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Histone H3 Phosphorylation is Under the Opposite Tonic Control of Dopamine D2 and Adenosine A2A Receptors in Striatopallidal Neurons

Jesus Bertran-Gonzalez[1,2,3], Kerstin Håkansson[4], Anders Borgkvist[4], Theano Irinopoulou[1,2,3], Karen Brami-Cherrier[1,2,3], Alessandro Usiello[5,6], Paul Greengard[7], Denis Hervé[1,2,3], Jean-Antoine Girault[1,2,3], Emmanuel Valjent[1,2,3,4] and Gilberto Fisone*[,4]

[1]Inserm, UMR-S 839, Paris, France; [2]Université Pierre et Marie Curie, Paris, France; [3]Institut du Fer à Moulin, Paris, France; [4]Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden; [5]CEINGE Biotecnologie Avanzate, Naples, Italy; [6]Università del Molise, Campobasso, Italy; [7]Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY, USA

The antipsychotic agent haloperidol regulates gene transcription in striatal medium spiny neurons (MSNs) by blocking dopamine D2 receptors (D2Rs). We examined the mechanisms by which haloperidol increases the phosphorylation of histone H3, a key step in the nucleosomal response. Using bacterial artificial chromosome (BAC)-transgenic mice that express EGFP under the control of the promoter of the dopamine D1 receptor (D1R) or the D2R, we found that haloperidol induced a rapid and sustained increase in the phosphorylation of histone H3 in the striatopallidal MSNs of the dorsal striatum, with no change in its acetylation. This effect was mimicked by raclopride, a selective D2R antagonist, and prevented by the blockade of adenosine A2A receptors (A2ARs), or genetic attenuation of the A2AR-associated G protein, $G\alpha_{olf}$. Mutation of the cAMP-dependent phosphorylation site (Thr34) of the 32-kDa dopamine and cAMP-regulated phosphoprotein (DARPP-32) decreased the haloperidol-induced H3 phosphorylation, supporting the role of cAMP in H3 phosphorylation. Haloperidol also induced extracellular signal-regulated kinase (ERK) phosphorylation in striatopallidal MSNs, but this effect was not implicated in H3 phosphorylation. The levels of mitogen- and stress-activated kinase 1 (MSK1), which has been reported to mediate ERK-induced H3 phosphorylation, were lower in striatopallidal than in striatonigral MSNs. Moreover, haloperidol-induced H3 phosphorylation was unaltered in MSK1-knockout mice. These data indicate that, in striatopallidal MSNs, H3 phosphorylation is controlled by the opposing actions of D2Rs and A2ARs. Thus, blockade of D2Rs promotes histone H3 phosphorylation through the A2AR-mediated activation of $G\alpha_{olf}$ and inhibition of protein phosphatase-1 (PP-1) through the PKA-dependent phosphorylation of DARPP-32.
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INTRODUCTION

The medium spiny neurons (MSNs) of the striatum are targeted by many psychoactive substances, including drugs used in the treatment of schizophrenia and other psychotic disorders. One major problem associated with the use of conventional antipsychotic drugs, such as haloperidol, is their propensity to generate extrapyramidal side effects, such as parkinsonism and tardive dyskinesia. These complications are most likely related to the ability of these drugs to antagonize dopamine D2 receptor (D2R)-mediated transmission. A better understanding of the molecular mechanisms, by which blockade of D2Rs affects striatal function, is therefore of high clinical relevance.

In the striatopallidal MSNs, D2Rs activate a $G\alpha_{i/o}$ protein coupled negatively to adenylyl cyclase (Kebabian and Calne, 1979; Stoof and Kebabian, 1981). Therefore, blockade of D2Rs promotes cAMP signaling. This effect depends on the expression, in striatopallidal MSNs, of the adenosine A2A receptors (A2ARs) (Fink *et al*, 1992; Schiffmann *et al*, 1991), which are activated tonically and are coupled to $G\alpha_{olf}$-dependent stimulation of adenylyl cyclase (Corvol *et al*, 2001; Fredholm, 1977). Thus, pharmacological blockade or genetic inactivation of A2ARs prevents the ability of haloperidol and other D2R antagonists to increase cAMP-dependent phosphorylation of downstream targets, such as the dopamine- and cAMP-regulated phosphoprotein of

*Correspondence: Dr G Fisone, Department of Neuroscience, Karolinska Institutet, Retzius väg 8, Institutionen för Neurovetenskap, 17177 Stockholm, Sweden, Tel: +46 8 524 873 75, Fax: +46 8 320 988, E-mail: gilberto.fisone@ki.se
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32 kDa (DARPP-32), and the GluR1 subunit of the glutamate AMPA receptor (Håkansson *et al*, 2006; Svenningsson *et al*, 2000). In addition, A2ARs are required for changes in the expression of several genes, including immediate early genes, caused by the blockade of D2Rs (Chen *et al*, 2001).

Alterations in the chromatin structure, through changes in the state of phosphorylation, acetylation, or methylation of specific histones, are critically involved in the control of gene expression. Indeed, phosphorylation of histone H3 at Ser10 by various protein kinases, including mitogen- and stress-activated kinases 1 and 2 (MSK1 and 2), is an important component of the nucleosomal response, which promotes chromatin decondensation, thereby allowing access to DNA by transcription factors and increasing the expression of early response genes, such as *c-fos* and *c-jun* (Davie, 2003; Soloaga *et al*, 2003). In the hippocampus and the striatum, it has been shown that histone H3 can be phosphorylated by MSK1 after the activation of the extracellular signal-regulated kinases (ERKs) (Brami-Cherrier *et al*, 2005; Chwang *et al*, 2006; Chwang *et al*, 2007).

An earlier study showed that haloperidol induces phosphorylation of the acetylated form of histone H3 in the striatum and that this effect is blocked by the inhibition of cAMP-dependent protein kinase (PKA) (Li *et al*, 2004). In addition, it has been recently reported that haloperidol increases histone H3 phosphorylation selectively in striatopallidal neurons (Bertran-Gonzalez *et al*, 2008). These observations raise the possibility that the combined control of cAMP signaling through D2Rs and A2ARs orchestrates the nucleosomal response in striatopallidal MSNs. In this study, we have employed pharmacological tools and transgenic mice to examine the signaling pathways that link blockade of D2Rs to histone H3 modification in the nucleus. Our results indicate that haloperidol, acting as an antagonist at D2Rs, increases histone H3 phosphorylation in striatopallidal MSNs, by promoting A2AR–$G\alpha_{olf}$-mediated activation of cAMP–DARPP-32 signaling. We also show that, in contrast to what has been observed in the response to psychostimulants in striatonigral neurons, the effects of haloperidol on histone H3 phosphorylation are independent of ERK–MSK1 activation.

MATERIALS AND METHODS

Animals

Male mice, 7–8 weeks old, were maintained in a 12 h light–dark cycle, in stable conditions of temperature (22°C), with food and water *ad libitum*. All experiments were carried out in accordance with the guidelines of The Swedish Animal Welfare Agency and The French Agriculture and Forestry Ministry (decree 87849, license A75-05-22). Swiss-Webster mice carrying *Drd1a*-EGFP or *Drd2*-EGFP bacterial artificial chromosome (BAC) transgenes, were generated by the GENSAT (Gene Expression Nervous System Atlas) program at The Rockefeller University (Gong *et al*, 2003). DARPP-32 T34A mutant, $Gnal^{+/-}$ ($G\alpha_{olf}$) and heterozygous and MSK1 knockout mice were generated as described in earlier studies (Belluscio *et al*, 1998; Svenningsson *et al*, 2003; Wiggin *et al*, 2002), and were backcrossed for at least 10 generations on a C57Bl/6 background.

Drugs

Haloperidol (0.5 mg/kg, Sigma-Aldrich, Sweden) was dissolved in saline containing 5% (vol/vol) acetic acid, and the pH was adjusted to 6.0 with 1 M NaOH. Raclopride (0.3 mg/kg, Sigma-Aldrich, France) was dissolved in 0.9% NaCl. KW6002 (3 mg/kg) and SL327 (50 mg/kg), gifts from Dr Edilio Borroni (Hoffmann-La Roche, Basel, Switzerland), were suspended by sonication in a solution of 5% (vol/vol) Tween 80 in saline and administered 5 and 30 min before haloperidol, respectively. All drugs were administered intraperitoneally (ip). The mice were habituated to handling and saline injection for three consecutive days before the experiment.

Tissue Preparation and Immunofluorescence

The mice were anesthetized rapidly with pentobarbital (500 mg/kg, ip, Sanofi-Aventis, France) and perfused transcardially with 4% (weight/vol) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.5). Their brains were post-fixed overnight in the same solution and stored at 4°C. Sections, 30 μm thick, were cut with a vibratome (Leica, France) and stored at −20°C in a solution containing 30% (vol/vol) ethylene glycol, 30% (vol/vol) glycerol, and 0.1 M sodium phosphate buffer, until they were processed for immunofluorescence. Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.25 M Tris and 0.5 M NaCl, pH 7.5), incubated for 5 min in TBS containing 3% H_2O_2 and 10% methanol, (vol/vol), and then rinsed three times for 10 min each in TBS. After 20 min incubation in 0.2% Triton X-100 in TBS, sections were rinsed three times in TBS again. Finally, they were incubated overnight at 4°C with the different primary antibodies. NaF (0.1 mM) was included in all the buffers and incubation solutions. Different histone H3 modifications were identified using rabbit polyclonal antibodies against phospho-Ser10-H3, acetyl-Lys14-H3, and phospho-Ser10-acetyl-Lys14-H3 (1 : 500, Upstate Ltd, UK). $G\alpha_{olf}$ protein levels in wild-type and $Gnal^{+/-}$ mice were assessed using rabbit polyclonal antibodies (1 : 500) (Herve *et al*, 2001). Activated ERK was detected with rabbit polyclonal antibodies (1 : 400, Cell Signaling Technology, Danvers, MA) and a mouse monoclonal antibody (1 : 400, Promega, Charbonnière, France) against diphospho-Thr202/Tyr204-ERK1/2. MSK1 was identified using a rabbit polyclonal antibody (1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with primary antibodies, sections were rinsed three times for 10 min in TBS and incubated for 45 min with goat Cy3-coupled (1 : 400, Jackson Laboratory, Bar Harbor, ME) and/or goat A488/A633 (1 : 400, Invitrogen AB, Sweden) secondary antibodies. Sections were rinsed for 10 min twice in TBS and in TB (0.25 M Tris) before mounting in Vectashield (Vector Laboratories, Burlingame, CA).

Immunofluorescence Analysis

Single- and double-labeled images from each region of interest were obtained bilaterally using sequential laser scanning confocal microscopy (Leica SP2 and Zeiss LSM). Neuronal quantification was performed in 375 × 375 μm images by counting Cy3-immunofluorescent nuclei (for P-AcH3 and P-ERK immunostaining). Cell counts were

done by an observer unaware of the treatment received by the mice. For the analysis of MSK1 expression in striatonigral and striatopallidal neurons, the average fluorescence intensity of each individual MSK1-immunoreactive nucleus was assessed automatically in *Drd1a*-EGFP and *Drd2*-EGFP mice samples, according to the colocalization of the EGFP signal. A home-written program based on Metamorph software (Molecular Devices, Sunnyvale, CA) was used to compute all the parameters.

Statistical Analysis

Data from the quantifications of P-H3, P-AcH3, and AcH3 in *Drd2*-EGFP mice (means ± SEM, $n = 3$–4) were analyzed using the two-way ANOVA, and *post hoc* comparisons between groups were made using the Bonferroni multiple comparison test. In the other P-AcH3 and P-ERK assessments, data (means ± SEM, $n = 2$–5) were analyzed using the one-way ANOVA and the Newman–Keuls *post hoc* multiple comparison test. The comparisons between MSK1 intensities in striatonigral or striatopallidal neurons (means ± SEM, $n = 310$–452) were performed using the unpaired *t*-test. In all cases, significance threshold was set at $p < 0.05$.

RESULTS

Haloperidol Induces a Sustained Phosphorylation of Histone H3 in Striatopallidal MSNs

MSNs represent the vast majority of striatal neurons and form two distinct efferent pathways, which exert opposite regulations on thalamo-cortical projection neurons (Gerfen, 1992). The MSNs of the direct pathway project to the substantia nigra pars reticulata and entopeduncular nucleus, whereas the MSNs of the indirect pathway innervate the external globus pallidus, which projects to the subthalamic nucleus (Gerfen, 1992). Striatonigral MSNs predominantly express dopamine D1 receptors (D1Rs), whereas striatopallidal MSNs contain D2Rs and A2ARs (Fink *et al*, 1992; Gerfen, 1992; Schiffmann *et al*, 1991).

Drd1a-EGFP and *Drd2*-EGFP mice provide a very efficient way to identify distinct neuronal populations in the striatum (Gong *et al*, 2003). Using these mice, it was shown that haloperidol induced histone H3 phosphorylation selectively in striatopallidal neurons (Bertran-Gonzalez *et al*, 2008). Here, we further investigated the action of haloperidol on histone modifications by comparing its effects on phosphorylation and acetylation of histone H3 (Figure 1). Immunofluorescence analysis in *Drd1a*-EGFP

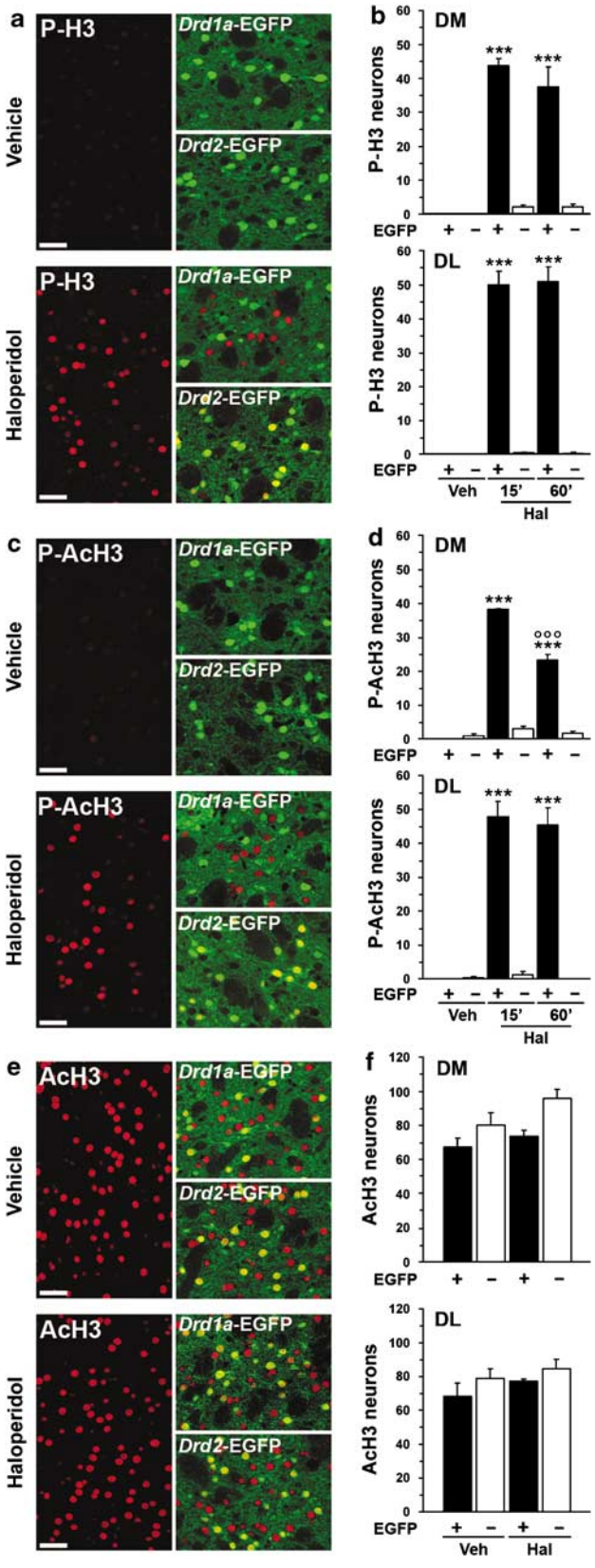

Figure 1 Effect of haloperidol on histone H3 phosphorylation and acetylation in striatal MSNs. Mice expressing EGFP in striatonigral (*Drd1a*-EGFP) or striatopallidal (*Drd2*-EGFP) MSNs were treated with vehicle or haloperidol and perfused 15 min (15′; a–f), or 60 min later (60′; b, d). (a, c, e). Confocal sections of the dorsal striatum, showing immunofluorescence (red) for phospho-Ser10-histone H3 (P-H3, a), phospho-Ser10-acetyl-Lys14-histone H3 (P-AcH3, c), and acetyl-Lys14-histone H3 (AcH3, e) alone (left panels), or in combination with EGFP fluorescence (green; right panels). (b, d, f) Quantification of P-H3- (b), P-AcH3- (d), andAcH3- (f) immunoreactive neurons among EGFP-positive (EGFP+) or EGFP-negative (EGFP−) neurons in the dorsomedial (DM) and dorsolateral (DL) striata of vehicle (Veh)- or haloperidol (Hal)-treated *Drd2*-EGFP mice (***$p < 0.001$ vs Veh; °°°$p < 0.001$ vs Hal 15′). Scale bars: 40 μm.

and *Drd2*-EGFP confirmed that the administration of haloperidol (0.5 mg/kg) induced a rapid (15 min) and prolonged (60 min) increase in the levels of Ser10 phosphorylated histone H3 in the D2R, but not in the D1R-expressing neurons of the dorsomedial and the dorsolateral striatum (Figure 1a and b). Similar results were obtained using an antibody against the Ser10 phosphorylated form of Lys14-acetylated histone H3 (Figure 1c and d). In contrast, haloperidol did not modify the number of neurons immunoreactive for acetyl-Lys14 histone H3 (Figure 1e and f), nor did it affect the number of acetyl-Lys12 histone H4-positive neurons (data not shown). As the phospho-acetylated form of histone H3 is the most functionally significant (Cheung *et al*, 2000; Salvador *et al*, 2001), antibodies against this form were used in the rest of the study.

Although haloperidol is an excellent D2R antagonist, it also binds to other receptors, including D1Rs (Missale *et al*, 1998). To confirm that the effects of haloperidol actually resulted from the blockade of D2R, we compared its action with that of raclopride, a highly selective D2 antagonist (Missale *et al*, 1998). Raclopride induced a robust phosphorylation of Lys14-acetylated histone H3 in D2R-expressing neurons of the dorsal striatum (Supplementary Figure 1). The effect of raclopride was less persistent than that of haloperidol (Supplementary Figure 1b), a difference most likely related to its shorter half-life (Farde *et al*, 1988; Kudo and Ishizaki, 1999).

Haloperidol-Induced Phosphorylation of Histone H3 Depends on A2ARs

In the striatum, haloperidol promotes cAMP–PKA signaling by removing the inhibition exerted by D2Rs on adenylyl cyclase (Kebabian and Calne, 1979; Stoof and Kebabian, 1981). This effect depends on the basal activation of A2ARs, which are selectively expressed in striatopallidal MSNs (Fink *et al*, 1992; Schiffmann *et al*, 1991), and are primarily responsible for the synthesis of cAMP in these neurons through the activation of $G\alpha_{olf}$ (Corvol *et al*, 2001; Zhuang *et al*, 2000). As shown in Figure 2, blockade of A2ARs, achieved with the selective antagonist KW6002 (3 mg/kg), dramatically reduced the increase in the number of P-AcH3-immunoreactive neurons produced by haloperidol. These results strongly support the hypothesis that the blockade of D2Rs promotes the phosphorylation of Lys14-acetylated histone H3 through disinhibition of an A2AR-triggered signaling cascade in striatopallidal MSNs. They also indicate that, in these neurons, the state of phosphorylation of histone H3 is regulated in an opposite way by dopamine, acting on D2Rs, and adenosine, acting on A2ARs.

Haloperidol Increases the Phosphorylation of Histone H3 Through $G\alpha_{olf}$

In striatal MSNs, receptor-mediated activation of adenylyl cyclase depends on the stimulation of the GTP-binding protein, $G\alpha_{olf}$ (Corvol *et al*, 2001; Zhuang *et al*, 2000). Therefore, we analyzed whether phosphorylation of histone H3 induced by haloperidol was altered in the *Gnal*$^{+/-}$ mice carrying a heterozygous mutation of the gene encoding for $G\alpha_{olf}$. In these animals, $G\alpha_{olf}$ expression is reduced by about

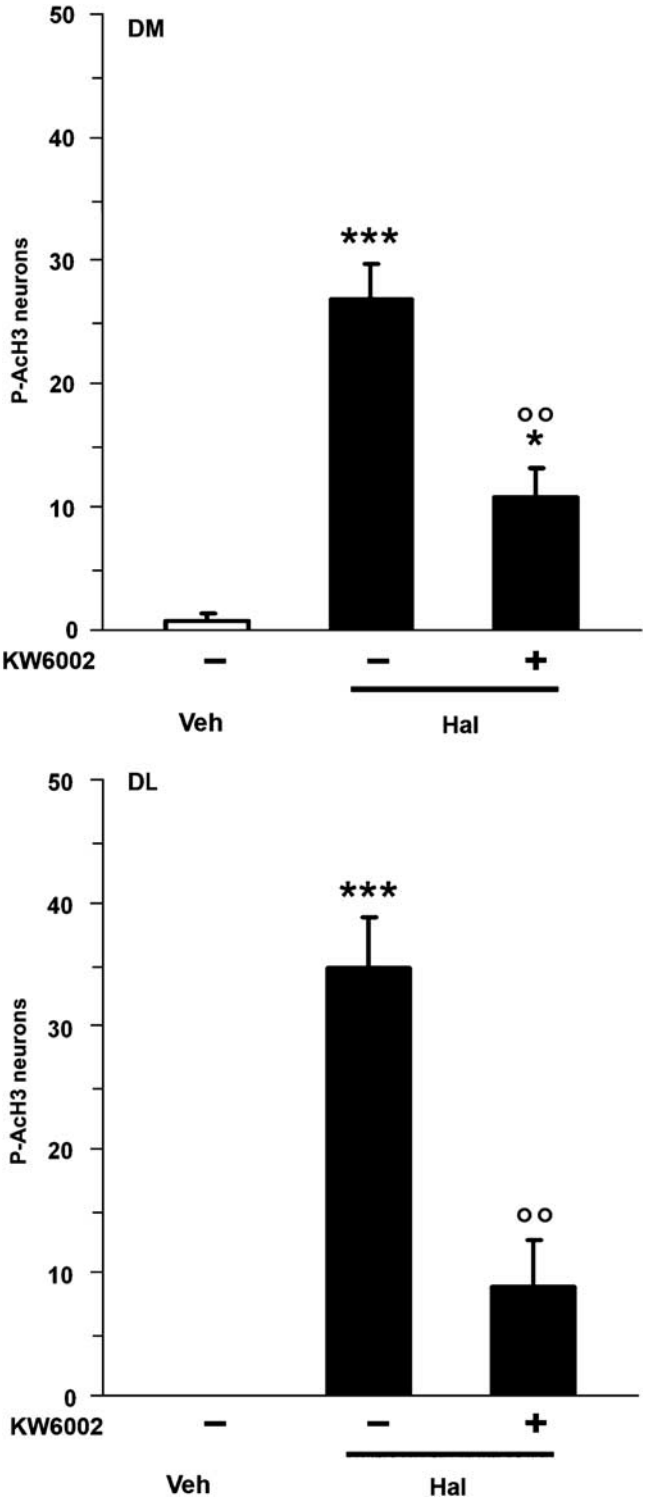

Figure 2 Haloperidol-induced histone H3 hosphorylation is prevented by the blockade of A2ARs. Wild-type mice were treated with haloperidol alone, or in combination with the A2AR antagonist KW6002 (injected 5 min prior to haloperidol) and perfused 15 min later. Quantification of phospho-Ser10-acetyl-Lys14-histone H3 (P-AcH3) immunoreactive neurons in the dorsomedial (DM) and dorsolateral (DL) striata of mice treated with vehicle (Veh), haloperidol (Hal), or haloperidol plus KW6002 (*$p<0.05$, ***$p<0.001$ vs vehicle; °°$p<0.01$ vs Hal).

50% (cf. Figure 3a), leading to an impaired A2AR-mediated activation of adenylyl cyclase (Corvol *et al*, 2001; Zhuang *et al*, 2000). We found that, after the administration of haloperidol (0.5 mg/kg), the number of P-AcH3-positive neurons was strongly reduced in the dorsal striatum of the *Gnal*$^{+/-}$ mice, as compared with wild-type mice (Figure 3b and c). These results indicate that increase in the phosphorylation of Lys14-acetylated histone H3, produced by blockade of D2Rs, depends on a signaling pathway in which Gα_{olf} is a limiting factor.

Involvement of DARPP-32 in Haloperidol-Induced Phosphorylation of Histone H3

The administration of D2R antagonists, including haloperidol, results in a PKA-dependent phosphorylation of DARPP-32 on Thr34 (Svenningsson *et al*, 2000), which is thus converted into a potent inhibitor of protein phosphatase-1 (PP-1) (Hemmings *et al*, 1984). DARPP-32-mediated suppression of PP-1 activity plays a key role in the regulation of the state of phosphorylation of many proteins targeted by the cAMP–PKA cascade (Greengard, 2001). Moreover, DARPP-32 is critical for the regulation of histone H3 phosphorylation produced by cocaine (Stipanovich *et al*, 2008). To examine the involvement of DARPP-32 in the control of the phosphorylation of Lys14-acetylated histone H3 exerted by haloperidol, we employed knock-in mice expressing a mutant form of DARPP-32, in which the PKA phosphorylation site, Thr34, is inactivated by substituting with an Ala (T34A mutant mice). In these mice, the ability of haloperidol to stimulate the phosphorylation of acetylated histone H3 in the dorsal striatum was strongly reduced (Figure 4a and b). Therefore, we concluded that, in striatopallidal MSNs, the blockade of D2Rs reduces the dephosphorylation of histone H3 through DARPP-32-mediated inhibition of PP-1.

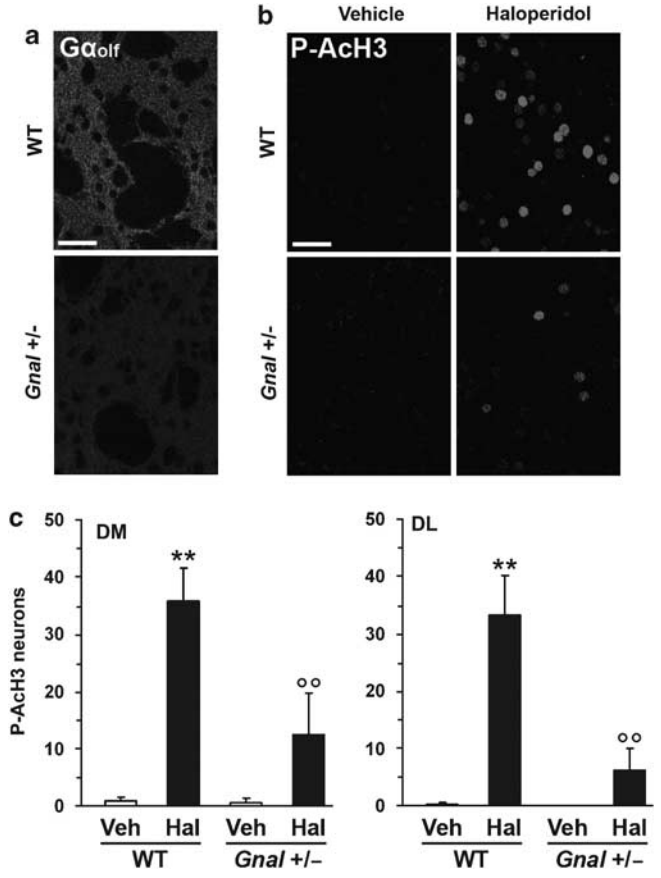

Figure 3 Gα_{olf}-mediated signaling is required for haloperidol-induced histone H3 phosphorylation. (a) Gα_{olf} immunoreactivity in single confocal sections of the dorsal striatum from a wild-type (WT) or a *Gnal* heterozygous (*Gnal*$^{+/-}$) mouse. Note the decrease in Gα_{olf} immunoreactivity in the striatum of the *Gnal*$^{+/-}$ mouse. (b, c) The WT and the *Gnal*$^{+/-}$ mice were treated with haloperidol and perfused 15 min later. (b) Phospho-Ser10-acetyl-Lys14-histone H3 (P-AcH3) immunoreactivity in single confocal sections of the dorsal striatum of the WT or the *Gnal*$^{+/-}$ mice. (c) Quantification of P-AcH3 immunoreactive neurons in the dorsomedial (DM) and dorsolateral (DL) striata of the WT and the *Gnal*$^{+/-}$ mice 15 min after the administration of vehicle (Veh) or haloperidol (Hal) (**$p<0.01$ vs WT Veh; °°$p<0.01$ vs WT Hal). Scale bars: 40 μm.

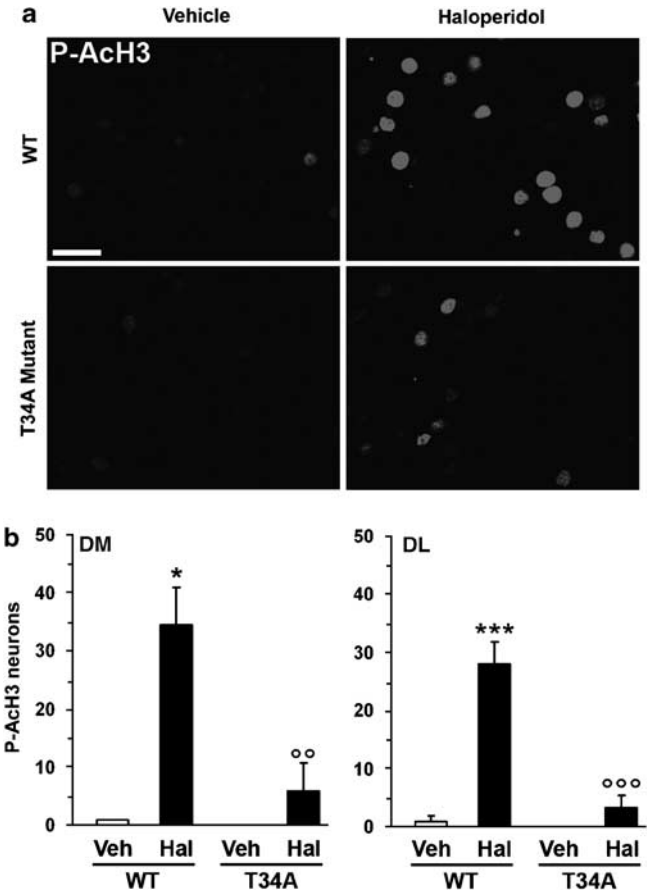

Figure 4 Mutation of Thr34 to Ala in DARPP-32 prevents haloperidol-induced histone H3 phosphorylation. Wild-type (WT) or T34A DARPP-32 mutant mice were treated with vehicle or haloperidol and perfused 15 min later. (a) Phospho-Ser10-acetyl-Lys14-histone H3 (P-AcH3) immunoreactivity in single confocal sections of the dorsal striatum from WT or T34A mutant mice. (b) Quantification of P-AcH3 immunoreactive neurons in the dorsomedial (DM) and dorsolateral (DL) striatum, 15 min after the administration of vehicle (Veh) or haloperidol (Hal) to WT or T34A DARPP-32 mutant mice (T34A) (*$p<0.05$, ***$p<0.001$ vs WT Veh; °°$p<0.01$, °°°$p<0.001$ vs WT Hal). Scale bar: 40 μm.

Haloperidol-Induced Phosphorylation of Histone H3 is Independent of ERK Activation

It is known that ERK, acting through its downstream target MSK1, elicits *in vivo* phosphorylation of histone H3 (Brami-Cherrier *et al*, 2005; Chwang *et al*, 2007). It has also been shown that the administration of haloperidol induces ERK phosphorylation in the striatum (Gerfen *et al*, 2002; Pozzi *et al*, 2003; Valjent *et al*, 2004), and that this effect occurs selectively in striatopallidal MSNs (Bertran-Gonzalez *et al*, 2008). In the striatum, the activation of ERK is mediated in part through the activation of the cAMP pathway (Santini *et al*, 2007; Valjent *et al*, 2005). Therefore, it was logical to hypothesize that the haloperidol-induced phosphorylation of Lys14-acetylated histone H3 might result from A2AR–cAMP-mediated activation of ERK. Examination of haloperidol-induced ERK phosphorylation in *Drd1a*-EGFP and *Drd2*-EGFP mice confirmed, as reported earlier (Bertran-Gonzalez *et al*, 2008), that ERK activation occurred selectively in striatopallidal MSNs (Figure 5a). Similar results were obtained with raclopride, indicating that, as in the case of histone H3 modification, the effect of haloperidol resulted from the blockade of D2R (Figure 5a). However, the effects produced by the D2R antagonists were limited to a small number of neurons and were restricted to the dorsomedial part of the striatum (Figure 5a). Next, we examined the effect of haloperidol in the presence or absence of SL327, a drug that blocks ERK by inhibiting the mitogen-activated protein kinase/ERK kinase (MEK). We found that the administration of 50 mg/kg of SL327 abolished haloperidol-induced ERK phosphorylation (Figure 5b and d), without affecting the concomitant

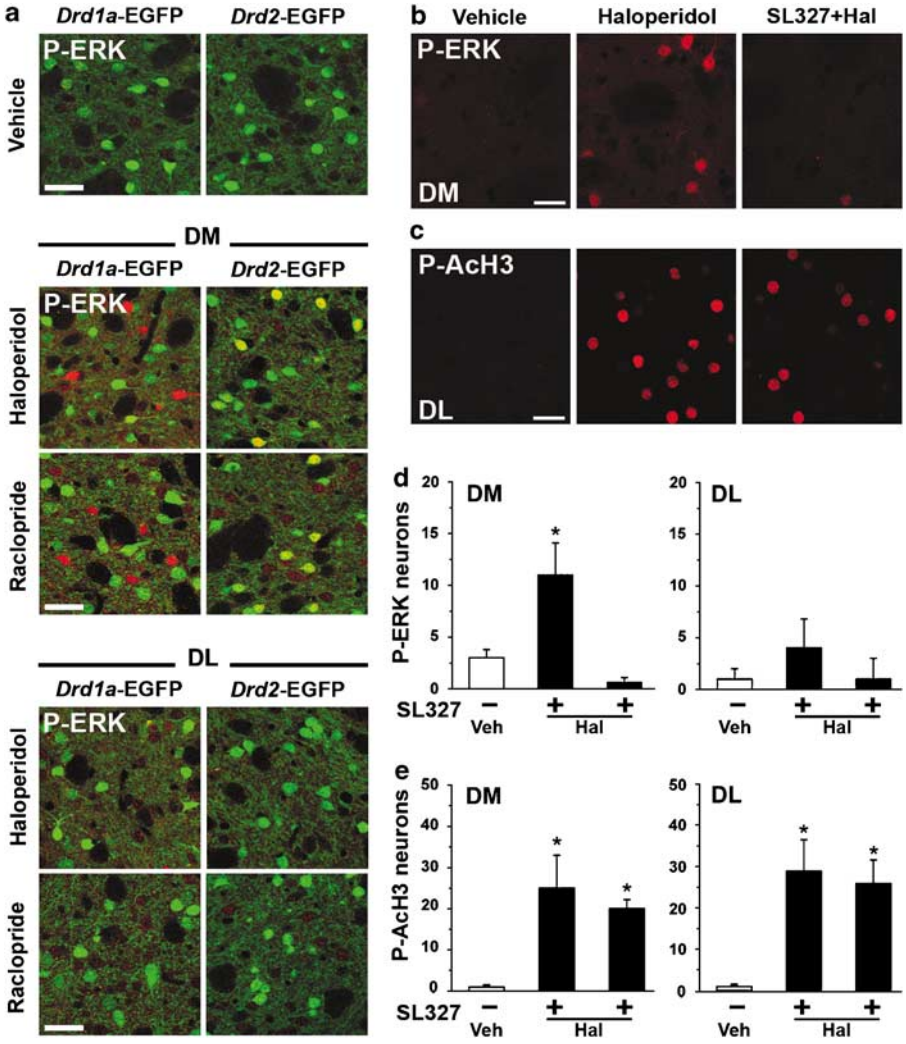

Figure 5 Histone H3 phosphorylation induced by blockade of D2Rs is independent of ERK activation. (a) Mice expressing EGFP in striatonigral (*Drd1a*-EGFP) or striatopallidal (*Drd2*-EGFP) MSNs were treated with vehicle, haloperidol, or raclopride, and perfused 15 min later. EGFP fluorescence (green) in single confocal sections of the dorsomedial (DM) or dorsolateral (DL) striatum is shown in combination with fluorescence (red) for diphospho-Thr202/Tyr204-ERK1/2 (P-ERK). Note, in haloperidol- and raclopride-treated mice, the relatively low number and prevalent localization in the DM of P-ERK-positive MSNs (single-labeled *Drd1a*-EGFP mice and double-labeled in *Drd2*-EGFP mice). (b–e) Wild-type mice were treated with haloperidol alone or in combination with the MEK inhibitor SL327 (injected 45 min prior to haloperidol) and perfused 15 min later. P-ERK (b, d) and phospho-Ser10-acetyl-Lys14-histone H3 (P-AcH3) (c, e) immunoreactivity was determined in single confocal sections of the DM striatum. (d, e) Quantification of striatal P-ERK (d) and P-AcH3 (e) immunoreactive neurons in the DM and DL striata of mice treated with vehicle (Veh) or haloperidol (Hal) alone or in combination with SL327 (*$p<0.05$ vs vehicle; °$p<0.05$ vs Hal). Scale bars: 40 μm.

increase in histone H3 phosphorylation observed in the dorsal striatum (Figure 5c and e). These results demonstrated that the effects of haloperidol on H3 phosphorylation, in contrast with those of cocaine (Brami-Cherrier *et al*, 2005), were ERK-independent.

MSK1 Expression is Lower in D2R-Expressing than in D1R-Expressing Neurons

MSK1 plays a critical role in the phosphorylation of histone H3 in several regions of the brain, including the striatum (Brami-Cherrier *et al*, 2005) and the hippocampus (Chwang *et al*, 2007). However, MSK1-immunoreactivity has been previously detected in only about 60% of the striatal neurons (Heffron and Mandell, 2005), prompting the question of the implication of MSK1 in haloperidol-induced H3 phosphorylation. To address this question, we studied the distribution of MSK1 immunoreactivity in *Drd1a*-EGFP and *Drd2*-EGFP mice (Figure 6). As reported earlier (Heffron and Mandell, 2005), MSK1 immunoreactivity was restricted to the nuclei (Figure 6a). Interestingly, there was a clear heterogeneity in labeling intensity (Figure 6a). The comparison of MSK1 immunoreactivity and EGFP fluorescence in *Drd1*-EGFP and *Drd2*-EGFP mice showed that the intensely labeled nuclei belonged almost invariably to the D1R-expressing neurons (Figure 6b). Quantitative study of immunolabeling in the two neuronal populations showed that the mean level of MSK1 immunoreactivity was lower in the D2R-neurons than in the D1R-neurons (Figure 6c). However, it is important to note that at least some MSK1 immunoreactivity was detected in all EGFP-expressing neurons in both the transgenic lines. In line with these results, a small but significant increase in phospho-MSK1-positive striatopallidal neurons occurs in response to haloperidol (Bertran-Gonzalez *et al*, 2008). Thus, our observations revealed that, although MSK1 was present in virtually all striatal MSNs, its expression levels were lower in D2R- than in D1R-containing neurons, raising the question of the contribution of MSK1 to the effects of haloperidol on H3 phosphorylation in striatonigral neurons.

Haloperidol-Induced Phosphorylation of Histone H3 in Striatopallidal MSNs is Independent of MSK1

To examine the role of MSK1 in the effects of haloperidol, we used MSK1 knockout mice, in which phosphorylation of histone H3 in response to cocaine is abolished (Brami-Cherrier *et al*, 2005). In these mice, the haloperidol-induced phosphorylation of histone H3 was virtually identical to that observed in the wild-type mice (Figure 7a and c). As expected, the effect of haloperidol on the number of phospho-ERK-positive neurons in the dorsomedial striatum was also unchanged (Figure 7b).

Our results showed that, after the administration of haloperidol, phospho-acetylated-histone H3-positive MSNs largely outnumbered phospho-ERK-positive MSNs (see Figure 5). However, double-labeling experiments indicated that haloperidol-induced phosphorylation of ERK and histone H3 were both detected in a modest number of neurons (Figure 7d). Therefore, we tested the role of MSK1 in the phosphorylation of histone H3 in this subset of

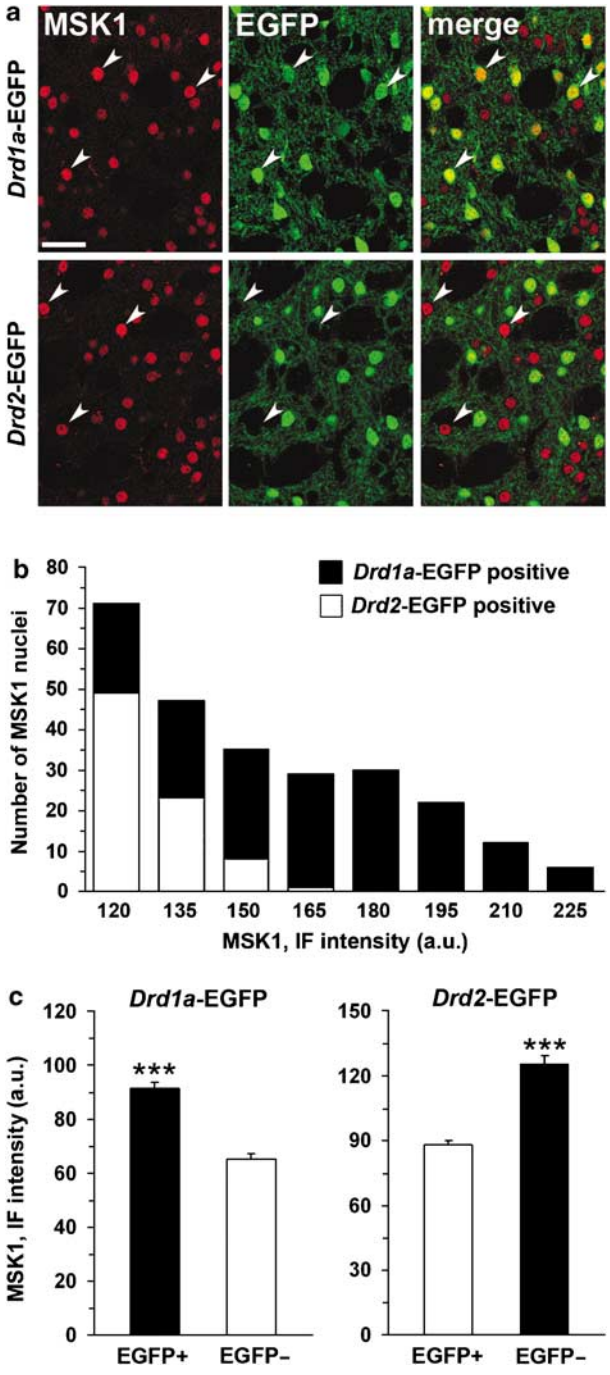

Figure 6 Expression of MSK1 in striatonigral and striatopallidal MSNs. (a) MSK1 immunoreactive nuclei (red) are colabeled with EGFP (green) in the striatum of *Drd1a*-EGFP or *Drd2*-EGFP mice in a double fluorescence analysis. Nuclei intensely labeled with MSK1 antibodies colocalized with EGFP in *Drd1a*-EGFP but not in *Drd2*-EGFP mice (arrowheads). Single confocal sections, scale bar: 40 μm. (b) Quantitative analysis of MSK1 fluorescence intensity in *Drd1a*-EGFP and *Drd2*-EGFP mice. Distribution histograms of intensely labeled MSK1 nuclei (average normalized immunofluorescence intensity > 120 arbitrary units (a.u.)) colocalized with EGFP in *Drd1a*-EGFP mice (black bars) and in *Drd2*-EGFP mice (white bars). (c) Mean values of MSK1 immunofluorescence (a.u.) of neurons containing (EGFP+) or not (EGFP−) EGFP in *Drd1a*-EGFP and *Drd2*-EGFP mice (***$p < 0.0001$).

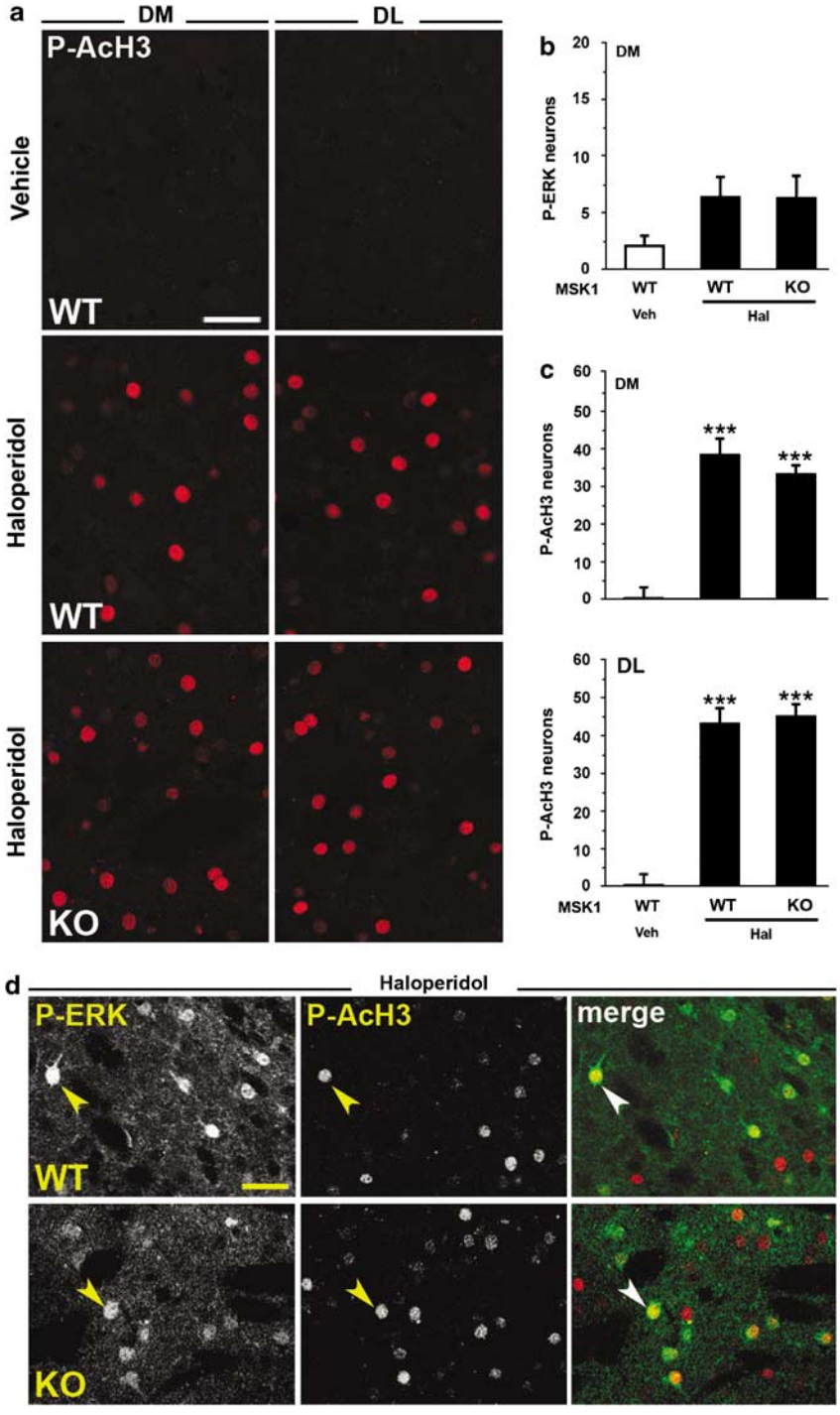

Figure 7 Haloperidol-induced histone H3 phosphorylation in striatopallidal MSNs is independent of MSK1. Wild-type (WT) or MSK1 knockout (KO) mice were treated with vehicle or haloperidol and perfused 15 min later. (a) Phospho-Ser10-acetyl-Lys14-histone H3 (P-AcH3) immunoreactivity in single confocal sections of the dorsomedial (DM) or dorsolateral (DL) striata of WT and MSK1 KO mice. (b, c) Quantification of (b) diphospho-Thr202/Tyr204-ERK1/2 (P-ERK) and (c) P-AcH3 immunoreactive neurons in the DM (b, c) and DL (c) striata of WT and MSK1 KO mice 15 min after the administration of vehicle (Veh) or haloperidol (Hal). No difference in P-AcH3 immunoreactivity was found between vehicle-treated WT mice and vehicle-treated MSK1 KO mice (the latter are not shown in the figure) (***$p < 0.001$ vs Veh WT). (d) Double immunofluorescence showing P-ERK (green) and P-AcH3 (red) in the DM of WT and MSK1 KO mice after haloperidol treatment. Arrowheads indicate colocalization of P-ERK and P-AcH3 immunoreactivity in the same MSNs. Scale bars: 40 μm.

striatopallidal MSNs. We found that concomitant phosphorylation of ERK and histone H3 was still present in the dorsomedial striata of MSK1 knockout mice (Figure 7d). In combination with the lack of effect of SL327 on H3 phosphorylation, these results showed clearly that haloperidol-induced histone H3 phosphorylation in striatopallidal MSNs occurred independently of the activation of the ERK–MSK1 cascade.

DISCUSSION

In this study, we have characterized the regulation of histone H3 exerted by blockade of D2Rs in striatopallidal MSNs. Our data show that the increase in histone H3 phosphorylation produced by haloperidol is mimicked by the highly selective D2R antagonist raclopride, involves A2AR–$G\alpha_{olf}$-mediated transmission, and requires PKA-dependent phosphorylation of DARPP-32, which leads to the inhibition of PP-1. In contrast, the effect of haloperidol on histone H3 phosphorylation is independent of ERK and MSK1 activation, which occurs only in a limited subset of striatopallidal MSNs.

Contribution of Adenosine A2ARs, $G\alpha_{olf}$ and DARPP-32 to Histone H3 Phosphorylation

In striatopallidal MSNs, cAMP signaling is controlled by the opposite actions of A2ARs, which increase cAMP production through $G\alpha_{olf}$, and D2Rs, which reduce cAMP production through $G\alpha_{i/o}$ (Corvol *et al*, 2001; Kebabian and Calne, 1979; Stoof and Kebabian, 1981). Thus, haloperidol and other D2R antagonists promote cAMP signaling by removing the inhibition exerted by D2Rs on adenylyl cyclase. This action depends on A2AR transmission, which maintains basal cAMP synthesis (Håkansson *et al*, 2006; Svenningsson *et al*, 2000).

We show that the increase in histone H3 phosphorylation produced by the administration of haloperidol is prevented by the pharmacological blockade of A2ARs. This finding indicates that, in the absence of D2R transmission, the tonic activation of A2ARs is able to increase the state of phosphorylation of histone H3. It also suggests that in striatopallidal MSNs, dopamine and adenosine exert opposing effects on nucleosomal response, an observation that is in line with their opposing actions on gene expression (Dragunow *et al*, 1990, Svenningsson *et al*, 1997).

The requirement of A2AR-mediated transmission in the regulation of histone H3, exerted by haloperidol, is further supported by the experiments performed in the *Gnal*$^{+/-}$ mice. We show that a reduction in the expression of $G\alpha_{olf}$, which dramatically decreases the ability of A2ARs to activate adenylyl cyclase (Corvol *et al*, 2007), prevents D2Rs antagonist from promoting histone H3 phosphorylation. This observation demonstrates the crucial role played by $G\alpha_{olf}$ in striatopallidal neurotransmission, and identifies this protein as a critical mediator for the actions of D2Rs antagonists, including antipsychotic drugs.

The observation that A2ARs and $G\alpha_{olf}$ are involved in the regulation of histone H3 phosphorylation, indicates the importance of the cAMP–PKA-signaling cascade in the control of this protein. In support of this view, this study also shows that PKA-dependent phosphorylation of DARPP-32 at Thr34 is an obligatory step in the phosphorylation of histone H3. This observation is in line with recent results showing the critical role played by nuclear accumulation of DARPP-32 in the control of histone H3 phosphorylation exerted by cocaine (Stipanovich *et al*, 2008). Thus, it appears that haloperidol enhances H3 phosphorylation through disinhibition of A2ARs, which leads to the stimulation of PKA and the concomitant suppression of PP-1 activity through phosphoThr34-DARPP-32. The idea that this mechanism occurs at the level of striatopallidal neurons is supported by recent data showing that haloperidol increases DARPP-32 phosphorylation at Thr34 specifically in this group of MSNs (Bateup *et al*, 2008).

Studies performed in striatal slices, have shown that D2Rs and A2ARs exert an opposite regulation on the state of phosphorylation of DARPP-32 (Lindskog *et al*, 1999). This observation is in agreement with the contrasting regulation exerted by haloperidol and KW6002 on histone H3 phosphorylation and indicates that, even in intact animals, the actions of these drugs are most likely exerted in the striatum, in which D2Rs and A2ARs are abundantly expressed on striatopallidal MSNs (Fink *et al*, 1992; Gerfen, 1992; Schiffmann *et al*, 1991).

ERK- and MSK1-Independent Regulation of Histone H3 Phosphorylation in Striatopallidal MSNs

Drugs able to promote cAMP-dependent signaling in the striatum, such as cocaine and L-DOPA, activate ERK and MSK1 through phosphorylation of DARPP-32 at Thr34 (Santini *et al*, 2007; Valjent *et al*, 2000). This activation of ERK and MSK1, which depends on D1Rs, mediates the concomitant increase in the state of phosphorylation of histone H3 (Brami-Cherrier *et al*, 2005; Santini *et al*, 2007). Based on this evidence, it has been proposed that the regulation of histone H3 in striatonigral MSNs involves the sequential activation of cAMP–PKA–DARPP-32 and ERK–MSK1 signaling (Girault *et al*, 2007). The idea of a critical role for ERK and MSK1 in the regulation of histone H3 is further supported by recent studies on histone phosphorylation in the hippocampus (Chwang *et al*, 2006; Chwang *et al*, 2007).

In contrast to the work mentioned above, several lines of evidence presented in this study show that ERK signaling is not involved in the phosphorylation of histone H3 produced by blockade of D2Rs. First, the modest and regionally restricted stimulation of ERK phosphorylation, observed in response to haloperidol, contrasts with the large and more widespread increase in histone H3 phosphorylation, suggesting that these events are functionally uncoupled. Second, and most importantly, neither pharmacological inhibition of ERK, nor genetic inactivation of MSK1, affects the increase in histone H3 phosphorylation produced by the blockade of D2Rs. These observations, together with the finding of lower expression of MSK1 in D2R- as compared with D1R-containing MSNs, suggest that ERK–MSK1 signaling is not necessary for histone H3 phosphorylation in striatopallidal neurons. Therefore, it is likely that, in these MSNs, A2AR-activated signaling pathways increase histone H3 phosphorylation independently of ERK–MSK1, possibly directly through PKA, or through other protein kinases. In support of this interpretation, PKA has been shown to phosphorylate chromatin *in vitro* (Taylor, 1982), and has been proposed to represent the physiological histone H3 kinase in non-neuronal cells (DeManno *et al*, 1999; Salvador *et al*, 2001). The involvement of phosphoThr34-DARPP-32, in the haloperidol-mediated regulation of histone H3, further suggests that PP-1 may be involved in histone dephosphorylation at Ser10.

Recent evidence showed the existence of important differences between striatonigral and striatopallidal MSNs with respect to several forms of synaptic plasticity (Cepeda *et al*, 2008; Day *et al*, 2006; Kreitzer and Malenka, 2007; Shen *et al*, 2007). The divergence in the role of ERK signaling and the mechanisms of regulation of chromatin remodeling, as reflected by the histone H3 phosphorylation, further shows the profound differences between these two populations of MSNs. Moreover, it sheds light on the molecular basis of the actions of antipsychotic drugs and on mechanisms implicated potentially in their extrapyramidal side effects.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that over the past 3 years P.G. has received compensation from Intracellular Therapies, Inc; PsychoGenics; Neurologix; Pfizer; Sanofi-Aventis.

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