slides were viewed using Nikon E800 microscope, images were captured with Nikon digital camera, and edited using Photoshop (v CS; Adobe Systems Inc., San Jose, CA, USA).

For immunofluorescence staining, sections were incubated for 1 h at room temperature with 1% BSA—1% normal donkey serum in Tris-buffered saline (TBST; 0.1 M Tris, pH 7.4, 0.9% saline, and 0.3% Triton X-100), then for 24 h in hPKCI antiserum (1:1000 dilution in 1% BSA/TBST), washed in TBST (three times of 5 min each), followed by 1 h incubation with Cy2-conjugated donkey anti-rabbit antibody (1:500 dilution; Jackson ImmunoResearch Lab Inc., West Grove, PA, USA). After wash, they were incubated for 24 h in a mixture of mouse anti-NeuN (1:1000 dilution in 1% BSA/TBST; Neuronal Nuclei, Chemicon) and goat anti-GFAP (1:500 dilution in 1% BSA/TBST; glial fibrillary acidic protein, Santa Cruz Biotechnology Inc.) and washed with TBST as above. The sections were then incubated with a mixture of Cy5-conjugated donkey anti-mouse antibody and Cy3-conjugated donkey anti-goat antibody (1:500 in 1% BSA/TBST; Jackson ImmunoResearch Lab Inc., West Grove, PA) for 60 min, washed in TBST as above, incubated with 2 nM DAPI (4'-6-diamidino-2-phenylindole, Molecular Probes) for 60 min, washed in TBS, and mounted. Slides were viewed under Nikon E800 microscope and images were captured on a Fluo View X confocal microscope (Olympus Instruments, CA, USA).

Statistical Analysis

Two-way analyses of variance (ANOVA) of genotype \times phase and genotype \times treatment were used to compare the results of the behavioral experiments. When ANOVA was significant ($p < 0.05$), *post hoc* comparisons between groups were made using Bonferroni test. Comparison of the parameters derived from the no net flux analysis was performed by a Student's *t*-test. Data are presented as mean \pm SEM. Statistical analyses were performed using Graphpad Prism version 4.00 for Windows (Graphpad Software, San Diego, CA, USA).

RESULTS

PKCI/HINT1^{-/-} Mice Display Decreased Spontaneous Locomotor Activity and Supersensitive Response to Amphetamine

Spontaneous locomotor activity measured during the light phase of the cycle (between 08:00 and 15:00 hours) and during the dark phase of the cycle (between 21:00 and 04:00 hours) which corresponds to the active phase, is shown in Figure 1. During the light phase, WT and KO mice displayed a lower level of spontaneous activity, measured as ambulatory and stereotypic movement for 60 min. In the dark phase, both genotypes exhibited an increase of locomotion as expected (light phase vs dark phase: WT: $p < 0.0001$ for acclimation and spontaneous; KO: $p < 0.0001$ for acclimation and $p < 0.01$ for spontaneous; Bonferroni). However, the KO mice consistently scored on average 40% lower than the WT in either the light or the dark phase (WT vs KO: stereotypy: $p < 0.01$ light phase acclimation and dark phase spontaneous; ambulation: $p < 0.0001$ dark phase acclimation and spontaneous; Bonferroni). These results indicate that the KO mice are hypolocomotive when tested

under habituated basal conditions (light/dark phases) or during exploration of a novel environment (acclimation phase) in comparison with the WT mice.

Rodent locomotor activity is known to be affected by CNS stimulants. The effect of AMPH on the locomotor activity of PKCI KO mice was examined in this study. As shown in Figure 2, AMPH (NIDA drug supply program) at a dose of 2.5 mg/kg increased locomotion in both WT and KO (ambulation: $p < 0.0001$ vs saline in KO; stereotypy: $p < 0.01$ vs saline in WT, $p < 0.0001$ vs saline in KO; Bonferroni). However, AMPH-evoked locomotor activity was significantly enhanced (≥ 3 -fold) in PKCI KO mice (WT vs KO: $p < 0.0001$ for ambulation and stereotypy). To determine whether this enhanced response was selective or occurred in response to other drug of abuse, locomotor activity in response to the MOR agonist morphine was assessed; the response to the GABA antagonist bicuculline was also examined. Morphine (NIDA drug supply program) and bicuculline (RBI, Natick, MA, USA) at a dose of 10 and 1 mg/kg, respectively, did not promote increase in locomotor activity as shown in Figure 2. The increase of AMPH-evoked activity in the KO mice was observed over a range of

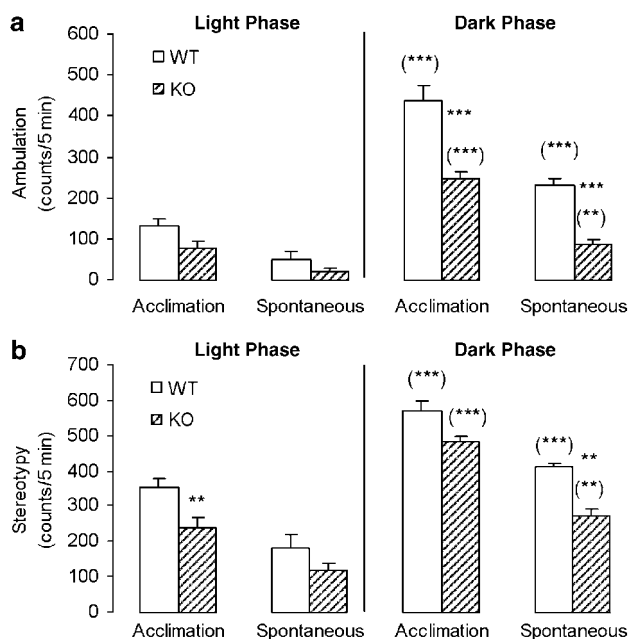


Figure 1 Spontaneous locomotion activity in the WT and PKCI/HINT1^{-/-} mice during light/dark phase of the cycle. Locomotion was monitored during the 30 min of acclimation to the novel environment followed by a 1 h period. Counts per 5 min were averaged over the 30 min of acclimation and the consecutive 1 h of spontaneous activity; mean \pm SEM are represented. (a) Ambulation during acclimation (for phase, $F_{(1,26)} = 119.21$, $p < 0.0001$; for genotype, $F_{(1,26)} = 30.07$, $p < 0.0001$; for interaction $F_{(1,26)} = 9.47$, $p = 0.0049$) and during the following 1 h of spontaneous activity (for phase, $F_{(1,26)} = 78.53$, $p < 0.0001$; for genotype, $F_{(1,26)} = 39.84$, $p < 0.0001$; for interaction $F_{(1,26)} = 17.94$, $p = 0.0003$). (b) Stereotypy during acclimation (for phase, $F_{(1,26)} = 44.85$, $p < 0.0001$; for genotype, $F_{(1,26)} = 12.77$, $p = 0.0014$; for interaction $F_{(1,26)} = 0.29$, $p = 0.5937$) and during the following 1 h of spontaneous activity (for phase, $F_{(1,26)} = 81.55$, $p < 0.0001$; for genotype, $F_{(1,26)} = 16.13$, $p = 0.0004$; for interaction $F_{(1,26)} = 1.74$, $p = 0.2111$). (**) $p < 0.01$ and (***) $p < 0.0001$ compare dark phase vs light phase; $p < 0.01$, (***) $p < 0.0001$ compare WT vs KO; WT and KO light phase $n = 9$; WT and KO dark phase $n = 6$; two-way ANOVA genotype \times phase, followed by Bonferroni.

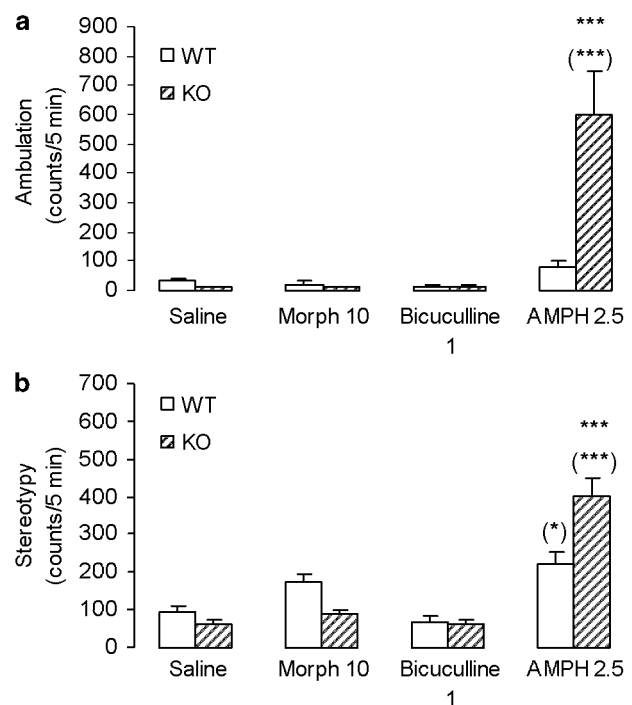


Figure 2 Acute morphine, bicuculline, and D-AMPH effect on ambulation and stereotypy. Saline (10 ml/kg i.p.), morphine (10 mg/kg i.p.), bicuculline (1 mg/kg i.p.), and D-AMPH (2.5 mg/kg i.p.) were administered after the period of acclimation and locomotion was measured during the following 120 min. Results are expressed as mean of the counts over the 120 min \pm SEM. (a) Ambulation (for treatment $F_{(3,56)} = 4.64$, $p = 0.0057$; for genotype $F_{(1,56)} = 2.59$, $p = 0.1131$; for interaction $F_{(3,56)} = 3.01$, $p = 0.0376$). (b) Stereotypy (for treatment $F_{(3,56)} = 17.5$, $p < 0.0001$; for genotype $F_{(1,56)} = 0.3$, $p = 0.587$; for interaction $F_{(3,56)} = 4.38$, $p = 0.0077$). (*) $p < 0.05$ and (***) $p < 0.0001$ compare saline vs treatment; (***) $p < 0.0001$ compares WT vs KO; $n = 5$ –12 for WT and $n = 6$ –14 for KO. Saline and bicuculline WT and KO $n = 6$; morphine WT $n = 5$, KO $n = 9$; AMPH WT $n = 12$, KO $n = 14$; two-way ANOVA genotype \times treatment, followed by Bonferroni.

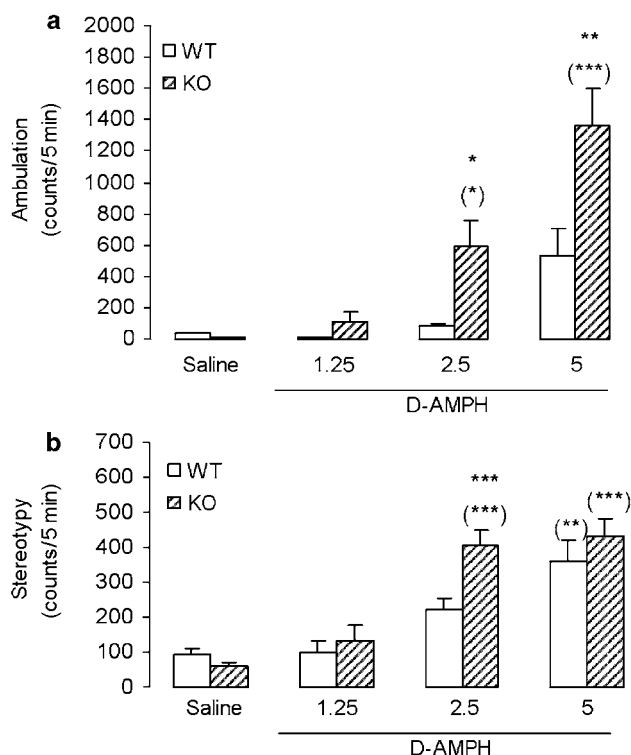


Figure 3 Dose-response to acute D-amphetamine in WT and PKCI/HINT1^{-/-} mice. Saline (10 ml/kg i.p.) or D-AMPH (1.25, 2.5, and 5 mg/kg i.p.) was administered after the period of acclimation and locomotion was measured during the following 120 min. (a) Ambulation (for treatment $F_{(3,57)}=15.44$, $p<0.0001$; for genotype $F_{(1,57)}=10.79$, $p=0.0018$; for interaction $F_{(3,57)}=3.25$, $p=0.0284$). (b) Stereotypy (for treatment $F_{(3,57)}=24.75$, $p<0.0001$; for genotype $F_{(1,57)}=7.02$, $p=0.01$; for interaction $F_{(3,57)}=4.15$, $p=0.0099$). (*) $p<0.05$, (**) $p<0.01$, and (***) $p<0.0001$ compare saline vs AMPH; * $p<0.05$, ** $p<0.01$, and *** $p<0.0001$ compare WT vs KO. Saline and AMPH 1.25 mg/kg WT and KO $n=6$; AMPH 2.5 mg/kg WT = 12 and KO = 14 and AMPH 5 mg/kg WT $n=6$ and KO $n=9$; two-way ANOVA genotype \times treatment, followed by Bonferroni.

AMPH doses as shown in Figure 3 (ambulation in KO: $p<0.05$ for AMPH 2.5 mg/kg vs saline, $p<0.001$ for AMPH 5 mg/kg vs saline; Bonferroni). To determine whether the enhancement of activity could be due to a decrease in stereotypy, this behavior was also assessed simultaneously. Similarly, AMPH produces a dose-related increase in stereotypy (stereotypy in KO: $p<0.0001$ for AMPH 2.5 and 5 mg/kg vs saline; Bonferroni). As shown in Figures 2 and 3, lack of PKCI/HINT1 is associated with an increase in both AMPH-evoked ambulation (WT vs KO: $p<0.05$ for AMPH 2.5 mg/kg; $p<0.01$ for AMPH 5 mg/kg; Bonferroni) and stereotypy (WT vs KO: $p<0.0001$ for AMPH 2.5 mg/kg; Bonferroni).

Amphetamine increases extracellular DA levels by stimulating its release and by reversing DA transport. Interactions of AMPH with other biogenic amines have also been reported. Therefore, in order to probe specifically the DA system, additional studies were conducted to determine whether PKCI/HINT1 deletion alters the locomotor activating effects of the selective DAT inhibitor, GBR 12909 (TOCRIS, Ellisville, MI, USA). Analogous to AMPH, a significant enhancement of GBR 12909-evoked locomotion

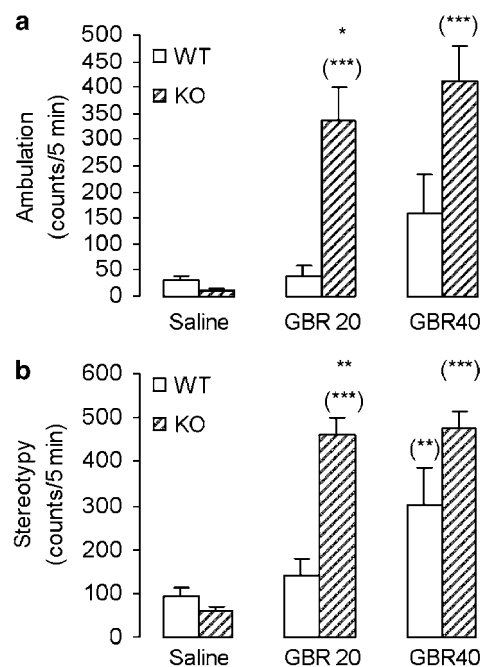


Figure 4 Dose-response to acute GBR 12909 in WT and PKCI/HINT1^{-/-} mice on locomotion. Saline (10 ml/kg i.p.) or GBR 12909 (20 and 40 mg/kg i.p.) was administered after the period of acclimation and locomotion was measured during the following 120 min. (a) Ambulation (for treatment $F_{(2,38)}=11.35$, $p<0.0001$; for genotype $F_{(1,38)}=14.67$, $p=0.0005$; for interaction $F_{(2,38)}=4.6$, $p=0.0162$). (b) Stereotypy (for treatment $F_{(2,38)}=25.22$, $p<0.0001$; for genotype $F_{(1,38)}=17.97$, $p=0.0001$; for interaction $F_{(2,38)}=7.82$, $p=0.0014$). (**) $p<0.01$ and (***) $p<0.0001$ compare saline vs GBR; * $p<0.05$ and ** $p<0.01$ compare WT vs KO; $n=7$ for WT and $n=9$ for KO; two-way ANOVA genotype \times treatment, followed by Bonferroni.

was seen from the KO mice. As shown in Figure 4, GBR 12909 elicited an increase in locomotion in both WT and KO mice at a dose of 40 mg/kg ($p<0.0001$ vs saline; Bonferroni). The dose of 20 mg/kg induced hyperlocomotion only in the KO animals (WT vs KO: $p<0.05$ for ambulation, $p<0.01$ for stereotypy; Bonferroni). These results indicate that mice without PKCI/HINT1 are supersensitive to AMPH and this enhanced response is likely mediated through the dopaminergic system, as it is mimicked by a selective DA transporter blocker.

Extracellular DA Levels in Nucleus Accumbens and Dorsal Striatum

DA projections to the striatum play an important role in the control of locomotor activity. Therefore, we used the technique of *in vivo* microdialysis in order to investigate the consequences of genetic deletion of the PKCI/HINT1 protein on basal extracellular DA dynamics as well as on AMPH-evoked alterations in DA levels of dialysate. Quantitative no net flux microdialysis revealed no significant differences in basal dialysate (DAdial) levels or in the estimated DAext) in the nucleus accumbens (Figure 5, $t=-0.47$, $df=16$, $p=0.65$ and $t=0.04$, $df=16$, $p=0.97$ for DAdial and DAext, respectively). Moreover, the DA extraction fraction (Ed), calculated as the slope of the no net flux regression line was unchanged in KO mice ($t=0.15$, $df=16$,

$p = 0.88$), suggesting that deletion of the PKCI/HINT1 did not alter the clearance of extracellular DA by the DA transporter (Smith and Justice, 1994; Chefer and Shippenberg, 2006). Similar negative results were obtained in the caudate putamen (DA_{dial}: $t = 0.58$, $df = 10$, $p = 0.58$; DA_{ext}: $t = 1.01$, $df = 10$, $p = 0.33$; Ed: $t = 0.81$, $df = 10$, $p = 0.43$). Conventional microdialysis revealed a marked increase in basal dialysate DA levels in response to AMPH. No significant difference between WT and KO mice in AMPH-evoked DA levels was observed in the dorsal striatum ($t = 0.58$, $df = 10$, $p = 0.58$). The maximal AMPH response was 1024 ± 104 and $1103 \pm 89\%$ of basal levels in WT and KO mice, respectively. Similarly, no genotype difference was observed in the nucleus accumbens (maximal AMPH response 405 ± 73 vs $403 \pm 47\%$ of baseline in WT and KO mice, respectively, $t = 0.02$, $df = 16$, $p = 0.98$). As the dose of AMPH used in microdialysis studies was that

producing an enhanced locomotor response in KO mice, the lack of genotype differences in the AMPH-evoked DA response suggests that the enhanced behavioral sensitivity to AMPH is due to changes at the postsynaptic rather than presynaptic level.

Apomorphine Induced a Differential Hyperlocomotor Activity in PKCI/HINT1^{-/-} Mice

To further explore the mechanism responsible for the behavioral supersensitivity to AMPH observed in the KO mice, we tested the locomotor response to the nonselective DA receptor agonist, apomorphine. A high dose (10 mg/kg) was used in order to probe postsynaptic DA receptor function. Data shown in Figure 6 revealed that the KO mice exhibited significantly higher (two- to threefold) locomotor activity as compared to WT in both total ambulation ($p < 0.01$; Bonferroni) and stereotyped behavior ($p < 0.0001$; Bonferroni). This result indicates that PKCI/HINT1 deletion is associated with postsynaptic DA receptor supersensitivity.

PKCI/HINT1 Brain Distribution and Neuronal Expression

Western blotting was used to determine the expression of PKCI/HINT1 in the cortex, cerebellum, midbrain, and

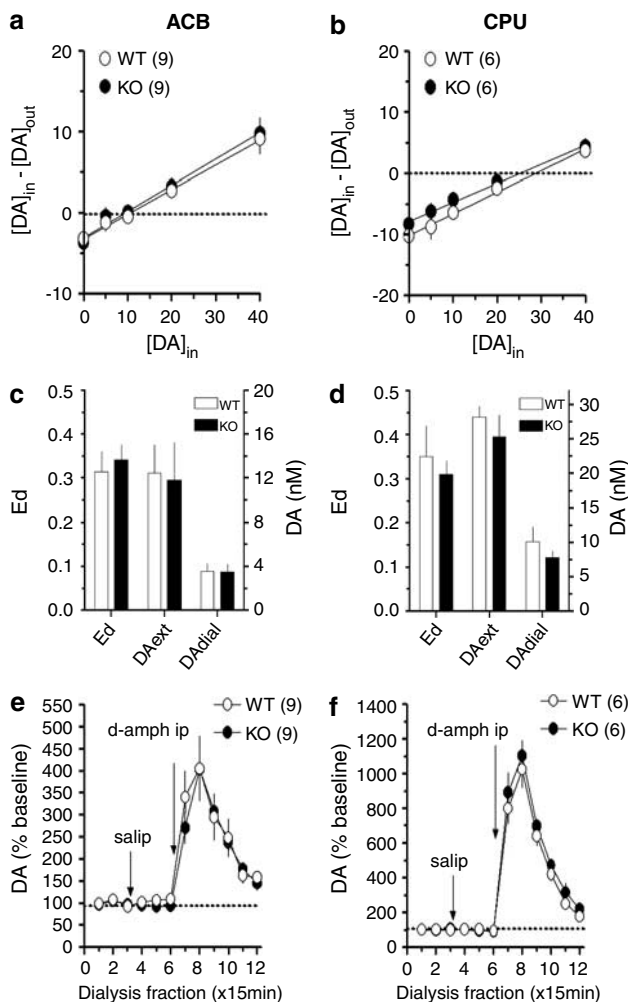


Figure 5 Basal and amphetamine-evoked extracellular DA in the nucleus accumbens and caudate-putamen of WT and PKCI/HINT1^{-/-} mice. No net flux microdialysis was used to assess the status of extracellular DA in the basal state. No significant genotype differences were observed in the estimated DA_{ext} (a, b) nor in the extraction fraction (Ed; c, d) suggesting that both release and uptake of DA are unchanged in the KO mice. In addition the amphetamine-evoked DA response did not differ in WT and KO animals (e, f). The same results were obtained in both, the nucleus accumbens (a, c, and e) and the caudate-putamen (b, d, and f).

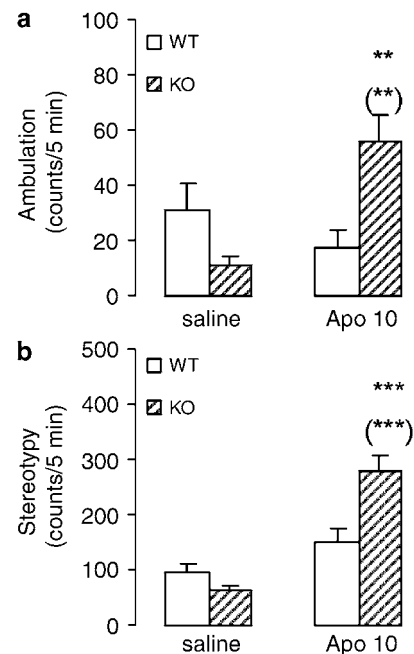


Figure 6 Apomorphine-induced hyperlocomotion in PKCI/HINT1^{-/-} mice. Saline (10 ml/kg i.p.) or apomorphine (10 mg/kg i.p.) was administered after the period of acclimation and locomotion was measured during the following 120 min. Results are expressed as mean \pm SEM of total scores. (a) Ambulation (for treatment $F_{(1,18)} = 4.26$, $p = 0.0538$; for genotype $F_{(1,18)} = 1.55$, $p = 0.2296$; for interaction $F_{(1,18)} = 14.6$, $p = 0.0013$). (b) Stereotypy (for treatment $F_{(1,18)} = 49.32$, $p < 0.0001$; for genotype $F_{(1,18)} = 5.91$, $p = 0.0257$; for interaction $F_{(1,18)} = 16.51$, $p = 0.0007$). (***) $p < 0.001$ and (****) $p < 0.0001$ compares saline vs Apo; ** $p < 0.01$ and *** $p < 0.0001$ compares WT vs KO. Saline WT and KO $n = 6$; APO WT and KO $n = 5$; two-way ANOVA genotype \times treatment, followed by Bonferroni.

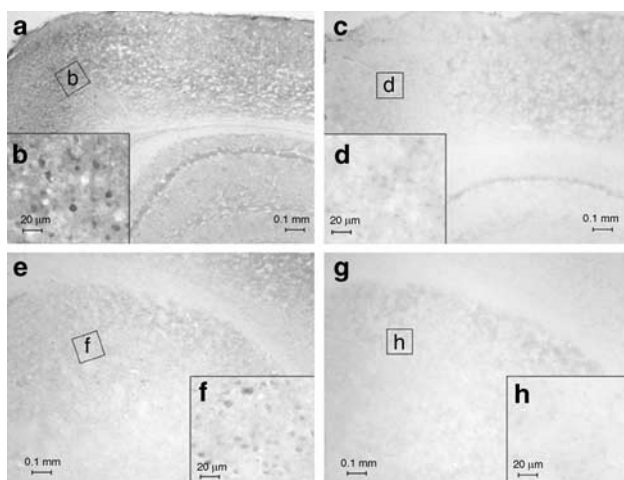


Figure 7 PKCI/HINT1 immunoreactivity in mouse brain region of frontal cortex and caudate putamen (striatum). (a, b and e, f) WT mouse; (c, d and g, h) KO mouse. PFA-fixed frozen brain coronal sections were treated with rabbit anti-PKCI polyclonal antiserum, dilution 1:10 000, followed by biotin-SP-conjugated donkey anti-rabbit IgG, dilution 1:1000. Dark blue staining represents PKCI/HINT1 positive cells. (a–d) frontal cortex; (e–h) caudate putamen (striatum).

spinal cord of KO and WT mice (data not shown). These experiments revealed a broad expression pattern of PKCI/HINT1 in brain and spinal cord of mice, suggesting an important role for PKCI/HINT1 in neurological system. In contrast, no expression was observed in KO mice confirming the absence of this gene. Immunohistochemical analysis of PKCI staining in WT mice revealed a localization pattern consistent with neurons and neuronal processes in various brain regions including frontal cortex and striatum (Figure 7). There was only low-level background staining in brain sections from PKCI/HINT1 gene knockout mice (Figure 7). PKCI/HINT1 staining could be clearly visualized within neurons and neuronal processes in quad-labeled fluorescent PFA-fixed frozen sections from various brain regions. A representative picture taken from thalamus is presented in Figure 8. PKCI/HINT1 immunoreactivity (green) was observed in neurons labeled with specific antineuronal marker antibody (anti-NeuN; blue), but was absent from astrocytes stained with specific anti-glia marker antibody (anti-GFAP; red). Although a low level of nonspecific background reactivity was noted as a light green color in sections from both WT and KO mice, this did not interfere with the observation of specific immunoreactivity.

DISCUSSION

The present studies demonstrate an important role of PKCI/HINT1 in modulating basal locomotor activity and the behavioral response to AMPH.

Furthermore, they suggest that lack of PKCI/HINT1 is associated with dysregulation of postsynaptic DA transmission.

The KO mice displayed decreased basal locomotor activity compared to WT controls both in a novel environment and after habituation. However, D-AMPH-

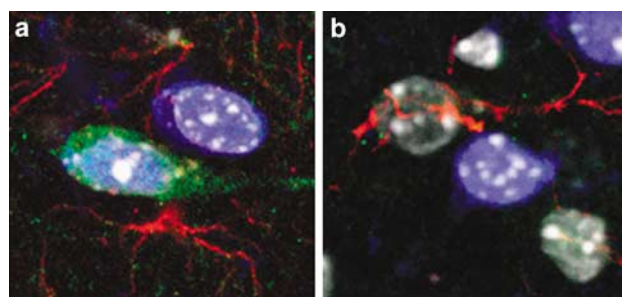


Figure 8 Quad-labeling in PFA-fixed frozen brain sections. (a) WT mouse; (b) KO mouse. PKCI/HINT1 was detected with anti-hPKCI polyclonal antiserum plus Cy2-conjugated donkey anti-rabbit antibody (green); neurons were identified with anti-NeuN monoclonal IgG plus Cy5-conjugated donkey anti-mouse antibody (blue); glial cells were identified with anti-GFAP polyclonal antiserum plus Cy3-conjugated donkey anti-goat antibody (red); and DAPI (white)-detected DNA in nuclei.

induced locomotor activity in KO mice is substantially higher than in WT animals suggesting that lack of functional PKCI/HINT1 protein results in hypersensitivity to the psychostimulant effects of AMPH. The lack of differences in locomotor activity in response to morphine and bicuculline also indicates that the enhanced locomotor activity in the KO is specific to AMPH. It is known that central DA systems are involved in the initiation and ongoing control of locomotion. The psychostimulant AMPH produces profound motor activation and this property is mediated at least in part by promoting the release of DA from presynaptic vesicles, as well as norepinephrine and serotonin. The primary targets of AMPH in the CNS are the monoamine transporters. Thus, specific monoamine transporter inhibitors will be useful tools to probe which of the three monoamines is responsible for enhanced LMA. In this study, a DA transporter-specific inhibitor, GBR 12909, induced a similar response of LMA in the PKCI/HINT1 KO mice as AMPH, therefore, demonstrated that DA is the most likely neurotransmitters involved in AMPH-induced LMA in our study.

AMPH-induced release of DA through the DA transporter (Sulzer *et al*, 1993; Schenk, 2002) is critical for AMPH-induced behavioral activation as mice lacking the DA transporter are insensitive to the locomotor stimulant effects of AMPHs (Giros *et al*, 1996). Thus, the enhanced behavioral sensitivity to AMPH in the KO mice might be explained by an enhanced AMPH-evoked DA overflow in NAc and CPu. However, the *in vivo* microdialysis data from this study did not support this possibility; there was no significant difference between WT and KO mice in AMPH-evoked DA levels observed in either the dorsal striatum or nucleus accumbens, suggesting that increased presynaptic DA release is not likely the cause for the behavioral supersensitivity to AMPH observed in the KO mice. Other explanations for this supersensitive behavior need to be explored. Apomorphine is a directly acting DA agonist. Low dose of apomorphine activates the presynaptic autoreceptors and decreases postsynaptic activity, whereas higher dose increase locomotion by activating postsynaptic DA receptors. In the present study, a relatively high dose of apomorphine (10 mg/kg) resulted in an enhanced locomotion in the KO mice. As previously reported (Halberda *et al*,

1997), selectively activation of D1- or D2-like receptors would produce different locomotor activity on mice. As a nonselective DA receptor agonist, apomorphine induced a modest increase of locomotor activity on the mPKCI KO mice but no change in control animals. This may well reflect a combined effect of apomorphine on both D1- and D2-like receptors.

Our findings from the LMA study, together with the results of the microdialysis study, suggest that changes at the postsynaptic level in the KO mice are more likely responsible for the observed increased sensitivity to AMPH. However, the specific postsynaptic modifications in the KO mice remain to be determined. Changes in the expression of DA receptors, alterations in receptor signal transduction, modification of the receptor phosphorylation, all can lead to functional consequences similar to what was observed in this study. Indeed, our laboratory results of GTP γ S binding assay using the KO mice brain tissues has indicated that both D1- and D2-like receptor activities are enhanced in the KO mice (unpublished data). Results from our previous study (Guang *et al*, 2004) also showed that PKCI/HINT1 inhibited PKC-related MOR phosphorylation. It is not clear whether the inhibition of MOR phosphorylation in PKCI/HINT1-expressing cells is due to direct inhibition of PKC activity or other alternative mechanism. Nevertheless, this result indicated that PKCI/HINT1 could play an important role in the regulation of phosphorylation of neurotransmitter receptors. Previous studies suggested that PKC-mediated phosphorylations of D₁ and D₂ receptors were involved in receptor internalization (Namkung and Sibley, 2004) and this rapid receptor sequestration is involved in agonist-induced receptor desensitization. Thus, PKCI/HINT1 may affect the postsynaptic sensitivity by altering the receptor phosphorylation state and/or interfering with receptor desensitization.

The existence of a tonically active MOR system that stimulates mesoaccumbens DA neurotransmission is well established (Spanagel *et al*, 1992; Herz, 1998). Our previous study had revealed that PKCI/HINT1 could attenuate PKC-mediated phosphorylation of MOR and regulate its function (Guang *et al*, 2004). The possibility exists that changes in opioid function in the PKCI/HINT1 KO can contribute to the supersensitivity to AMPH observed in these animals in the present study. Surprisingly, however, PKCI/HINT1 KO mice did not show increased sensitivity to morphine-induced locomotor activation. Therefore, this result seems not supporting the opioid involvement. However, it may also be explained by the strain variation among different mice as both stimulatory and depressive locomotor effects of morphine have been reported depending on the dose and the interval after the administration (Patti *et al*, 2005) and the strain of mice (Belknap *et al*, 1998; Patti *et al*, 2005).

The result of our brain immunohistochemical study of PKCI/HINT1 revealed that the protein is found in various regions including mesocorticolimbic and mesostriatal terminal regions; and is primarily localized to neurons, which is consistent with the result of *in situ* hybridization analysis of HINT1 mRNA (Vawter *et al*, 2004). A previous study on the intracellular localization of PKCI/HINT1 protein revealed that PKCI/HINT1 was present mainly in the nucleus with lesser amount in the cytoplasm. However, that intracellular localization study (Klein *et al*, 1998) was

carried out on non-neuronal cells. Our cellular distribution studies in neuronal cells have shown that mPKCI/HINT1 is primarily localized in the cytoplasm and neural processes. The possibility exists that the differential subcellular distribution in neuronal vs non-neuronal cells reflects differential functions for this protein in nervous vs peripheral tissues.

Finding the potential functional phenotype of a PKCI/HINT1 deficiency is also of significant clinical relevance, as this protein has been one of the candidate protein molecules whose expression is altered in schizophrenic patients (Vawter *et al*, 2001). PKCI/HINT1 was identified as one of the downregulated genes in brain samples of schizophrenic patients (Vawter *et al*, 2004). The observation that PKCI/HINT1 deficiency results in increased sensitivity to apomorphine and AMPH psychostimulant effects is consistent with an involvement of this protein in at least some of the symptoms of schizophrenia. Thus, the present results suggest that further research in the role of this protein on the pathophysiology of schizophrenia is clearly warranted. Several interesting questions arise for future research. An important issue is how the dopaminergic function is affected by PKCI/HINT1 and if the dopaminergic system is the only one to be involved. In addition it would be of interest to determine whether the PKCI/HINT1 KO mice could constitute an appropriate genetic model for schizophrenic research. It is recognized that there are some limitations in using conventional KO mice in studying the specific functions of a gene. Thus, the presence of a gene alteration in all the tissues naturally expressing the gene raises problems when attempting to assign a behavioral alteration to a specific brain structure or a pathway. Moreover, compensatory changes in other genes could occur in animals after genetic alteration. Therefore, using additional and complementary approaches to verify and further examine the function of PKCI/HINT1 in CNS would be required in order to gain a better understanding of PKCI/HINT1 protein and its role in normal brain function and schizophrenia.

In conclusion, this study has revealed an interesting neurological phenotype, the supersensitivity to AMPH, in PKCI/HINT1 KO mice. It has also provided evidence to suggest that DA postsynaptic transmission is the primary site affected by the lack of the PKCI/HINT1 gene in mice. These results suggest an important role of PKCI/HINT1 protein in CNS function, especially for DA neurotransmission, modulating basal locomotor activity and the behavioral response to AMPH.

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