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# Investigation of dopaminergic signalling in Meis homeobox 1 (*Meis1*) deficient mice as an animal model of restless legs syndrome

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## Summary

Restless legs syndrome (RLS) is a common neurological disorder in which sensorimotor symptoms lead to sleep disturbances with substantial impact on life quality. RLS is caused by a combination of genetic and environmental factors, and Meis homeobox 1 (*MEIS1*) was identified as the main genetic risk factor. The efficacy of dopaminergic agonists, including dopamine $D_2$ receptor (DRD2) agonists, for treating RLS led to the hypothesis of dopaminergic impairment. However, it remains unclear whether it is directly involved in the disease aetiology and what the role of *MEIS1* is considering its developmental and postnatal expression in the striatum, a critical structure in motor control. We addressed the role of *MEIS1* in striatal dopaminergic signalling in *Meis1*$^{+/-}$ mice, a valid animal model of RLS, and in *Meis1*$^{Drd2-/-}$ mice carrying a somatic null mutation of *Meis1* in Drd2$^{+}$ neurones. Motor behaviours, pharmacological exploration of DRD2 signalling, and quantitative analyses of DRD2$^{+}$ and DRD1$^{+}$ expressing neurones were investigated. Although *Meis1*$^{+/-}$ mice displayed an RLS-like phenotype, including motor hyperactivity at the beginning of the rest phase, no reduction of dopaminoceptive neurones was observed in the striatum. Moreover, the null mutation of *Meis1* in DRD2$^{+}$ cells did not lead to RLS-like symptoms and dysfunction of the DRD2 pathway. These data indicate that MEIS1 does not modulate DRD2-dependent signalling in a cell-autonomous manner. Thus, the efficiency of $D_2$-like agonists may reflect the involvement of other dopaminergic receptors or normalisation of motor circuit abnormalities downstream from defects caused by MEIS1 dysfunction.

**KEYWORDS**

animal behaviour, basal ganglia, genetic mouse models, hyperactivity, motor disorders, sex differences

## 1 | INTRODUCTION

Restless legs syndrome (RLS) is one of the most common sensorimotor neurological disorders, affecting 5%–10% of the population in Western countries. RLS diagnosis is based on evaluation of key sensorimotor symptoms, including an urge to move the legs particularly at the onset of the rest period and during the night, leading to severe sleep disturbances (Allen et al., 2014). In 80%–89% of patients, RLS is also associated with periodic limb movements (PLM) and eventually with hyperarousal (Michaud et al., 2002). Due to the lack

of knowledge on its physiopathology, there is no medication to cure RLS, but several types of symptomatic treatments have been used to date (During & Winkelman, 2019; Garcia-Borreguero et al., 2016; Winkelmann et al., 2018). In addition to α2δ ligands and opiates, the most commonly prescribed are dopamine $D_2$-like class receptor agonists, including dopamine $D_2$ receptor (DRD2) preferential agonists such as bromocriptine, and DRD3 preferential agonists such as pramipexole and ropinirole (During & Winkelman, 2019; Garcia-Borreguero et al., 2016; Winkelmann et al., 2018). Importantly, the latter two compounds bind with best affinity to DRD3 (inhibition constant [Ki] = 0.5 nM and Ki = 2.9 nM, respectively), but they can also bind DRD2 (Ki = 3.9 nM and Ki = 3.7 nM respectively) with an affinity similar to that of bromocriptine (Ki = 2.5 nM) (Coldwell et al., 1999; Kvernmo et al., 2006). Thus, from the pharmacological point of view, activation of DRD2 might be a convergent point of therapeutic effects. Although all these dopaminergic treatments are efficient for RLS and PLM, bromocriptine is less frequently administered due to more side-effects (Manconi et al., 2011; Walters et al., 1988; Winkelman et al., 2016). Moreover, dopaminergic agonists are used with caution because of the "augmentation" phenomenon of RLS symptoms, which can occur after about 6 months of chronic daily use (Earley & Allen, 1996).

The aetiology of RLS is complex, caused by a combination of genetic and environmental factors. In the majority of cases, early onset RLS is idiopathic and/or familial with the appearance of symptoms usually before the age of 45 years and slow progression (Allen & Earley, 2000; Winkelmann et al., 2002). The first genome-wide association study (GWAS) identified Meis homeobox 1 (*MEIS1*) as the major genetic risk factor for RLS (Winkelmann et al., 2007). This discovery has been then confirmed in several subsequent studies, including GWAS (Schormair et al., 2017; Spieler et al., 2014; Winkelmann et al., 2011) and other association studies (Kemlink et al., 2009; Vilariño-Güell et al., 2008; Yang et al., 2011). Several single-nucleotide polymorphisms (SNP) were identified in the non-coding regions of *MEIS1*, including rs12469063 positioned in a *MEIS1* intronic enhancer element (Schulte et al., 2011; Spieler et al., 2014). Accordingly, the risk allele of this SNP is associated with a ~50% reduction of enhancer activity. Moreover, rare genetic variants with strong effects could also contribute to the heritability of RLS. The most deleterious is a missense non-synonymous mutation Arg272His (p.R272H) affecting the first amino acid of the highly conserved DNA binding domain of *MEIS1*, which is essential for DNA binding (Schulte et al., 2014; Vilariño-Güell et al., 2009). Although the hypothesis of impaired dopaminergic signalling through $D_2$-like receptors is the most prevalent in RLS, it is not clear whether the dopaminergic system (including DRD2) is primarily affected in RLS and what the link with MEIS1 is.

*Meis1*$^{tm1Mtor}$ mice, heterozygous for an inactivating mutation of *Meis1* (Azcoitia et al., 2005), appeared useful for revealing the effects of reduced Meis1 expression on RLS-related phenotypes in mice, fulfilling some of the main criteria of a RLS animal model (Salminen et al., 2017, 2018; Schulte et al., 2014; Spieler et al., 2014). Although the diagnosis of RLS in human patients is established according to their subjective description of sensorimotor symptoms, it is accepted that an increase in locomotor activity in rodent models is an indirect parameter reflecting the urge to move (Allen et al., 2017; Baier et al., 2007). Accordingly, middle-aged *Meis1*$^{tm1Mtor}$ male mice have displayed spontaneous motor hyperactivity, particularly at the beginning of the rest phase, but without impairments of motor coordination or grip strength (Salminen et al., 2017). Such abnormalities were less consistent in female mice, which displayed only some hyperactivity in voluntary wheel-running at a young age (Salminen et al., 2017), but no spontaneous hyperactivity (Spieler et al., 2014). Moreover, analyses of heterozygous *Meis1*$^{tm1Mtor}$ mice revealed reduced sensitivity to the effects of the DRD2/3 agonist pramipexole on sensorimotor gating in pre-pulse inhibition (Salminen et al., 2017) and sleep (Salminen et al., 2018). Although such data support the potential involvement of dopaminergic systems in *Meis1*$^{tm1Mtor}$ phenotype, no apparent abnormalities in the number of dopaminergic neurones in the substantia nigra pars compacta and ventral tegmental area were detected (Salminen et al., 2017).

An alternative possibility of altered development, survival, or activity of dopaminoceptive neurones in the striatum, the key structure involved in the control of motor activity, has not been extensively investigated. Indeed, balanced signalling of direct and indirect pathways defined respectively by expression of DRD1 and DRD2 on striatonigral (sn) and striatopallidal (sp) medium spiny neurones (MSNs) is affected in many neurological diseases with motor abnormalities (Crittenden & Graybiel, 2011) and could also contribute to RLS physiopathology. A recent study by Lyu et al. reported hyperactivity of *Meis1*-deficient mice resulting from deletion of the eighth exon of the *Meis1* gene. This mouse model displayed decreased tyrosine hydroxylase protein expression associated with an increased dopaminergic turnover supporting abnormal signalling of dopaminergic neurones projecting to the striatum, although without any apparent changes in striatal DRD2 protein expression (Lyu et al., 2020). The mammalian striatum is also populated by aspiny neurones, including cholinergic interneurones expressing choline acetyltransferase (ChAT), which are important modulators of dopaminergic control of major striatal output pathways and relevant motor and cognitive functions (Semba et al., 1988).

Considering the potential developmental role of MEIS1 in the striatum (Rataj-Baniowska et al., 2015) and its striatal expression throughout adult life (Allen Brain Atlas), we hypothesised that compromised MEIS1 signalling may impact development and/or function or survival of DRD2+ spMSNs leading thereby to RLS-like motor symptoms.

In the present study, we addressed this hypothesis by studying the integrity of dopaminergic circuits in the striatum of *Meis1*-deficient mice carrying a heterozygous null mutation of *Meis1*, resulting from a knock-in of the cyan fluorescent protein (CFP) into the first exon of the *Meis1* gene, named hereafter *Meis1*$^{+/-}$ (González-Lázaro et al., 2014). As this model has never been used for research into RLS, we first validated and extended the characterisation of the RLS-like motor behaviour of this line. We next quantified the distribution of DRD1$^+$ and

DRD2$^+$ MSNs. Furthermore, we generated a somatic null mutation of *Meis1* in DRD2$^+$ neurones in *Meis1*$^{Drd2-/-}$ mice and investigated RLS-like motor behaviour along with the functionality of dopaminergic signalling in the striatum. To this end, catalepsy and cFos expression were explored after pharmacological challenge with haloperidol as a preferential DRD2 antagonist. Our data indicate that the RLS-like motor restlessness/hyperactivity observed in *Meis1*$^{+/-}$ mice does not involve cell-autonomous functions of MEIS1 in DRD2$^+$ neurones, suggesting that $D_2$-like agonists may act primarily through DRD3 receptors or downstream from MEIS1-affected cells or circuits.

## 2 | MATERIALS AND METHODS

The overall experimental design is summarised in Figure 1.

### 2.1 | Animals

#### 2.1.1 | Ethical committee agreement

The experiments described below were carried out following the European Community Council Directives of 24 November 1986 (86/609/EEC) and in compliance with the guidelines of the Centre National de la Recherche Scientifique (CNRS) and the French Agricultural and Forestry Ministry (decree 87,848). All protocols were approved by the local Ethics Committee (authorisation number: 2018081012086762) and accredited by the French Ministry for Superior Education and Research in accordance with the Directive of the European Union Council (2010/63/EU).

All mice were housed in individually ventilated cages (Techniplast, Italy) in groups of 3–4 mice per cage in 12/12 hr light/dark cycle (lights on from 07:00 to 19:00 hours). Water and food were available ad libitum.

#### 2.1.2 | Meis1$^{ECFP}$ mouse model

The he heterozygous line for *Meis1* null mutation was generated previously by knock-in of enhanced CFP (ECFP) into exon 1 followed by a stop codon, *Meis1*$^{tm2.1Mtor}$ (González-Lázaro et al., 2014). Experimental cohorts were obtained by crosses between wild-type (WT) and *Meis1*$^{+/-}$ mice maintained on the same mixed C57BL6N (87%) × CD1 (13%) genetic background for >10 generations. Null mutant mice are embryonic lethal as previously reported for the *Meis1*$^{tm1Mtor}$ line (Azcoitia et al., 2005).

For the behavioural and pharmacological experiments, the *Meis1*$^{+/-}$ mice were tested with their *Meis1*$^{+/+}$ (WT) littermate controls at the age of 35–45 weeks (indicated as 8 months). For the histological analysis, mice aged 4 and 8 months were used.

#### 2.1.3 | *Meis1*$^{Drd2-/-}$ mouse model and validation of *Meis1* exon 8 excision

Somatic *Meis1*$^{Drd2-/-}$ mutant mice were generated by crossing mice carrying floxed exon 8 of the *Meis1* gene (Kocabas et al., 2012) with transgenic line expressing Cre recombinase under the control of Drd2 promoter (Gene Expression Nervous System Atlas [GENSAT]) (Gong

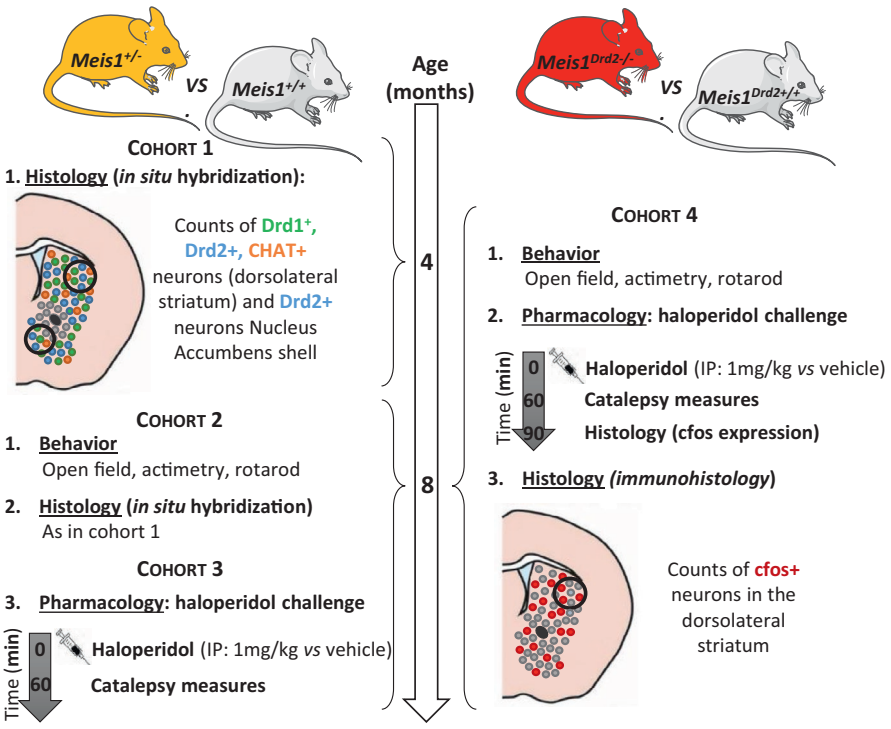



**FIGURE 1** Details for the experimental design. CHAT, choline acetyltransferase; Drd1, dopamine $D_1$ receptor; Drd2, dopamine $D_2$ receptor; IP, intraperitoneal; Meis1, Meis homeobox 1.Graphics adapted from Servier Medical Art under a Creative Commons licence (CC BY 3.0).

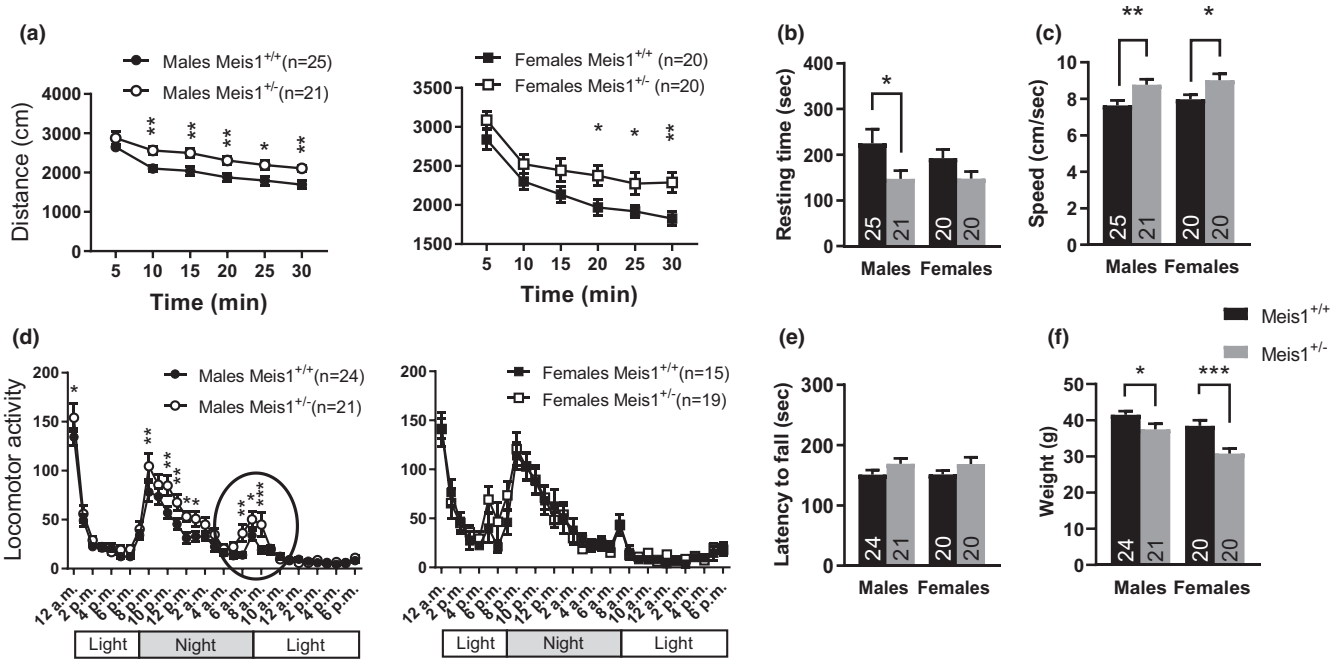



**FIGURE 2** Motor hyperactivity in 8-month-old *Meis1*$^{+/-}$ mice. (a) The mean distance travelled in the open field is shown for successive 5-min time-bins of the 30-min testing period for *Meis1*$^{+/-}$ and WT males (left panel) and females (right panel). (b) The average total resting time over the entire 30 min of testing in the open field was shown for each sex and genotype group. (c) The average movement speed in the open field arena over the 30-min testing period. (d) The spontaneous home-cage locomotor activity was monitored during 32 consecutive hours in actimetric cages. The number of infra-red beam breaks was used to express locomotor activity for males (left panel) and females (right panel). The hyperactivity at the beginning of the light phase/ resting period is indicated by a circle. (e) Motor coordination assessed by rotarod was expressed as the latency to fall (mean of the three trials). (f) Weights of the mice were scored after the rotarod test. The number of tested mice is provided in the legends of the graphs or in the corresponding bars of the histograms. Error bars correspond to *SEM*. Statistical differences with *Meis1*$^{+/+}$ mice are indicated for each group as $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ (ANOVA followed by post hoc Fisher's least significant difference test)

et al., 2003). Excision of exon 8 leads to loss of function of any putative Meis1 protein. Mice were on C57BL6N (97%) × CD1 (3%) genetic background. The *Meis1*$^{Drd2-/-}$ mice were tested with their *Meis1*$^{Drd2+/+}$ littermate controls carrying only floxed, but functional Meis1 gene at 10 months of age. The efficiency of excision of Meis1 exon 8 in *Meis1*$^{Drd2-/-}$ mice was validated using polymerase chain reaction (PCR) with a mix of three primers (P1, P2, P3; Figure 3a) in the striatum, where excision by Drd2-Cre is expected and in the cortex, where no Drd2-Cre is expressed and no excision should occur. The 261bp band diagnostic for excision event was amplified by P1 and P3 primers, which in WT or floxed mice do not amplify fragment of ~2.5 kb in our PCR conditions (Figure 3).

## 2.2 | Behavioural analyses

All behavioural tests were carried out according to the Standard Operating Procedure (SOP) established by the Mouse Clinical Institute (Mandillo et al., 2008). Naïve mice from all the different cohorts were subjected to three locomotor behavioural tests, successively done at the rate of one test/week for 3 consecutive weeks, and always in the order of presented in Figures 1 and 4. All experiments (except actimetry) were done between 09:00 and 16:00 hours. The number of mice used for each test is indicated in the legend or within the bar graphs.

### 2.2.1 | Open-field

To evaluate the novelty-induced motor activity, mice were placed in a squared arena with transparent walls (45 × 45 × 18 cm) for 30 min. Light intensity in the centre of the arena was set at 150 lux. Mouse movement was recorded and analysed using ActiTrack set-up and software (Bioseb, France).

### 2.2.2 | Actimetric cages

Spontaneous locomotor activity of each single mice was measured in individual actimetric cages (Imetronic, Pessac, France), for 32 consecutive hr starting at 11:00 and terminating at 19:00 hours of the next day. The light/dark cycle was maintained as in home-cage conditions with light on at 07:00 and light off at 19:00 hours.

Mouse activity was detected using infrared-light-beam frames suitable for monitoring horizontal and vertical (rearing) activities. Throughout the test period, all mice had ad libitum access to water and food.

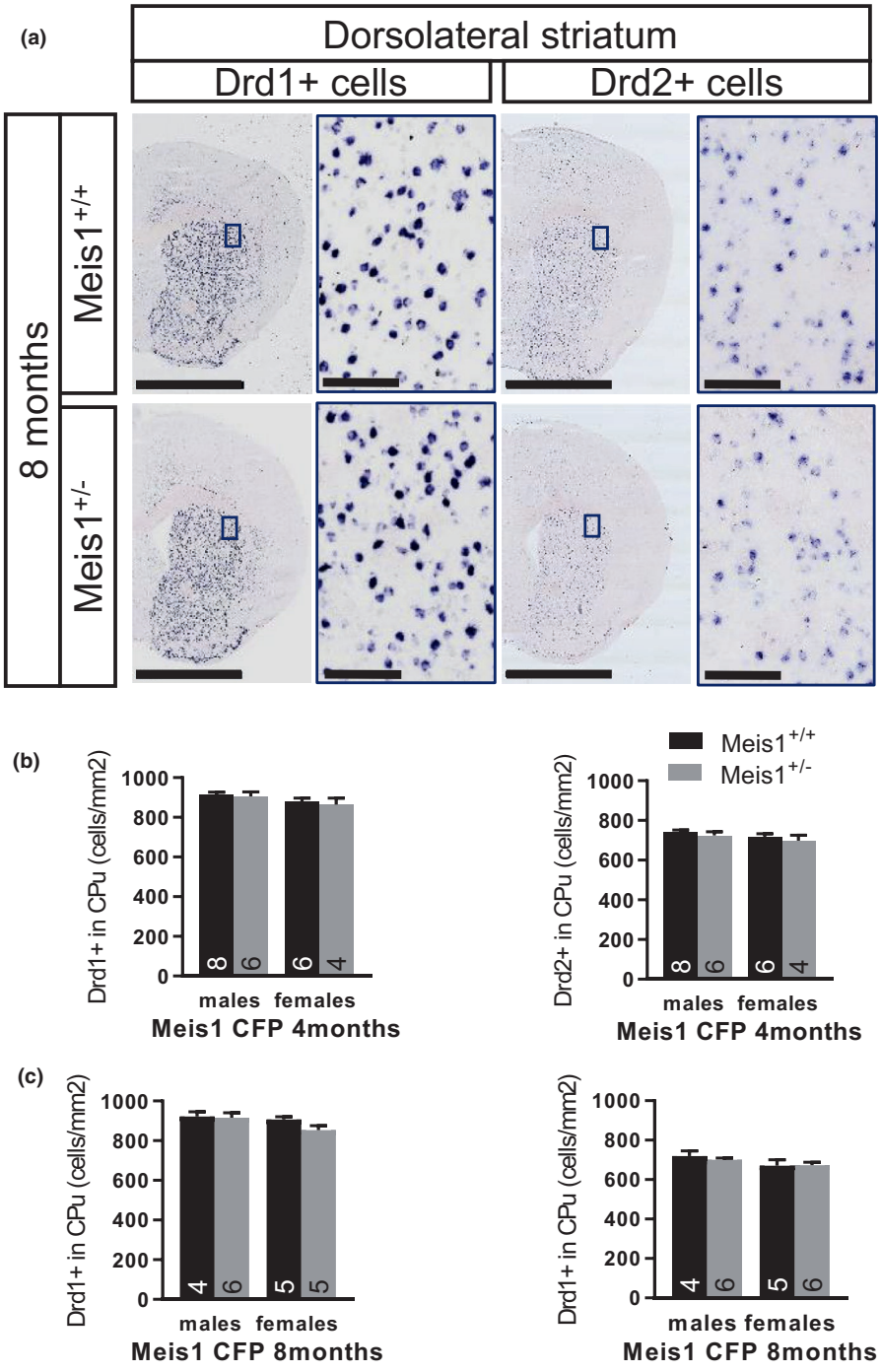



**FIGURE 3** The number of dopaminoceptive DRD1$^+$ and DRD2$^+$ medium spiny neurones (MSN) is conserved in the dorsolateral striatum of *Meis1*$^{+/-}$ mice. (a) Example of *in situ* detection of *Drd1*- and *Drd2*-expressing cells in the dorsolateral region caudate putamen (CPu) obtained after scanning of hybridised 8-month-old females *Meis1*$^{+/-}$ and *Meis1*$^{+/+}$ brain cryosections. For each *Drd1*$^+$ or *Drd2*$^+$ cell staining example, the left panel summarises the counting zone (indicated with box) in which positive MSNs were counted (scale bar 2.5 mm) whereas the right panel depicts an enlargement of the region of interest (ROI) (scale bar 100 μm). (b) The graphs show the number of *Drd1*$^+$ MSNs per mm$^2$ in the CPu of 4-month-old (left panel) and 8-month-old (right panel) *Meis1*$^{+/-}$ mice compared to their *Meis1*$^{+/+}$ littermates. (c) The graphs show the number of *Drd2*$^+$ MSNs per mm$^2$ in the CPu of 4-month-old (left panel) and 8-month-old (right panel) *Meis1*$^{+/-}$ mice compared to their *Meis1*$^{+/+}$ littermates. Results are plotted as mean ± *SEM* and ANOVA were used to compare mutant and WT, but no statistical differences between the two groups were found. The number of tested mice is provided in the legends of the graphs or in the corresponding bars of the histograms. CPu, dorsolateral striatum; *Drd1*$^+$ and *Drd2*$^+$, dopamine receptor Type 1 and 2; ISH, *in situ* hybridisation; MSN, medium spiny neurones

### 2.2.3 | Rotarod

The rotarod device (ROTA-ROD/RS, LE8200, LSI Letica Scientific Instruments, Bioseb, France) was used to investigate motor coordination and balance. Briefly, mice were placed on the cylinder of the rotarod for a 60-s habituation period, after which the cylinder was set to rotation with acceleration from 4 to 40 rpm in 5 min. The test consisted of three consecutive trials per mouse, separated by 15–20 min of recovery and there was no habituation phase in trials 2 and 3. The mean latencies to fall off the rotarod were then used for statistical analyses. After the test, the mice were weighed and the weight was considered for statistical analysis and standardisation of rotarod performance (see statistics). Indeed, besides the genotype, the weight of mice can influence the results of the rotarod test.

## 2.3 | Pharmacology and catalepsy test

Mice received intraperitoneal injections of 1 mg/kg haloperidol (Sigma, France) 60 min before the behavioural test. The catalepsy was tested by placing the forelimbs of the mouse on a horizontal bar fixed in a testing box at 4 cm above the ground. The time of complete immobility corresponding to the time required by the mouse to step

down from the bar was measured with a cut-off of 120 s for the catalepsy duration. Each mouse was tested twice: before the injection (spontaneous catalepsy) and then 60 min after the injection of haloperidol (haloperidol-induced catalepsy) or vehicle (control group). Each test consisted of three successive trials and the highest value was kept for the statistical analysis. Brains of treated mice were collected at 90 min after injection for further analysis.

## 2.4 | Histology

Mice were then killed by cervical dislocation, brains were frozen in cryoprotective media (OCT, Thermo Shandon Cryomatrix, Theermofisher Scientific, France) and stored at −80°C before further processing. Coronal cryosections of 14 μm were collected on Super-Frost Plus slides (MenzelGlaser) and stored at −20°C for analyses. The number of mice used for histological staining is indicated in the corresponding figures.

### 2.4.1 | *In situ* hybridisation

*In situ* hybridisations were carried according to previously published protocols using digoxigenin labelled riboprobes specific for *Drd1* (402 bp fragment of 3′ region of *Drd1*), *Drd2* (1681 bp fragment of 5′ region of *Drd2*), ChAT (full-length cDNA). Anti-digoxigenin antibody conjugated with alkaline phosphatase (anti-DIG, Roche 1093274, 1/2,000) followed by application of NBT (nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl phosphate)/levamisole solution were used for riboprobe detection.

### 2.4.2 | Immunofluorescence

Brain sections were post-fixed with 4% paraformaldehyde (v/v) and immunofluorescent detection of cFos was carried out using cFos antibody (mouse IgG1, Novus, 1:1,000) following permeabilisation in 0.1% Triton X100 in phosphate-buffered saline for 10 min and saturation of non-specific binding with 10% of heat-inactivated fetal calf serum for 1 hr. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, France). To control signal specificity secondary antibody was also applied without the corresponding primary antibodies in control brain sections. Slides were imaged using a Nanozoomer digital scanner (Hamamatsu Photonics, France).

### 2.4.3 | Cell counts

Cell number analyses were blinded to genotype. MSNs expressing $Drd1^+$, $Drd2^+$, and $ChAT^+$ were counted per $mm^2$ in the dorsolateral part of the striatum (caudate putamen [CPu]) and the ventral striatum (nucleus accumbens shell [NAccSh]). For each mouse, counting was done on at least three sections selected at corresponding bregma levels 1.15 and 0.5 for the dorsolateral striatum and between bregma 1.10 and 1.50 for the NAccSh. In each section three regions of interest (ROI) were selected semi-randomly in the dorsolateral region, as well as three ROI in the NAccSh, and mean data were used for the statistical analysis for each respective region. cFos-positive cells were counted using a similar approach and mean data were used for the statistical analysis.

## 2.5 | Statistics

To assess the effect of sex and genotype on the tested parameters, data were analysed using two-way analysis of variance (ANOVA) using sex and genotype as independent variables, or three-way repeated measures (RM) ANOVA in the case of the additional time factor. In case a difference between the sexes was observed for one parameter, each sex was also analysed by two-way RM ANOVA with genotype and time as variables. Post hoc Bonferroni's (histological and pharmacological analyses) or Fisher's least significant difference test (LSD; behavioural analyses) analyses were carried out in presence of a significant group effect or interaction in ANOVA analyses.

# 3 | RESULTS

## 3.1 | *Meis1* deficiency induces RLS-like motor dysfunction without affecting direct and indirect pathways in the striatum

*Meis1* deficiency in $Meis1^{tm1Mtor}$ heterozygous mice was shown to induce an RLS-like motor phenotype (Salminen et al., 2017). Although abnormal dopaminergic signalling in the striatum could underlie this phenotype, it has not been studied previously. To address this possibility we used a distinct $Meis1^{+/-}$ mouse line carrying a gene-inactivating knock-in of ECFP in the first exon of *Meis1* (González-Lázaro et al., 2014). We first investigated the motor behaviour of this model and its relevance to RLS and then quantified $DRD1^+$ and $DRD2^+$ MSNs in the striatum. Both $Meis1^{+/-}$ males and females displayed a significant increase in the distanced covered during the successive 5-min time bins of the 30-min testing period in the open-field test of novelty-induced activity. This was indicated by the main effect of *genotype* ($F$ (1, 82) = 13.39, $p < .001$) without *sex* effect ($F$ (1, 82) = 1.17, not statistically significant [ns]) or interaction *sex* × *genotype* × *time* ($F$ (5, 410) = 0.668, ns) in three-way ANOVA and post hoc analyses using Fisher's LSD test (Figure 2a). Two-way ANOVA analyses of the resting time and average speed of movement followed by post hoc Fisher's LSD test revealed also that hyperactivity phenotype resulted from both reduced time spent immobile ($F$ (1, 82) = 6.941, $p < .05$ for *genotype*, without *sex* effect $F$ (1, 82) = 0.4772, ns or interaction *sex* × *genotype* $F$ (1, 82) = 0.5011, ns; Figure 2b) and higher movement speed ($F$ (1, 82) = 13.94, $p < .001$ for *genotype*, without *sex* effect $F$ (1, 82) = 0.9527, ns or interaction

*sex* × *genotype* $F$ (1, 82) = 0.01839, ns; Figure 2c) over the entire 30 min of the test. However, no difference was observed between the two genotypes in rearing activity for both sexes during the successive 5-min time bins of the 30-min testing period (Figure S1a), as shown by the absence of *genotype* and *sex* effects in a three-way ANOVA (respectively $F$ (1, 82) = 0.6131, ns and $F$ (1, 82) = 1.668). These results were not confounded by altered anxiety state, as the percentage of time spent in the centre of the arena during the first 5 min was not affected in *Meis1*$^{+/-}$ mice (two-way ANOVA, $F$ (1, 82) = 0.7073, ns for *genotype* effect, $F$ (1, 82) = 3.448, ns for *sex* effect, $F$ (1, 82) = 0.6271, ns for *sex* × *genotype* interaction) and ranged between 16.6 ± 1.7% and 14.34 ± 1.8% of 5 min for WT and *Meis1*$^{+/-}$ males and 12.9 ± 0.8% and 12.8 ± 0.9% for WT and *Meis1*$^{+/-}$females, respectively.

Abnormal motor activity of *Meis1*$^{+/-}$ mice was also observed in a test of spontaneous locomotion in actimetric cages where the activity over the 32 hr testing period was sex and genotype-dependent as indicated by *genotype* × *sex* × *time* interaction in three-way ANOVA test ($F$ (30, 1,800) = 1.629, $p$ <.05). Two-way RM ANOVA analysis of locomotor activity for each sex throughout 32 hr of the testing period followed by post hoc Fisher's LSD analyses revealed that *Meis1*$^{+/-}$ males ($F$ (1, 41) = 6.415 for genotype, $p$ < .05 and $F$ (30, 1,230) = 1.713, $p$ < .01 for *genotype* × *time* interaction), but not females ($F$ (1, 19) = 0.006258 for genotype, ns and $F$ (30, 570) = 0.7699, ns for *genotype* × *time* interaction), displayed motor hyperactivity. Importantly, this hyperactivity was evident at the onset of the resting phase at the beginning of the light cycle (activity peak between 07:00 and 08:00 hours), but also at the beginning of the test and during the active dark phase of the light/dark cycle (Figure 2d).

*Meis1*$^{+/-}$ mice did not display altered locomotor coordination when tested in the rotarod test as indicated by the absence of statistical differences obtained with Fisher's LSD post hoc test following two-way ANOVA analyses ($F$ (1, 81) = 4.215, $p$ >.05 for *genotype*, $F$(1, 81) = 1.76*10$^{-5}$, ns for *sex*, $F$ (1, 81) = 0.0008812, ns for *sex* × *genotype* interaction) (Figure 2e). These latter measures had to be corrected for the weight due to weight differences between groups (mean 39.9 ± 1.3 g for WT males; 37.4 ±1.6 g for *Meis1*$^{+/-}$ males; 38.5 ±1.6 g for WT females, and 30.8 ±1.4 g for *Meis1*$^{+/-}$ females). Indeed, *Meis1*$^{+/-}$ males and females had reduced weight when compared to their WT sex-matched littermates as indicated by two-way ANOVA followed by Fisher's LSD post hoc test ($F$ (1, 81) = 18.1, $p$ < .001 for *genotype*, $F$ (1, 81) = 12.47 $p$ < .01 for *sex*, $F$ (1, 81) = 1.754, ns for *genotype* × *sex* interaction) (Figure 2f). The weight difference between *Meis1*$^{+/-}$ and WT mice may reflect increased activity of *Meis1*$^{+/-}$ mice, but also potential differences in food intake. To address this point, food intake during the 32 hrs of the actimetry test was analysed. Two-way ANOVA analyses did not reveal any significant difference ($F$ (1, 54) = 1.998, ns for *genotype*, $F$ (1, 54) = 3.83*10$^{-5}$, ns for *sex* effects, $F$ (1, 54) = 0.07743, ns for *sex* × *genotype* interaction). In total, *WT* males ate a mean of 6.0 ±0.6 g, *Meis1*$^{+/-}$ males ate 4.9g ±0.5 g, *WT* females ate 5.8 ±0.7 g and *Meis1*$^{+/-}$ females ate 5.1 ±0.5 g.

Considering that *Meis1* is expressed in developing and postnatal striatum (Allen Mouse Brain Atlas) and that its potential involvement in striatal development was suggested (Rataj-Baniowska et al., 2015), we investigated the possibility that *Meis1* haploinsufficiency may affect striatal development or striatal integrity during ageing.

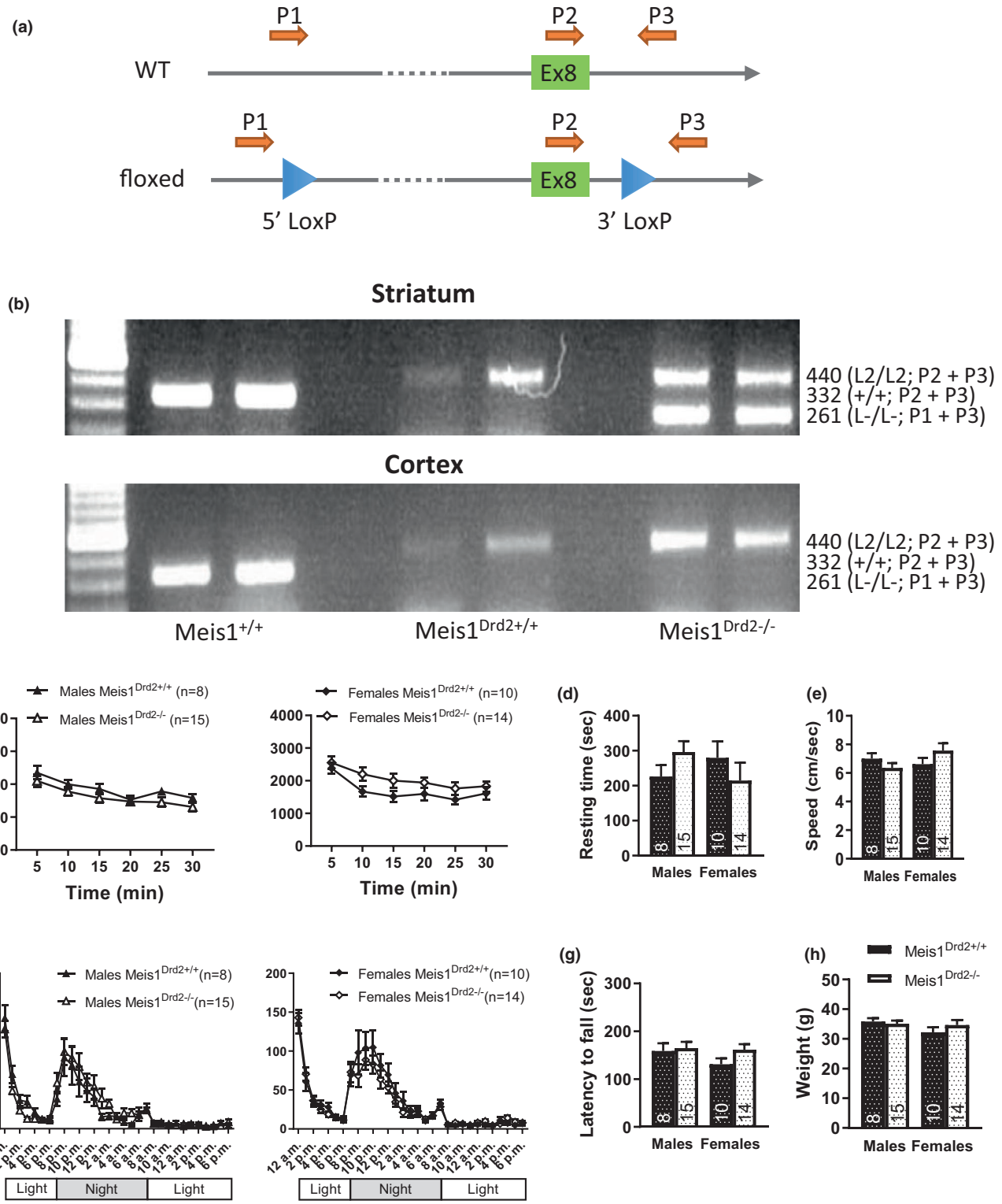
To this end, we used *in situ* hybridisation of Drd1$^{+}$ and Drd2$^{+}$ to perform quantitative analyses of snMSN and spMSN respectively in 4- and 8-month-old mice (Figure 3a–c). In 4-month-old mice, the number of Drd1$^{+}$ cells was ~890 cells/mm$^2$, whereas that of DRD2$^{+}$ cells was ~720 cells/mm$^2$ in CPu of both WT and *Meis1*$^{+/-}$ males and females, and no statistical differences were observed between any of the tested groups (Figure 3b). To investigate the potential role of MEIS1 in the maintenance of those cells, we also performed cell counts at 8 months of age, when the behavioural phenotype is evident. However, cell number did not change significantly to that observed at 4 months (~900 cells/mm$^2$ for DRD1$^{+}$ and ~690 cells/mm$^2$ for DRD2$^{+}$) and there was also no significant difference between WT and *Meis1*$^{+/-}$ males or females (Figure 3c).

To test whether MEIS1 may affect other striatal regions, we focussed on DRD2$^{+}$ spMSNs in the NAccSh (Figure S2a). The number of cells was somewhat higher than in CPu, attaining ~930 cells/mm$^2$ irrespective of genotype, sex, and age. Finally, to test whether MEIS1 may modify the formation of other cell types involved in the control of MSN signalling, we also quantified cholinergic interneurones by counting ChAT-positive cells. However, no difference was observed either for the 4- or 8-month-old *Meis1*$^{+/-}$ mice in the number of ChAT$^{+}$ interneurones in the dorsolateral part of the striatum, with ~26 cells/mm$^2$ and ~23 cells/mm$^2$ at 4 and 8 months, respectively, and independently of sex and genotype (Figure S2b,c).

### 3.2 | Somatic inactivation of *Meis1* in DRD2$^{+}$ cells does affect mouse behaviours and dopaminergic signalling

Although we did not observe any effect of *Meis1* deficiency on MSN content in the striatum, we cannot exclude that the functions of those neurones were affected in *Meis1*$^{+/-}$ mice and/or development or survival of those neurones could be compromised in absence of Meis1. Considering the beneficial effects of DRD2 receptor agonists, DRD2$^{+}$ spMSNs were of particular interest to us. Thus, to address these points and to increase the robustness of our approach we generated mice carrying a somatic null mutation of *Meis1* in DRD2$^{+}$ neurones by *Drd2*-Cre mediated inactivation of floxed *Meis1* exon 8 and compared *Meis1*$^{Drd2-/-}$ to *Meis1*$^{+/+}$ mice for their RLS-like behavioural performance and functionality of DRD2 signalling (Figure 4a).

To validate the excision of *Meis1* in the striatum of *Meis1*$^{Drd2-/-}$ mice, we performed PCR analyses. As expected, we confirmed excision of *Meis1* exon 8 in the striatum (261 bp band), the site of *Drd2*-Cre expression, but not in the cortex (Figure 4b). To investigate whether *Meis1* inactivation in DRD2$^{+}$ spMSNs leads to an RLS-like




phenotype, we analysed the behaviour of *Meis1*$^{Drd2-/-}$ using the same behavioural battery of tests as that used for *Meis1*$^{+/+}$ mice.

We did not observe any difference between *Meis1*$^{Drd2-/-}$ and *Meis1*$^{Drd2+/+}$ in novelty-induced activity in the open-field ($F$ (1, 43) = 0.1292, ns for *genotype* effect, $F$ (5, 215) = 1.166, ns for *sex* × *genotype* × *time*) (Figure 4c). No differences between groups were observed for the resting time (two-way ANOVA, $F$ (1, 43) = 2.322, ns for interaction *sex* × *genotype*; $F$ (1, 43) = 0.002781, ns for *genotype*; Figure 4d) and the average speed (two-way ANOVA, $F$ (1, 43) = 3.179, ns for interaction *sex* × *genotype*; $F$ (1, 43) = 0.1048, ns for *genotype*; Figure 4e), the parameters affected in *Meis1*$^{+/-}$ mice. *Meis1*$^{Drd2-/-}$ mice did not display any gross abnormalities in anxiety, as the mean percentage of time spent in the centre of the arena during the first 5 min of the test was not different between *Meis1*$^{Drd2+/+}$ (7.4

**FIGURE 4** The ablation of Meis1 only in DRD2$^{+}$ medium spiny neurones (MSN) does not affect motor behaviour in mice. (a) Map of the *Meis1* locus carrying floxed exon 8 and locations of primers used for polymerase chain reaction (PCR) to test Cre-mediated excision. (b) PCR validation of Cre-mediated excision was carried out with specific primer combinations as indicated on the right side of the panel next to the size (base pairs) of expected PCR fragments. From the left to the right, PCR were performed on DNA from two independent samples obtained from the striatum (top) and cortex (bottom) of the WT, Meis1$^{Drd2+/+}$ and Meis1$^{Drd2-/-}$ mice. (c) The mean distance travelled in the open field is shown for successive 5-min time-bins of the 30-min testing period for *Meis1*$^{Drd2-/-}$ and *Meis*$^{Drd2+/+}$ males (left panel) and females (right panel). (d) The average total resting time over the entire 30 min of testing in the open field was shown for each sex and genotype group. (e) The average speed of displacement in the open-field arena over the 30 min testing period. (f) The spontaneous home-cage locomotor activity was monitored during 32 consecutive hours in actimetric cages. The number of infra-red beam breaks was used to express locomotor activity for males (left panel) and females (right panel). (g) Motor coordination assessed in rotarod was expressed as the latency to fall (mean of the three trials). (h) Weights of the mice were scored after the rotarod test. Error bars correspond to *SEM*. The number of tested mice is provided in the legends of the graphs or in the corresponding bars of the histograms.

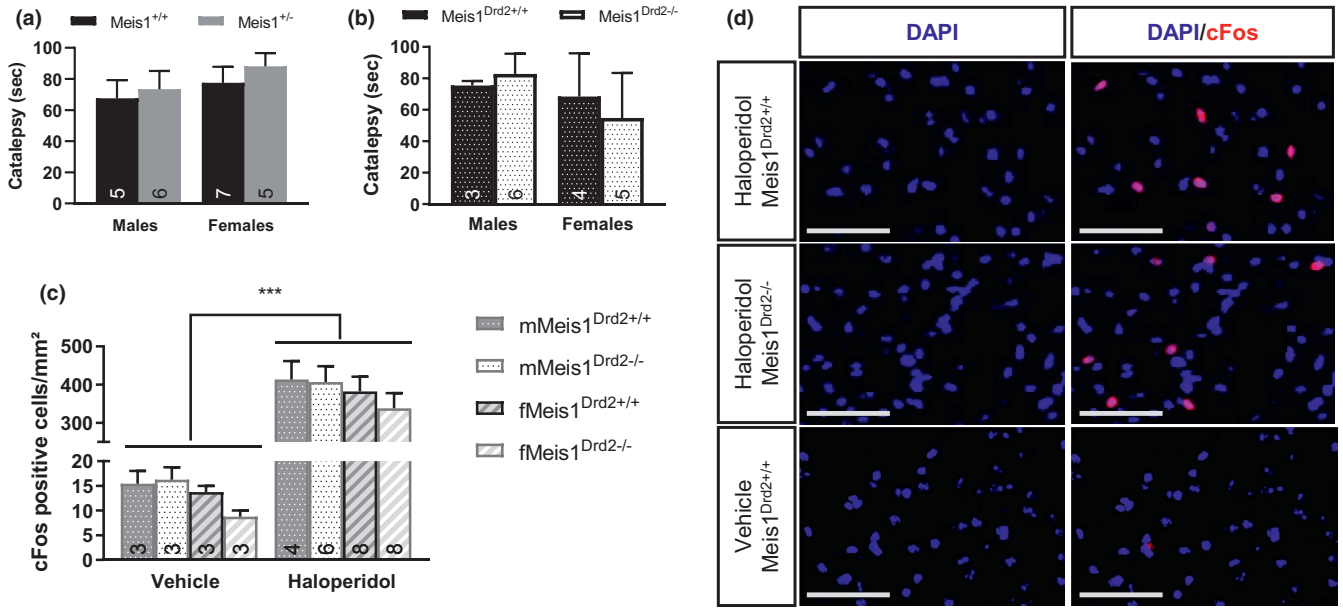



**FIGURE 5** The functions of Drd2$^{+}$ medium spiny neurones (MSN) are not affected in mice in the absence of *MEIS1*. (a) Haloperidol-induced-catalepsy of 8-month-old *Meis1*$^{+/-}$ male and female mice compared to their *Meis1*$^{+/+}$ littermates. (b) Haloperidol-induced-catalepsy of 12-month-old *Meis1*$^{Drd2-/-}$ male and female mice compared to their *Meis1*$^{Drd2+/+}$ littermates. The pharmacological response to haloperidol is expressed as the mean of the twohighest values of catalepsy. (c) cFos activation following haloperidol or vehicle injection is represented by number of cFos-positive cells contained per mm$^2$ within the dorsolateral part of the striatum. (d) Examples of cFos and 4',6-diamidino-2-phenylindole (DAPI) immunostaining on brain cryosections in the dorsolateral part of the caudate putamen following haloperidol or vehicle injections. The scale bar corresponds to 50 μm. Results are plotted as mean ± *SEM*. with *** corresponding to $p < .001$ (ANOVA and Bonferroni's post hoc test). The number of tested mice is provided in the corresponding bars of the histograms

± 1.3%) and *Meis1*$^{Drd2-/-}$ (6.6 ± 1.4%) males. This was supported by the absence of significant effect of *genotype* (two-way ANOVA; $F$ (1, 43) = 2.087, ns), or interaction *sex* × *genotype* ($F$ (1, 43) = 1.049, ns for *genotype*).

Consistent with the absence of hyperactivity phenotype in the open-field, *Meis1*$^{Drd2-/-}$ did not display any hyperactivity in the spontaneous locomotor activity assay in the actimetric cages. Statistical analyses revealed a circadian activity profile as peaks of activity were perfectly conserved between WT and mutant mice for both sexes (three-way ANOVA, $F$ (30, 1,290) = 1.279, ns for *genotype* × *sex* × *time* interaction; $F$ (1, 43) = 0.01651, ns for *genotype*; $F$ (1, 43) = 1.697, ns for *sex*; Figure 4f).

Finally, locomotor coordination and balance were not altered when investigated in rotarod. Accordingly, no statistical differences were observed in two-way ANOVA analyses of the corrected latency to fall ($F$ (1, 43) = 0.7969, ns for interaction *sex* × *genotype*; $F$ (1, 43) = 1.797, ns for *genotype*; $F$ (1, 43) = 1.271, ns for *sex*; Figure 4g). Weight differences clearly observed in *Meis1*$^{+/-}$ mice were not present (two-way ANOVA, $F$ (1, 43) = 1.146, ns for interaction *sex* × *genotype*; $F$ (1, 43) = 0.2993, ns for *genotype*; $F$ (1, 43) = 1.778, ns for *sex*; Figure 4h).

### 3.3 | Compromised Meis1 signalling does not affect functions of Drd2$^{+}$ neurones

Although *Meis1* haploinsufficiency in *Meis1*$^{+/-}$ mice did not affect MSN number and we did not identify any RLS-like behavioural

abnormalities in *Meis1*$^{Drd2-/-}$ mice, we cannot exclude that compromised or absent Meis1 signalling in these respective mouse models leads to subtle functional abnormalities in MSNs and in particular DRD2$^+$ spMSNs. To address this point, we used a pharmacological approach by challenging mutant mice with haloperidol (1 mg/kg), a DRD2 selective antagonist, and used catalepsy and cFos expression as functional readouts of DRD2 activity. *Meis1*$^{+/-}$ mice did not display any differences in spontaneous catalepsy before injection, which was ~2 s for all mice. Also after haloperidol treatment, no significant difference was seen between WT and *Meis1*$^{+/-}$ mice as indicated by two-way-ANOVA analyses ($F$ (1, 19) = 0.04728, ns, for *sex* × *genotype* interaction or $F$ (1, 19) = 0.562, ns for *genotype* effect; $F$ (1, 19) = 1.276, ns for *sex* effect Figure 5a). To further investigate whether complete loss of MEIS1 expression in DRD2$^+$ neurones might impact their function, we next tested *Meis1*$^{Drd2-/-}$ mice. We did not observe any difference in catalepsy scores before treatment (~2 s for latency to move for all mice) and 60 min after the haloperidol treatment (Figure 5b). Accordingly, two-way ANOVA analyses did not reveal any genotype-dependent differences in response to haloperidol ($F$ (1, 14) = 1.017, ns for *genotype* × *sex* interaction, $F$ (1, 14) = 0.08779 ns for *genotype*, $F$ (1, 14) = 2.838, ns for *sex*).

To further investigate DRD2 signalling, we also studied whether abolished MEIS1 signalling in DRD2$^+$ cells may affect downstream molecular targets of the DRD2 receptor. To this end, we also quantified the induction of cFos expression 90 min after haloperidol treatment in *Meis1*$^{Drd2-/-}$ mice (Figure 5c,d).

Haloperidol injection induced cFos expression in all mice, as indicated by the main effect of *treatment* (three-way ANOVA, mixed-effect, $F$ (1, 30) = 898.4, $p$ <.001), which was independent of the genotype ($F$ (1, 30) = 1.233 for genotype effect and $F$ (1; 30) = 0.7423 for *sex* × *genotype*) (Figure 5c).

## 4 | DISCUSSION

With the identification of *MEIS1* variants as a highly relevant risk factor for RLS, *Meis1* became an obvious target locus and gene for modelling of RLS (Schulte et al., 2014; Winkelmann et al., 2011). Considering bias towards the loss-of-function effect of such variants (Schulte et al., 2014) or reduced expression level of *Meis1* (Spieler et al., 2014), we studied *Meis1* mice heterozygous for a null mutation of *Meis1* due to insertion of ECFP into the first exon of the *Meis1* gene (González-Lázaro et al., 2014). We found that these mice display spontaneous motor hyperactivity, including the time during the onset of the light phase between 07:00 and 08:00 hours, which is a resting period for rodents. This most pertinent phenotype for RLS was present in 8-month-old *Meis1*$^{+/-}$ males, which also displayed an increase of motor activity during the active, dark phase of the light–dark cycle when measured in actimetric cages as well as in response to novelty in the open-field. Although *Meis1*$^{+/-}$ mutant females were also more active, they expressed this hyperactivity differently, as it was evident only in novelty-induced locomotion in the open-field at 8 months of age. Importantly, increased locomotion in *Meis1*$^{+/-}$ mice in the open-field reflected not only an increased speed of movement, but also reduced time spent without movement, which was especially evident for *Meis1*$^{+/-}$ males. This sex difference is striking and opposite to that observed in RLS patients, as women are more frequently affected by RLS (Berger & Kurth, 2007). Whereas the exact origin of the sex difference in humans is not known, the weaker phenotype in *Meis1*$^{+/-}$ female mice may reflect globally superior hormonal neuroprotection in mice due to highly frequent oestrous and tonic, relatively high levels of oestrogens throughout life as opposed to short, monthly oestrogen peaks in women. Finally, the hyperactive phenotype associated with restlessness, its circadian profile, and its sex dimorphism observed in *Meis1*$^{+/-}$ mice seems to be a robust and reliable RLS-relevant marker. It closely reproduced the phenotype reported for distinct *Meis1* deficiency models, including *Meis1*$^{tm1Mtor}$ mice carrying gene-inactivating knock-in of mutated human oestrogen receptor (ERT2) in-frame in the 12th exon of the *Meis1* gene (Salminen et al., 2017). In both models, no obvious motor coordination abnormalities were detected. Additionally, *Meis1*-deficient mice lacking the eighth exon of *Meis1* also displayed motor hyperactivity, which was restricted to the light period when analysed by spontaneous activity in running wheel, and observed throughout the 24 hr of the light–dark cycle when analysed in an open field (Lyu et al., 2020).

Whereas *MEIS1* has been identified as the main genetic risk factor for RLS, the aetiology and physiopathology of RLS are little understood (Schulte et al., 2014; Winkelmann et al., 2011). The observation that some dopaminergic agonists, such as pramipexole and bromocriptine, alleviate symptoms (at least during the first months of treatment) led to the hypothesis of dysfunctional dopaminergic signalling through dopamine D2-like receptors and in particular DRD2, one of the key dopamine receptors involved in motor control. Furthermore, balanced signalling of snMSNs and spMSNs expressing respectively DRD1 and DRD2 is a frequent cause of motor impairments. Considering the developmental and postnatal expression of MEIS1 in the striatum, we investigated the potential effect of *Meis1* deficiency on striatal neuroanatomy and dopaminergic signalling in mice. To this end, we counted DRD1$^+$ and DRD2$^+$ MSNs in *Meis1*$^{+/-}$ mice focussing on the motor and somatosensory dorsolateral (CPu) and ventral, limbic (NAccSh) part of the striatum. We did not observe any change in the number of DRD1$^+$ and DRD2$^+$ MSNs in pre-symptomatic (4-month-old) *Meis1*$^{+/-}$ mice, suggesting that the development of these neurones is not sensitive to low doses of MEIS1. The number of both cell types was also not affected in symptomatic (8-month-old) *Meis1*$^{+/-}$ mice, supporting the absence of their neurodegeneration. Finally, the number of ChAT$^+$ cholinergic interneurones, an important modulator of MSNs, was not altered in the *Meis1*$^{+/-}$ striatum. This observation is in agreement with a recent report of normal content of these interneurones in a distinct model of *Meis1* deficiency (Lyu et al., 2020). However, Lyu et al. reported that *Meis1* deficiency affected synchronous firing of these cells, which remains to be evaluated in the models we investigated here.

Although the number of MSNs was not affected, we speculated that their functionality might be affected in *Meis1*$^{+/-}$ mutant

mice. However, the haloperidol challenge did not reveal any obvious differences in catalepsy measures, used as a readout of DRD2 functionality, pointing to the absence of a direct link between dysfunctional DRD2 signalling and RLS-like motor symptoms in *Meis1*$^{+/-}$ mice. This was further supported by the absence of RLS-like motor hyperactivity/restlessness in almost 1-year-old *Meis1*$^{Drd2-/-}$ mice carrying a homozygous null mutation in DRD2$^{+}$ neurones. Importantly, these mice also displayed a normal response to haloperidol, as confirmed by analyses of catalepsy and cFos expression in the dorsolateral and ventral striatum. As haloperidol affinity is about 20-fold higher for DRD2 than DRD3, it is unlikely that we substantially activated DRD3 receptors at the doses used in the present study (Sokoloff et al., 1990). Thus, our observations point to the absence of any cell-autonomous role of MEIS1 in control of DRD2$^{+}$ spMSNs, which comprise a major component of the basal ganglia motor control circuit. Accordingly, we postulate that primary defects in RLS reside in other motor circuit(s), which are upstream or downstream of spMSN control. Thus, cortico-striatal or thalamo-striatal glutamatergic inputs controlled by distinct cerebellar nuclei could be of interest for future studies.

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## CONFLICT OF INTEREST

No conflict of interest.

## AUTHOR CONTRIBUTIONS

WK, JW, DL, MT designed the study and obtained funding, LC, VF, WK planned and performed experiments, LC, WK analysed and interpreted data, LC, WK, wrote the manuscript, WK supervised the study, LC and WK revised the manuscript with input from all authors.

## DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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