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ORIGINAL ARTICLE

Inducible ablation of dopamine D2 receptors in adult mice impairs locomotion, motor skill learning and leads to severe parkinsonism

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Motor execution and planning are tightly regulated by dopamine D1 and D2 receptors present in basal ganglia circuits. Although stimulation of D1 receptors is known to enhance motor function, the global effect of D2 receptor (D2R) stimulation or blockade remains highly controversial, with studies showing increasing, decreasing or no changes in motor activity. Moreover, pharmacological and genetic attempts to block or eliminate D2R have led to controversial results that questioned the importance of D2R in motor function. In this study, we generated an inducible *Drd2* null-allele mouse strain that circumvented developmental compensations found in constitutive *Drd2*$^{-/-}$ mice and allowed us to directly evaluate the participation of D2R in spontaneous locomotor activity and motor learning. We have found that loss of D2R during adulthood causes severe motor impairments, including hypolocomotion, deficits in motor coordination, impaired learning of new motor routines and spontaneous catatonia. Moreover, severe motor impairment, resting tremor and abnormal gait and posture, phenotypes reminiscent of Parkinson's disease, were evident when the mutation was induced in aged mice. Altogether, the conditional *Drd2* knockout model studied here revealed the overall fundamental contribution of D2R in motor functions and explains some of the side effects elicited by D2R blockers when used in neurological and psychiatric conditions, including schizophrenia, bipolar disorder, Tourette's syndrome, dementia, alcohol-induced delusions and obsessive-compulsive disorder.

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INTRODUCTION

Motor planning and action selection are tightly regulated by neural circuits of the basal ganglia.[1,2] Dopamine is a key modulator of these circuits by acting on two distinct striatofugal populations of medium spiny neurons (MSNs) that together exert a fine-tuned control of movement and motor performance. MSNs of the direct pathway (dMSNs) mainly express Gs-coupled D1 dopamine receptors (D1Rs) and project to the internal globus pallidus (GP) and the pars reticulata of the substantia nigra, whereas MSNs of the indirect pathway (iMSNs) express Gi/Go-coupled D2Rs and project to the external GP and the ventral pallidum (for a review, see Gerfen and Surmeier[3]). Selective *in vivo* optogenetic stimulation of dMSNs elicits hyperlocomotion, whereas stimulation of iMSNs induces akinesia, demonstrating that these two parallel pathways have opposite roles on motor function.[4] Although a large body of evidence indicates that administration of D1R agonists increase locomotor activity, presumably by enhancing the excitability of dMSNs, the effect of D2R agonists on locomotion remains highly controversial, with studies showing increasing, decreasing or no changes in motor activity. Differences in D2R-mediated effects have been found for the same agonist given to mice or rats[5] and also to mice from different inbred or outbred strains.[6] This level of discrepancy probably results from the coexisting multiple subpopulations of striatal D2Rs each of which has differential, and even opposite, roles within the basal ganglia circuits. D2Rs are found not only in iMSNs dendrites but also in cholinergic interneurons,[7,8] in presynaptic corticostriatal glutamatergic terminals[9] and in presynaptic terminals of midbrain dopamine neurons where they act as inhibitory autoreceptors.[10,11] Although the use of conditional mutagenesis, cell-specific activation of engineered light-activated channels and selective stimulation of designed receptors is beginning to solve the participation of the individual components of this elaborate functional ensemble,[4,11–15] the global participation of D2R across the wide spectrum of motor functions still remains as a relevant challenge owing to the frequent use of dopamine agonists and antagonists in prevalent neurological and psychiatric conditions, such as Parkinson's disease (PD), schizophrenia and bipolar disorder.

In contrast to the divergent effects observed with D2R agonists, the use of D2R blockers induce dose-dependent reductions in motor performance in all mouse and rat strains and at high doses provokes catatonia.[16] A similar effect is observed in psychiatric patients receiving neuroleptics that may exhibit drug-induced parkinsonism and, at higher doses, catatonia.[17] Thus the complete blockade of D2R impairs all motor skills similarly to what is observed in rodents and primates lacking midbrain dopamine neurons or in dopamine-deficient mice.[18] However, we were surprised that mutant mice lacking D2R (*Drd2*$^{-/-}$) were proficient to execute complex motor coordination tasks[19] and only showed a mild reduction in spontaneous locomotor activity.[19–21] Thus reconciliation of the results obtained using pharmacological and

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genetic approaches has become an interesting challenge as this discrepancy questioned the importance of D2R-mediated effects in motor function. Alternative hypotheses that could explain this conundrum are: (1) *Drd2*$^{-/-}$ mice develop an alternative genetic program that partially compensates for the lack of D2R and/or (2) D2R compounds are not sufficiently selective and also target other receptors involved in motor performance.

In this study, we have undertaken a conditional genetic approach to directly evaluate in adult mice the importance of D2R in various aspects of motor behavior: (1) spontaneous locomotion, (2) acquisition of novel motor skills, and (3) execution of a previously learned motor task. To circumvent possible developmental compensations, we generated an inducible *Drd2* null-allele novel mouse strain (ind*Drd2*$^{f/f}$) that allowed us to study the functional consequences of losing D2R at different ages in comparison to constitutive *Drd2*$^{-/-}$ mice. Our conditional *Drd2* knockout (KO) model allowed us to recognize the fundamental contribution of D2R to normal motor functions and to question the use of constitutive KO animal models to assess gene function during adulthood.

MATERIALS AND METHODS

Generation of *Drd2* inducible mutant mice

Mutant mice with targeted loxP sites flanking *Drd2* exon 2, *Drd2*$^{tm1.1Mrub}$ (*Drd2*$^{f/f}$),[10] were crossed with transgenic mice carrying a tamoxifen (TAM)-inducible cre recombinase gene driven by a ubiquitously active CAG enhancer-promoter Tg(CAG-cre/Esr1*)5Amc (CAG-CreERT) mice[22] and obtained *Drd2*$^{tm1.1Mrub}$ Tg(CAG-cre/Esr1*)5Amc (*Drd2*$^{f/f}$::CAG-CreERT) mice, a *Drd2*-inducible mutant strain dubbed ind*Drd2*$^{f/f}$. This conditional mutant strategy has been successfully used by several laboratories,[23–25] including ours,[26] to ablate genes in different brain areas. Both parental strains had been previously backcrossed to C57BL/6J for 10 generations to homogenize their genetic background ($n = 10$). We established a mouse colony by mating *Drd2*$^{f/f}$ with ind*Drd2*$^{f/f}$ mice that yields littermates of both genotypes in equal amounts, and *Drd2*$^{f/f}$ were used as controls. At 3 months of age, both *Drd2*$^{f/f}$ and ind*Drd2*$^{f/f}$ mice received 50 mg kg^{-1} TAM (Sigma-Aldrich, St. Louis, MO, USA) or vehicle (Sesame oil, Sigma-Aldrich) for 10 days along a 2-week period. Ind*Drd2*$^{f/f}$ mice receiving TAM at 3 or beyond 6 months of age were named *Drd2*KO@3mo or *Drd2*KO@6mo, respectively. All groups were compared with constitutive *Drd2* null mutant mice, *Drd2*$^{-/-}$, previously generated in our laboratory during the generation of *Drd2*$^{f/f}$ mice,[10] also backcrossed to C57BL/6J for 10 generations ($n = 10$). Only male mice were used except when noted. For more details on this section, see Supplementary Information.

RESULTS

Inducible ablation of D2R in adult mice produces severe motor impairments

Drd2 inducible mutant mice (ind*Drd2*$^{f/f}$) were treated at 3 months of age with 50 mg kg^{-1} TAM for 10 days during 2 consecutive weeks to eliminate their functional *Drd2* alleles, becoming *Drd2*KO@3mo mice (Figure 1a). As control groups, we treated *Drd2*$^{f/f}$ (Cre-less) mice with TAM and mice of both strains with vehicle. Three weeks after concluding the TAM treatments, we determined the level of *Drd2* expression in mice from each group. *In situ* hybridization using a [^{35}S]-labeled *Drd2* exon 2 antisense riboprobe on brain coronal sections at the level of the striatum (Figure 1b) and midbrain (Supplementary Figure S1) showed a marked reduction of *Drd2* mRNA levels in *Drd2*KO@3mo mice. Similar results were obtained by binding autoradiography using the radiolabeled D2R antagonist [^{3}H]-nemonapride in which the density of D2R showed to be much lower than in all the control groups but still appreciable in comparison to *Drd2*$^{-/-}$ mice (Figure 1c). A relative quantitative reverse transcriptase–PCR analysis performed with striatal samples of all groups determined that the level of *Drd2* expression in *Drd2*KO@3mo mice was reduced by 85% (Figure 1d). In addition, semiquantitative densitometry performed on [^{3}H]-nemonapride-binding autoradiographies showed that the level of striatal D2R decreased 75% compared with the control groups (Figure 1e).

Three weeks after concluding the treatments, we evaluated the functional motor consequences in mice losing D2R as adults. *Drd2*KO@3mo mice showed markedly reduced spontaneous locomotor activity in an open field compared with the control groups, despite showing normal habituation parameters. Differences in distance travelled were accentuated along the 3 days (Figure 2a). Interestingly, locomotor activity of inducible *Drd2*KO@3mo mice was much lower than that observed in constitutive *Drd2*$^{-/-}$ mice. The hypolocomotion determined in *Drd2*KO@3mo mice resulted from a reduced frequency of movement initiations rather than reduced ambulatory velocity that was similar among all groups (one-way analysis of variance (ANOVA) for day 1- Group: $F(4, 56) = 1.1852$, $P = 0.32733$; ambulatory velocity: *Drd2*$^{f/f}$: (43.88 ± 2.03) cm s^{-1}, *Drd2*$^{f/f}$+TAM: (44.65 ± 1.94) cm s^{-1}, ind*Drd*$^{f/f}$: (44.95 ± 1.87) cm s^{-1}, *Drd2*KO@3mo: (40.80 ± 2.13) cm s^{-1}, *Drd2*$^{-/-}$: (40.76 ± 1.74) cm s^{-1}). A detailed analysis of locomotor activity measured in 5-min bins showed a remarkable reduction in the ambulatory distance travelled by *Drd2*KO@3mo mice in comparison to all other groups during the first 5 min of the 3 consecutive days (Figure 2b). This difference was absent in constitutive *Drd2*$^{-/-}$ mice suggesting that the motivational effect elicited by a novel environment is blunted in mice losing their D2R as adults. At day 3 we found that *Drd2*KO@3mo mice displayed significantly reduced locomotor activity compared with *Drd2*$^{-/-}$ mice along all 5-min bins. To evaluate spontaneous catatonia, we briefly trained mice to mount their front paws on a horizontal bar elevated 6 cm from a sitting platform and measured the time they spent immobile before returning to their normal posture. We found that, different from control and *Drd2*$^{-/-}$ mice, *Drd2*KO@3mo mice displayed long-lasting spontaneous catatonia showing once again, a great phenotypic difference between mice constitutively lacking D2R (*Drd2*$^{-/-}$) and mice with induced D2R ablation (*Drd2*KO@3mo) in adulthood (Figure 2c).

We then tested the effects of the D2R antagonist haloperidol and the indirect dopamine agonist amphetamine on locomotor activity of mice of all groups. Haloperidol (0.3 mg kg^{-1}, s.c.) induced a marked reduction in locomotor activity of mice of the three control groups but failed to produce any significant effect in *Drd2*$^{-/-}$ or *Drd2*KO@3mo mice, suggesting that the remaining population of D2R still present in this latter group of mice lacks functional relevance (Figure 2d). In this experiment and the following, baseline activity of *Drd2*KO@3mo and *Drd2*$^{-/-}$ mice receiving saline were not different probably because as the test series progress (Figure 1a) mice become familiar with the open field and exploratory activity may wane. DL-amphetamine (3 mg kg^{-1}, s.c.), induced a 1.2–2.0-fold increase in the distance travelled during 30 min after the injections by mice from the 3 control groups. Similarly, amphetamine elicited a 2 fold increase in *Drd2*$^{-/-}$ mice. In contrast, amphetamine failed to induce a significant effect in *Drd2*KO@3mo mice (repeated-measures ANOVA–*Post-hoc* Fisher analysis, $P = 0.45$). Together, these results indicate that D2R are essential for amphetamine-mediated hyperlocomotion and evidence once more that *Drd2*$^{-/-}$ mice develop compensatory mechanisms that do not appear to exist in mice rendered D2R-deficient as adults.

Impaired motor coordination and inability to learn new motor skills after ablating D2R in adult mice

Motor coordination tasks depend on the proper function of basal ganglia circuits and, in particular, on dopamine neurotransmission. Thus we compared the balance, coordination and motor-planning ability of untrained control and D2R-deficient mice in a motor task performed on a horizontal elevated rod rotating at 16 r.p.m. Under

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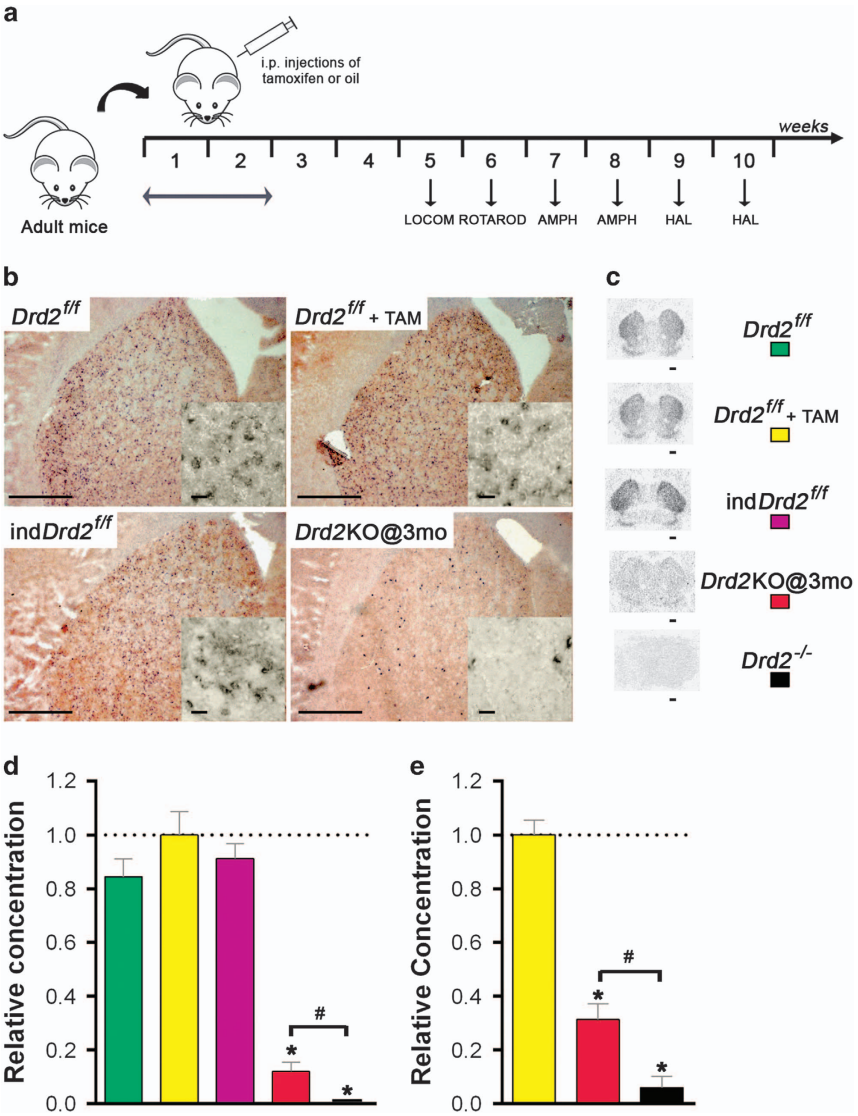

Figure 1. Successful ablation of D2 receptor (D2R) in adulthood. (**a**) Scheme of the progression of the experiment. (**b**) *In situ* hibridization for *Drd2* mRNA expression. Scale bars represent 1 mm. Inset shows higher magnification. Scale bars represent 0.125 mm. (**c**) [^{3}H]-nemonapride binding autoradiography. Scale bars represent 1 mm. (**d**) *Drd2* mRNA expression normalized to 18S rRNA, relative to average of control mice (*Drd2*$^{f/f}$+TAM (tamoxifen)). Analysis of variance (ANOVA) $F(4,25) = 47.491$, $P < 0.0001$. *Post-hoc* Fisher analysis $^*P < 0.0001$ compared with controls, $^\#P < 0.05$ compared with *Drd2*$^{-/-}$ mice. (**e**) Semiquantitative [^{3}H]-nemonapride-binding autoradiography, relative to *Drd2*$^{f/f}$+TAM mice. ANOVA $F(2,9) = 69.69$, $P < 0.0001$. *Post-hoc* Fisher analysis $^*P < 0.0001$ compared with control mice, $^\#P < 0.05$ compared with *Drd2*$^{-/-}$ mice.

these conditions, control mice were able to negotiate the rotarod displaying a low number of falls (Figure 3a) and long-lasting latency to the first fall (Figure 3b) when tested in an accelerating rod (4–40 r.p.m.). In contrast, *Drd2*KO@3mo and *Drd2*$^{-/-}$ mice exhibited more difficulties in coping with this task with many more falls during the 5 min tested and lower latencies to the first fall.

Dopamine has a fundamental role in the transition from goal-oriented to habitual behavior, which is driven by practicing and repeating a motor task. To determine the importance of D2R in motor learning, we trained mice from all groups to negotiate an accelerated rotarod in a 3 trials per day schedule performed during 5 consecutive days. All control groups improved their performance on the rotarod evidenced by a day-by-day increase in the latency to the first fall (Figure 3c). *Drd2*$^{-/-}$ mice also showed a significant improvement that persisted with practice, whereas *Drd2*KO@3mo mice failed to exhibit any progress in this motor task (Figure 3c). Finally, we compared the ability of naive mice on the rotarod (16 r.p.m.) with their performance after receiving a 5-day training on the accelerated rod (3 trials per day). We found that, while *Drd2*$^{f/f}$ and *Drd2*$^{-/-}$ mice considerably improved their motor skills after the practice, *Drd2*KO@3mo mice failed to significantly improve their performance (Figure 3d). Together, these results indicate that the loss of D2R in adult mice induces an irreversible decline in motor performance and learning, revealing an essential role of central D2R in motor function.

Late-onset loss of D2R impairs the execution of a previously well-learned motor routine

After demonstrating the essential role of D2R in learning a novel motor task, we wanted to evaluate the importance of D2R during the execution of a previously learned motor routine. To this end,

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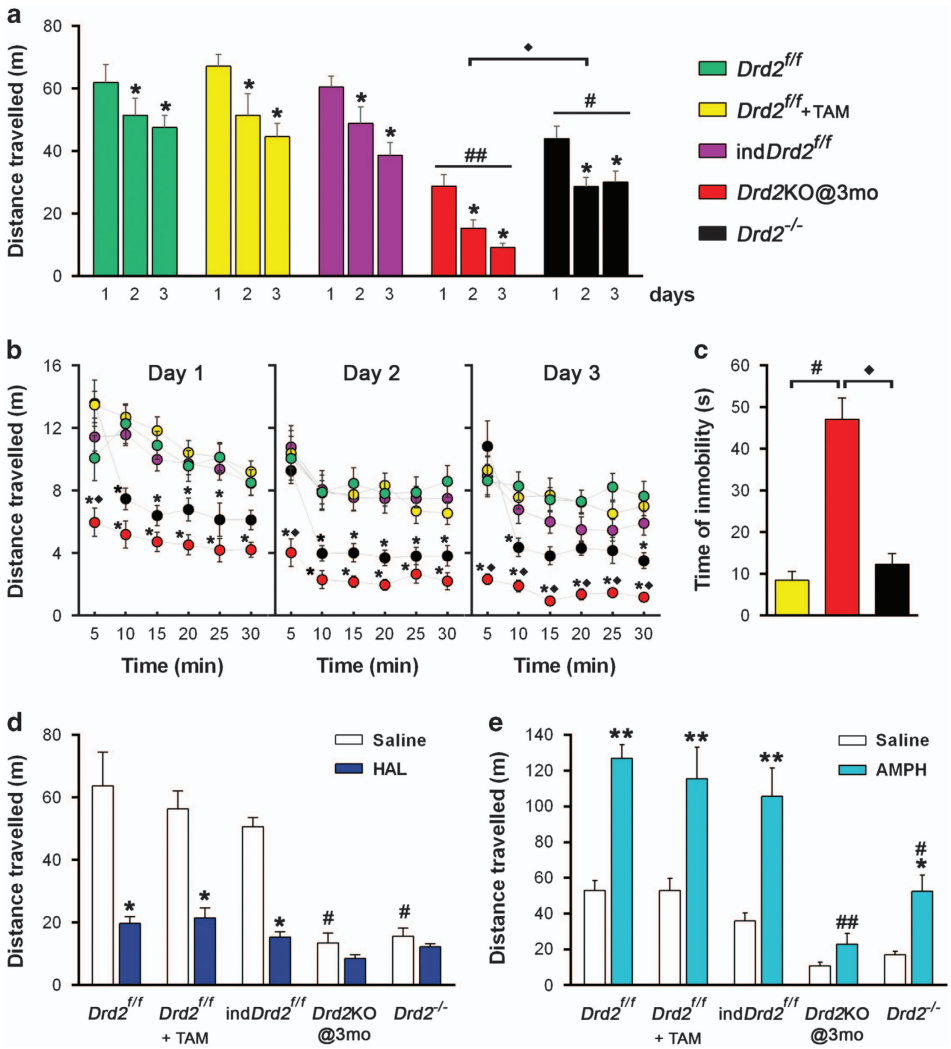

Figure 2. Enhanced impairment in spontaneous locomotor activity and spontaneous catatonia after losing D2 receptor (D2R) in adulthood. (**a**) Locomotor activity in an open field for 30 min along 3 consecutive days. Repeated-measures analysis of variance (ANOVA). Group: $F(4,63) = 17.578$, $P < 0.00001$; Time: $F(2,126) = 73.311$, $P < 0.0001$; Interaction: $F(8,126) = 1.1520$, $P = 0.33360$. *Post-hoc* Tukey's analysis: $^{*}P < 0.05$ compared with day 1 within group; $^{\#}P < 0.001$, $^{\#\#}P < 0.0001$ compared with the control groups; $^{\blacklozenge}P < 0.005$ compared with *Drd2*$^{-/-}$. (**b**) Detailed locomotor activity in 5-min bin on each day. Day 1. Group: $F(4,64) = 16.041$, $P < 0.0001$. Time: $F(5,320) = 22.636$, $P < 0.0001$. Interaction: $F(20,320) = 3.915$, $P < 0.0001$. Day 2. Group: $F(4,62) = 13.12$, $P < 0.0001$. Time: $F(5,310) = 30.79$, $P < 0.0001$. Interaction: $F(20,310) = 2.181$, $P < 0.005$. Day3. Group: $F(4,65) = 17.34$, $P < 0.0001$. Time: $F(5,325) = 20.88$, $P < 0.0001$. Interaction: $F(20,325) = 3.917$, $P < 0.0001$. *Post-hoc* de Fisher analysis. $^{*}P < 0.05$ vs control groups, $^{\blacklozenge}P < 0.05$ compared with *Drd2*$^{-/-}$ mice. (**c**) The spontaneous immobility after being placed on a bar has been evaluated only in three of the groups. ANOVA $F(2,13) = 34.30$, $P < 0.0001$. *Post-hoc* Fisher analysis $^{\#}P < 0.0001$ compared with *Drd2*$^{f/f}$+TAM (tamoxifen) mice; $^{\blacklozenge}P < 0.0001$ compared with *Drd2*$^{-/-}$ mice. (**d**) Locomotion after 20 min of 0.3 mg kg^{-1} haloperidol (HAL) or saline injections. Repeated-measures ANOVA. Group: $F(4,31) = 11.637$, $P < 0.0001$. Drug: $F(1,31) = 67.979$, $P < 0.0001$. Interaction: $F(4,31) = 7.6232$, $P < 0.001$. *Post-hoc* Fisher analysis $^{*}P < 0.0001$ compared with the saline within group; *post-hoc* Tukey $^{\#}P < 0.005$ compared with the control groups. (**e**) Locomotor activity measured during 30 min immediately after 3 mg kg^{-1} amphetamine (AMPH) or saline injections. Repeated-measures ANOVA. Group: $F(4,35) = 10.381$, $P < 0.0001$. Drug: $F(1,35) = 61.813$, $P < 0.0001$. Interaction: $F(4,35) = 3.0032$, $P < 0.05$. *Post-hoc* Fisher analysis $^{*}P < 0.05$, $^{**}P < 0.0005$ compared with the saline within group; *post-hoc* Tukey $^{\#\#}P < 0.001$, $^{\#}P < 0.05$ compared with the control groups.

we trained naive mice of all genotypes on the rotarod during 5 consecutive days. As expected, all groups showed great motor coordination improvement following this training schedule (Figure 3e, left panels). Upon conclusion of the 5-day training, all mice were treated with TAM or vehicle as in the experiments above (Figure 1a), and 3 weeks later, they were all evaluated again using an identical protocol for 5 consecutive days. Interestingly, all the control groups reinitiated their motor performance at the same high level they had previously achieved during training (Figure 3e, right panels of the first three groups). During the posttreatment experimental phase, *Drd2*$^{-/-}$ mice also maintained their previous high-level performance on the rotarod (Figure 3e, shadow panel of the *Drd2*$^{-/-}$ group). In contrast, *Drd2*KO@3mo mice completely lost the ability to negotiate the rotarod they had gained before the induction of D2R ablation. Furthermore, instead of improving along the consecutive days, their performance worsened day-by-day after TAM treatment (Figure 3e, right panel, *Drd2*KO@3mo group) with latencies to the first fall that were as low as those observed with *Drd2*KO@3mo mice evaluated after having been treated with TAM as adults (Figure 3c). These results demonstrate that D2R are essential to execute previously learned motor routines.

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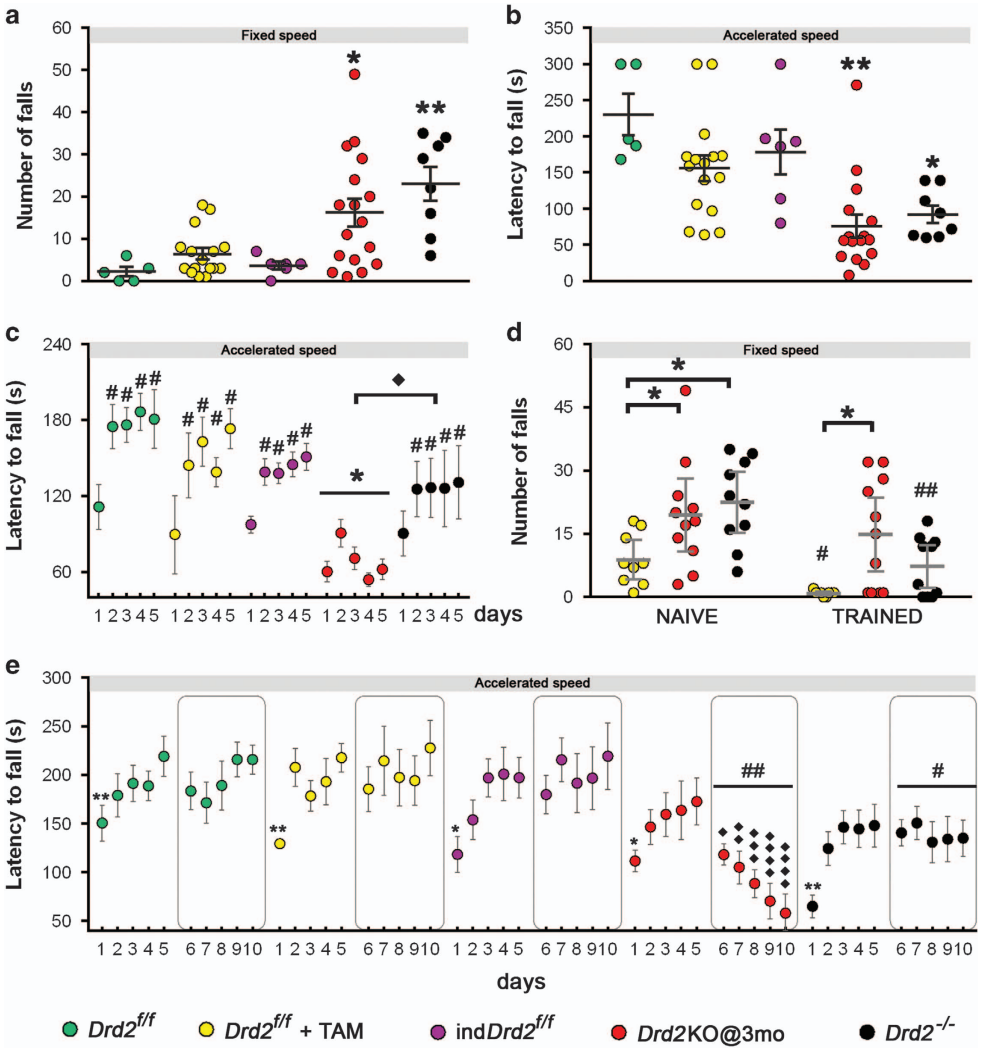

Figure 3. Impaired motor skills learning and execution of well-learned motor routines. Mice tested on a rotating rod at 16 r.p.m. without previous training. (**a**) Number of falls in 180 s. Analysis of variance (ANOVA) $F(4,47) = 7.152$, $P < 0.0001$. *Post-hoc* Fisher analysis $^{*}P < 0.01$, $^{**}P < 0.005$ vs control groups. (**b**) Later, mice were tested on an accelerated rotating rod (4-40 rpm in 5 min). ANOVA: $F(4,46) = 8.031$, $P < 0.0001$. *Post-hoc* Fisher analysis $^{*}P < 0.05$, $^{**}P < 0.005$ vs control groups. (**c**) Extensive training for 5 consecutive days on an accelerating rod (4–40 r.p.m. in 5 min). Figure shows average latency to fall per day. Repeated-measures ANOVA. Interaction: $F(16,136) = 1.8669$, $P < 0.05$. Group: $F(4,34) = 5.775$, $P < 0.005$. *Post-hoc* Fisher analysis: $^{*}P < 0.005$ compared with control mice, $^{\blacklozenge}P < 0.05$ compared with $Drd2^{-/-}$ mice; Days: $F(4,136) = 18.976$, $P < 0.0001$. *Post-hoc* Bonferroni analysis. $^{\#}P < 0.05$ compared with day 1 within group. (**d**) A new group of mice were tested on a rotating rod at 16 r.p.m. before (Naive) and after (Trained) intensive training on an accelerated rod (4–40 r.p.m. in 5 min) for 5 consecutive days. Repeated-measures ANOVA. Group: $F(2,27) = 5.6855$, $P < 0.01$. Training: $F(1,27) = 29.986$, $P < 0.0001$. Interaction $F(2,27) = 3.5181$, $P < 0.05$. *Post-hoc* Fisher analysis. TRAINED: $^{\#}P < 0.05$, $^{\#\#}P < 0.00005$ compared with the NAIVE within group; $^{*}P < 0.05$ compared with $Drd2^{f/f}$+TAM (tamoxifen) mice within training phase. (**e**) Mice were trained on an accelerating rod for 5 consecutive days (days 1–5) and were then treated with TAM or vehicle, accordingly. They were re-tested after 3 weeks (days 6–10). Repeated-measures ANOVA. Interaction: $F(36,234) = 3.056$, $P < 0.0001$. Training: $F(9,234) = 10.17$, $P < 0.0001$. *Post-hoc* Bonferroni analysis $^{*}P < 0.05$, $^{**}P < 0.005$ comparing days 1–4 to day 5; $^{\blacklozenge}P < 0.05$, $^{\blacklozenge\blacklozenge}P < 0.01$, $^{\blacklozenge\blacklozenge\blacklozenge}P < 0.0001$, $^{\blacklozenge\blacklozenge\blacklozenge\blacklozenge}P < 0.00005$ comparing days 6–10 to last day before treatment (day 5 within group). Group: $F(4,26) = 5.292$, $P < 0.005$. Repeated-measures ANOVA after treatment (days 6–10). Interaction $F(16,104) = 3.828$, $P < 0.0001$. Training $F(4,104) = 1.777$, $P = 0.1391$. Group $F(4,26) = 6.496$, $P < 0.0001$. *Post hoc* Fisher analysis $^{\#}P < 0.05$, $^{\#\#}P < 0.0005$ compared with the control groups.

Gait and postural abnormalities after ablating D2R in older mice

To determine the functional consequences of D2R ablation at a more advanced age, we evaluated motor behaviors of other cohorts of $Drd2^{f/f}$, $Drd2^{-/-}$ and ind$Drd2^{f/f}$ mice treated with TAM at 6–8 months of age. Three weeks after the treatment, spontaneous locomotor activity was recorded during 3 consecutive days in a novel open field. *Drd2*KO@6mo and $Drd2^{-/-}$ mice showed reduced locomotion and normal habituation, similar to the profiles observed in young adults (Figure 4a). As found in younger adult mice (Figure 2c), *Drd2*KO@6mo mice displayed high scores of spontaneous catatonia contrary to control $Drd2^{f/f}$ and $Drd2^{-/-}$ mice (Figure 4b). When trained on a rotarod during 5 consecutive days, control $Drd2^{f/f}$ and $Drd2^{-/-}$ mice showed significant improvement in their performance, whereas *Drd2*KO@6mo mice failed to show any improvement on the rotarod (Figure 4c). Moreover, on the fifth day, mice with D2R ablation as older adults showed a significant worsening in this motor coordination task (Figure 4c), different from what was observed when TAM

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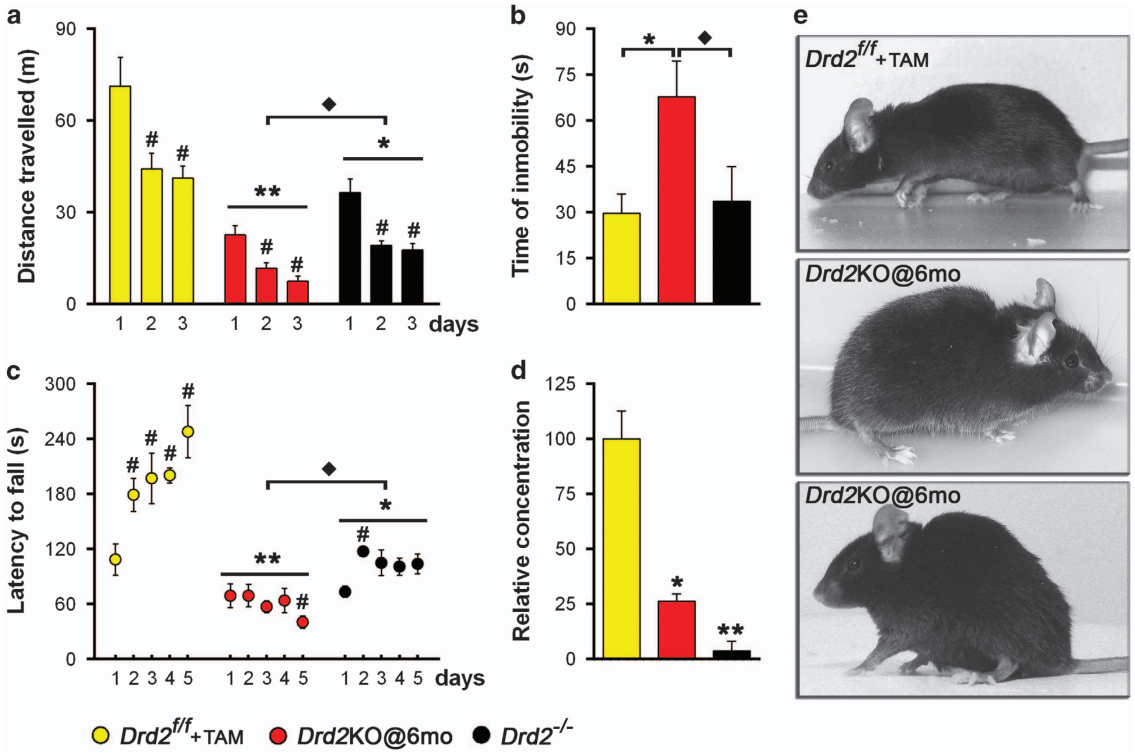

Figure 4. Abnormal corporal posture of mice after losing D2 receptor (D2R) in old age. Mice were treated and tested after reaching the age of 6 months. *Drd2*$^{f/f}$ mice were treated with tamoxifen (TAM) and included as controls. (**a**) Spontaneous locomotor activity in aged mice for 3 consecutive days. Repeated-measures analysis of variance (ANOVA) interaction: $F(4,24) = 1.8755$, $P = 0.14750$; Time: $F(2,24) = 32.25$, $P < 0.0001$; *Post-hoc* Fisher analysis #$P < 0.05$ compared with day 1 within group. Group: $F(2,12) = 38.044$, $P < 0.0001$. *Post-hoc* Fisher analysis *$P < 0.005$, **$P < 0.0001$ compared with *Drd2*$^{f/f}$+TAM mice, ◆$P < 0.005$ compared with *Drd2*$^{-/-}$. (**b**) Spontaneous catatonia in aged mice. ANOVA $F(2,12) = 4.4588$, $P < 0.05$. *Post-hoc* Fisher analysis *$P < 0.05$ compared with *Drd2*$^{f/f}$+TAM mice, ◆$P < 0.05$ compared with *Drd2*$^{-/-}$ mice. (**c**) Extensive training on an accelerating rod for 5 consecutive days. Repeated-measures ANOVA. Interaction: $F(8,48) = 9.1549$, $P < 0.0001$. Time: $F(4,48) = 8.6202$, $P < 0.0001$. *Post-hoc* Fisher analysis #$P < 0.05$ compared with day 1 within group. Group: $F(2,12) = 33,026$, $P < 0.0001$. *Post-hoc* Fisher analysis *$P < 0.0005$, **$P < 0.00005$ compared with *Drd2*$^{f/f}$+TAM mice, ◆$P < 0.05$ compared with *Drd2*$^{-/-}$ mice. (**d**) Semiquantitative [^{3}H]-nemonapride-binding assay, relative to *Drd2*$^{f/f}$+TAM mice. ANOVA $F(2,8) = 34.792$, $P < 0.0005$. *Post-hoc* Fisher analysis *$P < 0.0005$, **$P < 0.00005$ compared with *Drd2*$^{f/f}$ TAM mice. (**e**) *Drd2*KO@6mo mice (middle and bottom panels) walked taking very short steps with the paws remaining very close to the body with a constant stooped posture. Neither *Drd2*$^{-/-}$ nor control mice (top panel) show any abnormal postural feature even at advanced ages.

treatment was initiated at 3 months of age (Figure 3c). Note that in all the groups the latency to fall was slightly reduced in comparison with 3–4-month-old mice (Figure 3c). Analysis of striatal D2R density by binding autoradiography in these older mice (Figure 4d) showed similar results as those previously observed in younger adults (Figure 1e). Although the motor deficits observed in *Drd2*KO@6mo mice were quantitatively similar to those observed in *Drd2*KO@3mo, *Drd2*KO@6mo mice displayed a severe phenotypical profile reminiscent of a PD animal model that was unique to this group of mice (Supplementary Video S1). *Drd2*KO@6mo displayed a hunched posture (10 out of 10; Figure 4e and Supplementary Video S2), accompanied in most cases by gait abnormalities evidenced by abnormal walking synchronicity between front and rear paws (7 of 10 mice, Supplementary Video S3) and resting tremor (5 of 10 mice, Supplementary Video S4). However, *Drd2*$^{-/-}$ and *Drd2*KO@3mo mice never showed any of these postural abnormalities even when evaluated after 6 months of age. The PD traits exhibited by *Drd2*KO@6mo mice were significant when compared with control and *Drd2*$^{-/-}$ mice (Fisher's exact test for abnormal gait: $P < 0.01$ *Drd2*KO@6mo vs *Drd2*$^{f/f}$+TAM, $P < 0.005$ *Drd2*KO@6mo vs *Drd2*$^{-/-}$ mice; hunched posture: $P < 0.001$ *Drd2*KO@6mo vs *Drd2*$^{f/f}$+TAM, $P < 0.0001$ *Drd2*KO@6mo vs *Drd2*$^{-/-}$ mice; tremor: $P < 0.05$ *Drd2*KO@6mo vs *Drd2*$^{f/f}$+TAM, $P < 0.05$ *Drd2*KO@6mo vs *Drd2*$^{-/-}$ mice).

D2R ablation in adulthood induces parkinsonian-like oscillatory synchronization in the basal ganglia

A neuropathophysiological hallmark of PD,[27,28] also found in dopamine-depleted rodents and monkeys,[29–31] is the presence of increased synchronized oscillatory activity of multiple frequency bands along the basal ganglia nuclei of the indirect striato–GPe (external GP)–subthalamic nucleus pathway.[32–34] A striking form of pathological synchronization along the indirect pathway takes place in the classical 6-OHDA rat model of PD, where GPe neurons abnormally synchronize to anesthesia-induced slow oscillations.[34–37] To determine whether ablation of D2R suffices to alter synchronization along the indirect pathway, we simultaneously recorded local field potentials in the motor cortex and spike discharges of GPe neurons by means of a 24-channel silicon probe in anesthetized mice (Figures 5a–c). In control *Drd2*$^{f/f}$ mice, we found that most GP neurons are not coupled to cortical activity and most of the remaining GP neurons recorded were coupled in-phase (Figure 5d). In *Drd2*KO@3mo mice, however, the population of synchronized neurons remarkably increased, with their vast majority showing an antiphase coupling (180 °) and a minor population of phase-coupled neurons. Constitutive *Drd2*$^{-/-}$ mice showed a similar phenotype, although less pronounced (Figure 5d), in line with the milder behavioral phenotype observed

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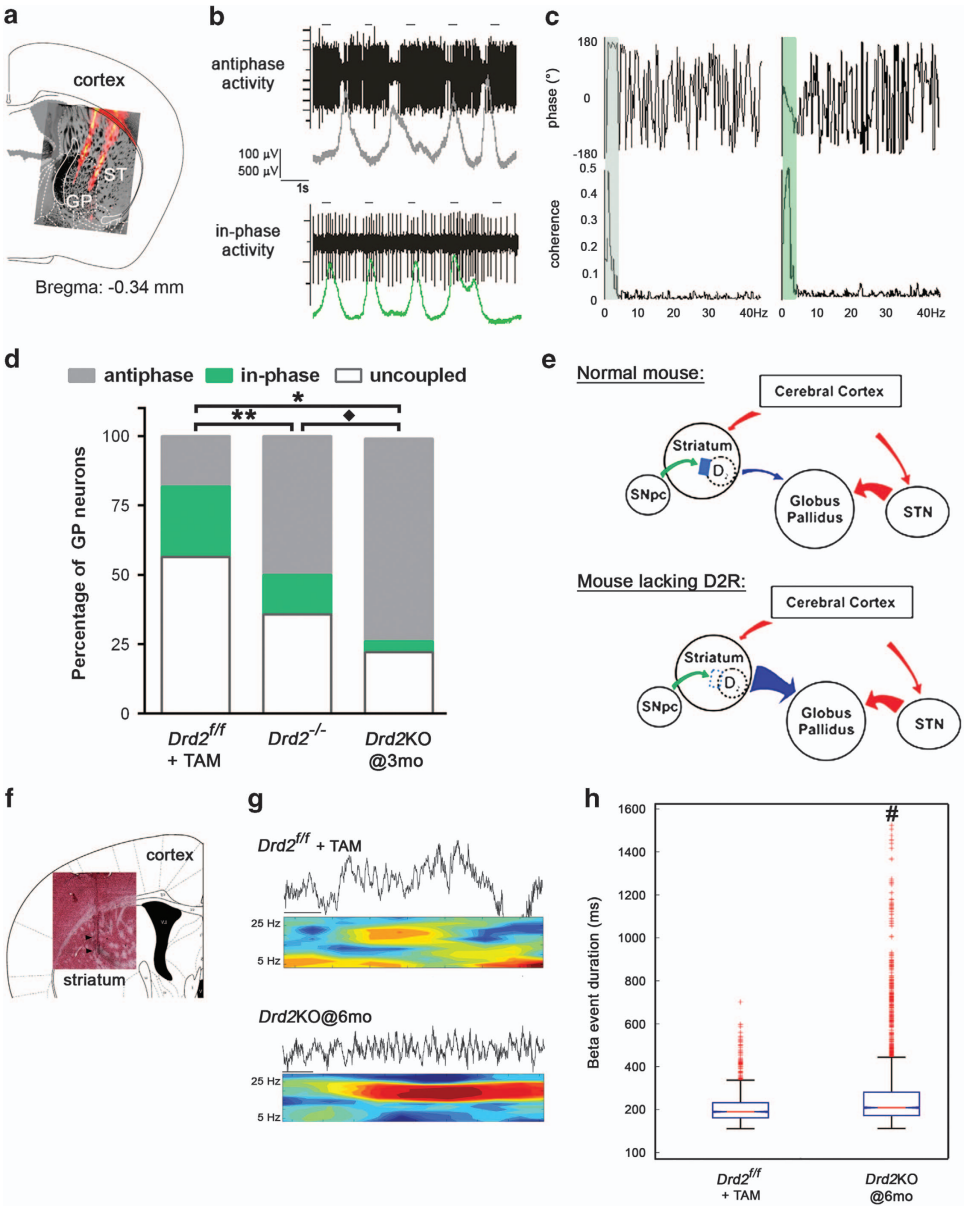

Figure 5. Increased synchronicity and beta band activity in mice after losing D2 receptor (D2R) in adulthood. (**a**) Globus pallidus (GP) neurons were recorded with a 24-channel silicon probe in mice under urethane anesthesia, and cortical activity was assessed through an intracortical macroelectrode. (**b**) GP neurons may display decreases (above) or increases (below) in firing (black lines) during peaks of cortical activity. In our cortical recordings, the upward deflection of the local field potential is related to depolarization and firing of pyramidal neurons. (**c**) Phase relationship between GP firing and cortical activity as assessed with coherence and phase spectra. Antiphase activity reveals corticostriatal inhibitory influence over the GP. (**d**) D2R ablation increases the proportion of GP neurons showing antiphase oscillations in the GP. Chi-square test: $^{*}P < 0.0001$, $^{**}P < 0.00001$ compared with control mice *Drd2*$^{f/f}$+TAM (tamoxifen) mice and $^{\blacklozenge}P < 0.05$ compared with *Drd2*$^{-/-}$ mice. Data are from 55 neurons recorded from 4 *Drd2*$^{f/f}$+TAM mice, 56 neurons recorded from 5 *Drd2*$^{-/-}$ mice and 68 neurons recorded from 5 *Drd2*KO@3mo mice. (**e**) Basal ganglia circuit showing the main connections of the GP in mice. Cortical influence is conveyed to the GP through the subthalamic nucleus (STN; excitatory) and the striatal D2R medium spiny neurons (inhibitory). Dopamine diminishes striatal influence over the GP in normal conditions (top). D2R ablation would increase cortico-striatal inhibitory influence on GP activity (bottom). (**f**) Schematic drawing showing the striatal area from which the local field potential recordings were taken with tetrodes. (**g**) Beta events were detected with a custom-made software routine. Examples of beta events (black traces: local field potential) and their time frequency representation. Timescale: 200 ms. (**h**) *Drd2*KO@6mo mice showed an excess of long beta events ($^{\#}P < 0.001$, Kruskal–Wallis test).

in these animals. Moreover, GP neurons showed a higher average firing rate in constitutive (41.8 ± 2.4 spikes s^{-1}) and *Drd2*KO@3mo mice (42.6 ± 2.5 spikes s^{-1}), compared with control mice (26.6 ± 1.1 spikes s^{-1}; one-way ANOVA, $F(2,228) = 26.2$ $P < 0.0001$). This is in line with findings in animal models of PD, which show that GP firing rate changes are inconsistent with those predicted by classical basal ganglia models.[38] Overall, these data show that D2R ablation in adult mice induces changes in cortico-pallidal synchronization, reminiscent of chronic striatal DA depletion. These changes are more pronounced than those observed in *Drd2*$^{-/-}$ that probably activate developmental compensations on downstream basal ganglia structures (Figure 5e).

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Beta oscillations are particularly evident during bradykinetic episodes[39–41] and, interestingly, diminish with L-DOPA treatment as voluntary movement improves.[31,42,43] Although a deficit in striatal D2R stimulation has been proposed to explain increased beta oscillations in the indirect pathway,[44] this hypothesis has never been directly tested. To determine whether D2R ablation is able to increase beta oscillations in freely moving mice, we implanted tetrodes in the dorsal striatum of control and *Drd2*KO@6mo adult mice to record local field potentials while mice explored an open field (Figure 5f). As expected, locomotor activity was lower in *Drd2*KO@6mo mice with immobility scores of 57.95% ± 0.06% that were significantly higher than in control mice (42.05% ± 0.03%, $P < 0.05$). Beta oscillation activity appeared sporadically both in control (1.29 ± 0.08 events min^{-1}) and *Drd2*KO@6mo mice (1.45 ± 0.08 events min^{-1}), with no clear connection with ambulation. However, beta events lasted longer in *Drd2*KO@6mo mice (Figure 5g). More specifically, beta events showed an excess of long duration events (> 200 ms) in *Drd2*KO@6mo mice (Figure 5h).

Altogether, these results show that the bradykinesia observed after ablating D2R in adult mice correlates with increased synchronization between the frontal cortex and the GP (Figure 5d) and with increased striatal beta activity (Figure 5h). The overall motor performance of control, constitutive $Drd2^{-/-}$ and mice rendered D2R-deficient as adults correlates well with the percentage of non-synchronized GP neurons but not with the density of striatal D2R, suggesting that, whereas $Drd2^{-/-}$ mice are able to partially compensate for the lack of the early expression of *Drd2*, inducible D2R mutant mice are a more genuine model to directly study the functional importance of D2R in motor behavior of adult mice.

DISCUSSION

In this study, we investigated the importance of D2R in adult mice during the execution and learning of motor tasks. Pharmacological efforts performed during the past three decades to solve this matter failed probably because D2R compounds are not fully selective as they also target D3R and D4R, as well as other structurally related G-protein coupled receptors expressed in brain circuits involved in motor function.[45–48] Molecular genetic attempts to selectively inactivate D2R have also been unsatisfactory owing to the activation of compensatory mechanisms that partially circumvented the loss of *Drd2* expression.[19,49] Furthermore, pharmacological and genetic approaches have led to irreconcilable results such that D2R blockade by neuroleptics induces akinesia and catatonia, whereas $Drd2^{-/-}$ mice only exhibit mild bradykinesia.[19] To solve this discrepancy, we designed a novel mouse model that allowed us to inactivate D2R function at any time and studied motor behaviors and electrophysiological phenotypes triggered by the ablation of D2R in adult mice. We found that mice rendered D2R-deficient as adults display severe motor problems, including fewer locomotor-initiation events, spontaneous catatonia, serious difficulties to perform a simple motor coordination task and a blunted ability to execute a previously learned motor routine. These phenotypes differ from those displayed by constitutive $Drd2^{-/-}$ mice and, conversely, are similar to the effects elicited by classical D2R blockers on wild-type animals or patients receiving neuroleptics. Thus this molecular genetic strategy uncovered the overall essential role of D2R in motor performance of adult mice.

A clear advantage of the conditional genetic approach used in this study is the ability to by-pass the activation of alternative developmental programs that may occur along the early formation and maturation of brain circuits and during the establishment of the whole body growth plan. This is particularly important while studying D2R as $Drd2^{-/-}$ mice are known to have an impaired growth hormone axis and, consequently, are dwarfs.[12,49,50] Induction of D2R ablation in adult mice, however, assured normal size allowing us to better assess quantitative motor parameters that in part depend on body size. Although the TAM treatment used in this study did not completely eliminate *Drd2* expression, the residual level (~20% of striatal *Drd2* mRNA) appears to have minimal functional relevance given the spontaneous catatonia displayed by *Drd2*KO@3mo mice and the lack of effect that the D2R blocker haloperidol and the D1/D2 indirect agonist amphetamine produced in the locomotor activity of these mice. The large phenotypic difference observed between adult mice carrying constitutive or inducible *Drd2* null mutations highlights the importance of allelic inactivation timing when investigating the function of a gene product such as the D2R. Thus the developmental compensatory mechanisms activated in $Drd2^{-/-}$ mice put aside the simplistic idea that constitutive D2R-deficient mice are just mice without D2R. Our results show that studying motor function in mice that developed and matured normal brain circuits and lose their D2R as adults has clinical relevance as the use of antipsychotics often triggers a motor-deficit syndrome known as drug-induced parkinsonism.[51] Thus temporal control of genetic inactivation provides a useful alternative to study gene function at particular time points of the animal life and adds another dimension to the understanding of how complex circuits operate in the brain, as we and others showed during the recent years by generating cell-type-specific *Drd2* mutant mouse lines to investigate the role of D2R in particular subsets of neurons[10,12,13,15,52] or pituitary cells.[14] Temporal regulation of *Drd2* allele inactivation gave us also the possibility to examine the relative importance of D2R as mice age. Using this approach, we found that the later D2R ablation is induced, the more severe the motor disabilities displayed by the conditional mutant mice are. In fact, loss of D2R in 6–8-month-old mice yielded a variety of motor deficits that were more severe than those found in mice of the same age rendered D2R-deficient at 2 months of age. In mice rendered D2R-deficient at an older age, we observed posture abnormalities reminiscent of clinical features typically observed in PD such as abnormal gait and posture, resting tremor and akinesia. The correlation found between the age at which D2Rs are eliminated and the severity of the motor symptoms indicates that, as the brain matures, plastic compensatory mechanisms wane, and the essential role of the D2R emerges more strikingly. This result adds to the actual debate concerning the age at which the human brain reaches full maturity since modern imaging studies show that myelination, synaptic pruning and connectivity continue developing further after previously considered. It is tempting to speculate that fully matured brains become plastically locked rendering older individuals more vulnerable for disability and illness. This study has also revealed the predominant outcome of inactivating simultaneously all D2R, which are expressed in a variety of neuronal types that orchestrate motor function in the basal ganglia: (1) D2 autoreceptors located in the terminals of nigrostriatal DA neurons, (2) presynaptic D2R present in glutamatergic corticostriatal terminals, (3) D2R expressed in cholinergic striatal interneurons, and (4) somatodendritic D2R present in iMSN striatopallidal GABAergic neurons. The first three of these D2R subpopulations exert a tight inhibitory control on basal ganglia output and their ablation predicts the induction of hyperlocomotion, as we clearly observed in D2 autoreceptor-deficient mice.[10] Lack of D2R in cholinergic interneurons or glutamatergic cortical terminals would probably result in a similar phenotype owing to the expected extra release of acetylcholine[53,54] or glutamate[9] in the striatum. In contrast, ablation of D2R present in iMSNs predicts hypolocomotion owing to the loss of DA-mediated inhibition of GABAergic striatopallidal output, the phenotype found here in *Drd2*KO@3mo mice. Thus, the net effect of eliminating all D2R reveals the critical importance of maintaining a normal activity of both the D1R-dMSN and D2R-iMSN striatal outputs (Figure 5e).

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In agreement, concomitant stimulation of D1R and D2R has a synergistic role in motor behavior,[55,56] whereas recent studies show that D1R-dMSN and D2R-iMSN co-activate during voluntary movement.[57]

We have also taken advantage of inducible inactivation of *Drd2* alleles to determine the behavioral consequences of D2R deficiency in goal-directed routines, the formation of motor habits and the execution of previously learned motor skills.[58] Both *Drd2*$^{-/-}$ mice as well as those losing D2R as adults showed poor motor skills to negotiate a rotarod. Following a 5-day training schedule, *Drd2*$^{-/-}$ mice showed considerable improvement in the execution of this task, whereas in *Drd2*KO@3mo mice did not, indicating that they were unable to learn a new motor coordination skill. Furthermore, when D2R ablation was induced in previously trained highly proficient mice, their performance worsened severely along the consecutive sessions, suggesting a combination of motor and motivational deficits. Our data indicate that the loss of D2R stimulation in adult mice is incompatible with learning and executing motor routines and even impairs the execution of previously acquired motor skills. These observations are consistent with what is observed in the clinic with PD patients that show deficits in acquiring and automatizing new motor routines even after intensive training.[59–61]

In addition to the behavioral deficits clasically associated with D1R/D2R imbalance, including the onset of parkinsonism, we also found that mice rendered D2R-deficient as adults displayed typical electrophysiological hallmarks present in PD patients, animal models of PD[28] and in animals treated with neuroleptics. Increased synchronization throughout the cortico-basal ganglia loop is partly due to poor filtering of cortical inputs in the striatum[27,29,35,36,62] so that the inhibitory input into the GPe is increased. We recorded an increased number of synchronized antiphase events in the GPe of *Drd2*$^{-/-}$ mice that were even greater in *Drd2*KO@3mo mice, establishing an inverse correlation between motor performance and antiphase synchronization among the three genotypes. In addition, we also found that the duration of oscillatory activity event of the beta frequency was increased in *Drd2*KO@6mo mice, in agreement with findings showing enhanced beta activity in PD patients and animal models of PD.[27] Overall, D2R ablation induces Parkinson-like oscillatory activity in the basal ganglia which is more pronounced when *Drd2* null-alleles are generated in adult mice, further suggesting the existence of an alternative developmental program in the basal ganglia that attenuates the consequences of D2R loss in *Drd2*$^{-/-}$ mice. Altogether, we consider that these results have important clinical relevance as the extensive use of D2R blockers, mainly represented by classical and atypical neuroleptics, often leads to drug-induced parkinsonism, not only when used as antipsychotic drugs but also to treat several other neurological and psychiatric conditions, including Tourette's syndrome, dementia, alcohol-induced delusions, bipolar disorder and obsessive-compulsive disorder.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

EPB, MGM and MR designed the experiments. EPB, RCC, GLG, EC, MAB, VR and DN performed the experiments and analyzed the data. EPB, RCC, EC, VR and DN performed the behavioral and biochemistry assays. GLG and MAB performed electrophysiological recordings. EB and MR wrote the paper. MGM edited the manuscript. MR perceived and directed the project.

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