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Genetic Knockdown of mGluR5 in Striatal D1R-Containing Neurons Attenuates L-DOPA-Induced Dyskinesia in Aphakia Mice

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Abstract

L-DOPA is the main pharmacological therapy for Parkinson's disease. However, long-term exposure to L-DOPA induces involuntary movements termed dyskinesia. Clinical trials show that dyskinesia is attenuated by metabotropic glutamate receptor type 5 (mGluR5) antagonists. Further, the onset of dyskinesia is delayed by nicotine and mGluR5 expression is lower in smokers than in non-smokers. However, the mechanisms by which mGluR5 modulates dyskinesia and how mGluR5 and nicotine interact have not been established. To address these issues, we studied the role of mGluR5 in D1R-containing neurons in dyskinesia and examined whether nicotine reduces dyskinesia via mGluR5. In the aphakia mouse model of Parkinson's disease, we selectively knocked down mGluR5 in D1R-containing neurons (aphakia-mGluR5$^{KD\text{-}D1}$). We found that genetic downregulation of mGluR5 decreased dyskinesia in aphakia mice. Although chronic nicotine increased the therapeutic effect of L-DOPA in both aphakia and aphakia-mGluR5$^{KD\text{-}D1}$ mice, it caused a robust reduction in dyskinesia only in aphakia, and not in aphakia-mGluR5$^{KD\text{-}D1}$ mice. Downregulating mGluR5 or nicotine treatment after L-DOPA decreased ERK and histone 3 activation, and FosB expression. Combining nicotine and mGluR5 knockdown did not have an added antidyskinetic effect, indicating that the effect of nicotine might be mediated by downregulation of mGluR5 expression. Treatment of aphakia-mGluR5$^{KD\text{-}D1}$ mice with a negative allosteric modulator did not further modify dyskinesia, suggesting that mGluR5 in non-D1R-containing neurons does not play a role in its development. In conclusion, this work suggests that mGluR5 antagonists reduce dyskinesia by mainly affecting D1R-containing neurons and that the effect of nicotine on dyskinetic signs in aphakia mice is likely via mGluR5.

Keywords Basal ganglia · Dyskinesia · mGluR5 · Nicotine · Parkinson's disease

Introduction

Parkinson's disease (PD) is characterized by the loss of dopaminergic neurons in the *substantia nigra pars compacta* and is associated with a reduction of dopaminergic tone in the striatum that correlates with the motor symptomatology. The dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) provides the most effective symptomatic treatment for PD [1, 2]. However, chronic L-DOPA administration together with the progression of PD induces motor side effects, such as dyskinesia. Strong evidence demonstrates that the dopamine D1 receptor (D1R) is critical for the development of dyskinesia [3–5]. For instance, L-DOPA and D1R agonists induce dyskinesia [6], and increased activation of D1R is associated with enhanced expression of extracellular-signal-activated kinases (ERK), phospho-acetylated histone 3 (pAcH3) and FosB [7–12]. These findings indicate that reduction of D1R-dependent signaling may help to alleviate dyskinetic symptoms [8, 13, 14]. Previous evidence demonstrated that glutamatergic and cholinergic transmission modulate the D1R signaling pathway [15, 16]. In fact, glutamatergic transmission in the basal ganglia is increased in dyskinesia [17–20]. Interestingly, this overactivation could be reversed in animal

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models by modulating the metabotropic glutamate receptor type 5 (mGluR5) [21].

The mGluR5 is highly expressed in striatal D1R- and D2R-containing neurons but also in cholinergic, parvalbumin, and somatostatinergic interneurons as well as in corticostriatal and thalamostriatal pathways [22, 23]. Several lines of evidence implicate mGluR5 in the development of dyskinesia [24–29]. First, L-DOPA treatment increases mGluR5 binding in the striatum of dyskinetic MPTP-treated monkeys and 6-OHDA-lesioned rats [28, 30]. Second, clinical and preclinical studies show that mGluR5 antagonists have moderate to mild antidyskinetic effects but at the expense of the therapeutic efficacy of L-DOPA [31–33]. Because mGluR5 is so widely distributed on striatal neurons, it is possible that these effects reside in different populations. Therefore, before dismissing mGluR5 as a potential therapeutic target for treatment or prevention of dyskinesia, it is important to determine if the antidyskinetic role of mGluR5 antagonists reside in a specific striatal population.

Previous studies have shown that dyskinesia is less frequent in PD patients who smoke compared to those who do not [34]. Although tobacco smoke contains many chemical components, it is possible that nicotine plays a role, since previous studies show that nicotine has antidyskinetic effects in rodents and non-human primates [35–38]. Nicotine actions are mediated by nicotinic acetylcholine receptors (nAChR) that are highly expressed in the striatum and modulate dopaminergic transmission. In the striatum, the two most abundant nicotine receptors are the β2* (α4β2*, α6β2*) and the α7 [39]. These receptors modulate the release of dopamine and glutamate in the striatum [3, 40]. Interestingly, previous studies showed that α7 receptor-specific binding is increased in the putamen of dyskinetic MPTP-treated monkeys and PD patients [41]. It is noteworthy that mGluR5 expression is downregulated in smokers, specifically in the caudate nucleus and putamen, suggesting that nicotine may be the cause of this downregulation [42]. Moreover, other previous studies suggest that the antidyskinetic effect of nicotine may be the result of targeting glutamatergic inputs [18, 43, 44].

Because D1R-containing neurons are strongly implicated in the development of dyskinesia, our aim in this study was to investigate the role of mGluR5, in direct pathway neurons, in dyskinesia. To produce a parkinsonian mouse model with D1R-specific reduction of mGluR5, we crossed transgenic mice, with a selective reduction of mGluR5 in D1R-containing neurons ($mGluR5^{KD\text{-}D1}$), with aphakia mice, a genetic model of PD [45, 46]. To understand the function of mGluR5 in direct pathway neurons in dyskinesia, we compared the effect of L-DOPA treatment in these mice to aphakia mice [46]. We also studied the dyskinetic effect of nicotine in these mice to determine if mGluR5 on D1R-containing neurons plays a role.

Methods

Mice

Female and male mice (22–30 g) were housed under 12-h light-dark cycle with food and water ad libitum, temperature 22 °C, and humidity 44%. $mGluR5^{KD\text{-}D1}$ mice were generated by homologous recombination and kindly provided by Dr. Bilbao and Dr. Spanagel, from the Central Institute of Mental Health, Manheim Germany [45]. Aphakia mice and C57BL/6J mice were obtained from our own facility. Aphakia mice with a selective knockdown of mGluR5 in D1R-containing neurons were created by crossing aphakia mice with $mGluR5^{KD\text{-}D1}$ mice. All mice were on a C57BL/6J background, and the genotype was determined by PCR analysis of tail-tip DNA as described previously [45, 46].

Drugs

L-DOPA, benserazide, and 1-methyl-3-(4-methylpyridin-3-yl)-6-(pyridin-2-ylmethoxy)-1H-pyrazolo-[3,4-b]pyrazine (PF470) were purchased from Sigma–Aldrich (Spain). (−)-Nicotine ditartrate was purchased from Tocris-Bioscience (UK).

Nicotine and L-DOPA Treatment

Half of the mice were randomly assigned to control (sweetened water) or nicotine groups. Nicotine was supplied in drinking water (300 μg/mL in 2% saccharine vehicle) for 8 weeks. This concentration corresponds to a level similar to that found in long-time smokers [3]. Chronic nicotine treatment in the drinking water was initiated a month before the chronic administration of L-DOPA. Mice received a daily injection of benserazide (10 mg/kg i.p.) followed, 20 min later, by L-DOPA (10 mg/kg i.p) (Fig. 1(a)) [47]. To assess the non-D1R role of mGluR5 in dyskinesia, a highly potent negative allosteric modulator, PF470 (1 mg/kg s.c.), was used (Ki = 0.9 nM vs MTEP Ki = 16 nM) together with benserazide.

Behavioral Analysis

Following injections, mice were placed individually in glass cylinders and were video-recorded for 4 min, beginning 30 min after L-DOPA or saline injection. Three and four paw dyskinesia (3–4 paw dyskinesia) was scored as described previously. Briefly, 3–4 paw dyskinesia was noted when mice stood on their hind paws and reared against the wall of the cylinder, moving both front paws and repeatedly lifting the hind paws up and down [47–49].

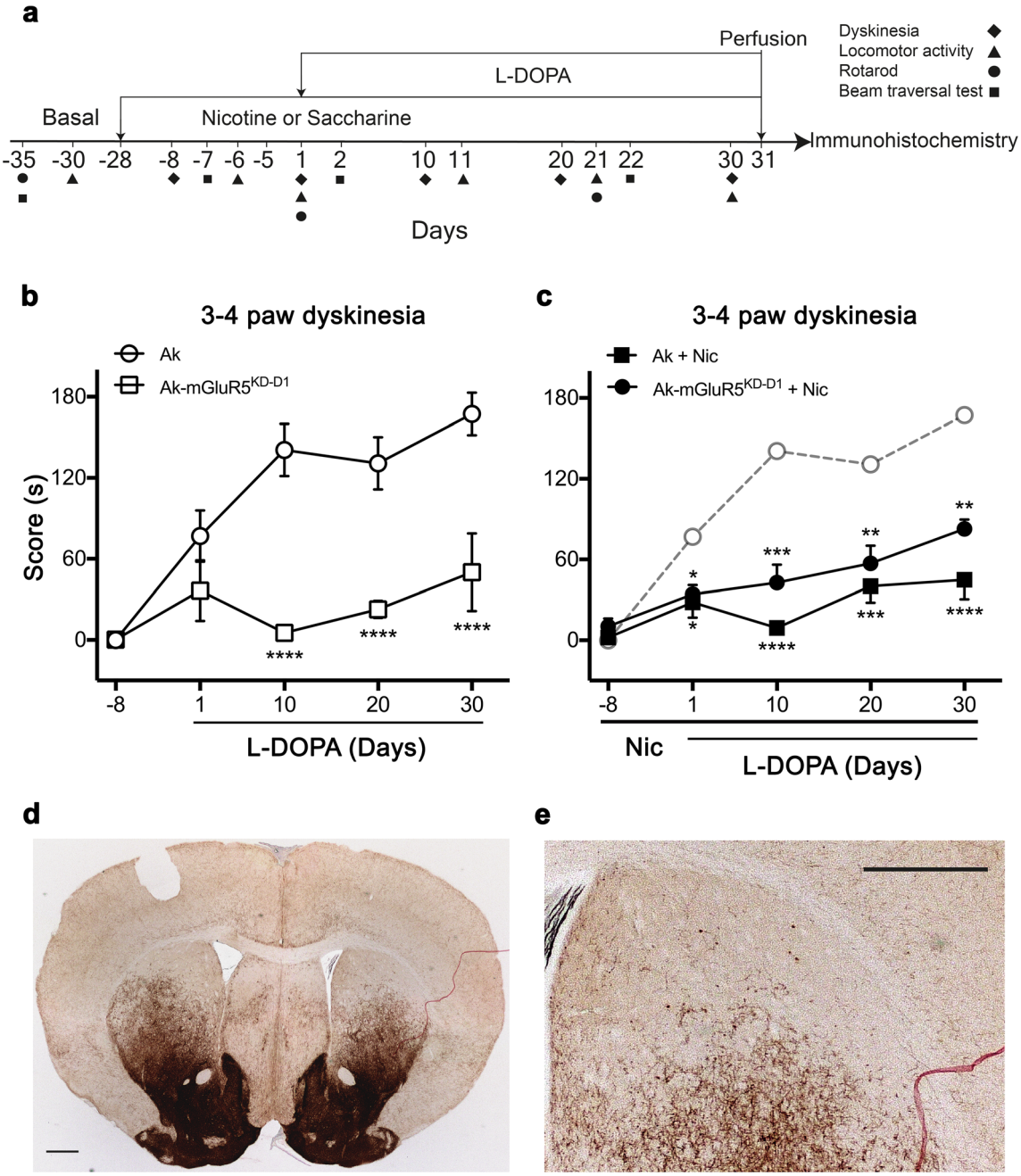


Fig. 1 Selective reduction of mGluR5 in D1R-containing neurons diminishes L-DOPA-induced dyskinesia in aphakia (Ak) mice. *a* Schematic view of the experimental timeline. *b*, *c* Scores of 3–4 paw dyskinesia in Ak and Ak-mGluR5^{KD-D1} mice treated with L-DOPA *b* and nicotine + L-DOPA *c*. *d* Coronal section from an Ak mouse immunostained for TH. *e* High magnification image from *d* showing a gradient of TH loss in dorsolateral striatum. Scale bar = 500 μm. Data are expressed as mean ± SEM, $n = 9–15$ per group. Two-way ANOVA followed by Bonferroni's post hoc test showed significant differences for *b* [genotype, $F_{1,102} = 48.95$, $p < 0.0001$; time, $F_{4,102} = 10.04$, $p < 0.0001$; interaction, $F_{4,102} = 5.21$, $p = 0.0007$] and for *c* [treatment, $F_{4,146} = 16.83$, $p < 0.0001$; time, $F_{2,146} = 40.3$, $p < 0.001$; interaction, $F_{8,146} = 4.35$, $p = 0.0001$]. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. L-DOPA-treated Ak (dotted line in *c*). Nic nicotine

Basal motor behavior was evaluated 1 to 2 weeks before L-DOPA administration. Locomotor activity was assessed in activity cages (AccuScan instruments, Inc.) as previously described [13]. We measured the total distance (cm) traveled during a 30-min period, beginning 30 min after L-DOPA injection. Motor coordination and balance were measured in the challenging beam traversal test as previously described [50]. Briefly, before treatments, mice were trained to cross the beam, and once trained, they were videotaped. We measured the total time it took the mice to cross the beam. The beam traversal test was evaluated 45 min after L-DOPA

injection, when dyskinesia is higher. Motor coordination was evaluated using the rotarod test (UgoBasile, Varese, Italy), following an accelerating protocol with increasing speed from 4 to 40 rpm over a 5-min period and measuring latency up to the first fall off the rod [47]. Mice were tested in six consecutive trials, 20 min apart. All behavioral experiments were assessed by observers who were blind to mouse genotypes and treatment.

Immunohistochemistry

One hour after the last L-DOPA injection, animals were anesthetized with an overdose of pentobarbital (Normon Labs, Madrid Spain) and perfused with saline solution, followed by 4% paraformaldehyde in phosphate-buffered saline. The brains were post-fixed overnight and transferred to a solution of 0.1 M phosphate buffer. The brains were cut into 30-μm coronal sections with a vibratome (Leica, Wetzlar, Germany), and immunohistochemistry was performed on free-floating sections. To stain sections of striatum, we used the standard avidin-biotin immunohistochemical protocol with the following primary antibodies: tyrosine hydroxylase (TH, 1:1000; Chemicon), FosB (1:15000; Santa Cruz Biotechnology), pERK (1:250; Cell Signaling Technology), and pAcH3 (1:10000; Millipore). For immunofluorescence, we used mGluR5 antibody (1:100; Alomone labs) revealed with Alexa fluor 647-conjugated secondary antibody (1:500; Invitrogen). Quantification of FosB, pAcH3, and pERK, TH and mGluR5 expression levels were carried out using ImageJ [51]. The number of immunolabeled cells were determined using two-three serial rostrocaudal sections (+ 1.10 to + 0.14 mm relative to bregma) per animal from the lesioned dorsolateral striatum as previously described. The digital images were obtained under a Leica microscope using a × 40 objective. The data are presented as the number of stained nuclei per square millimeter in the denervated striatum as previously reported [14]. We conducted fluorescence analyses of mGluR5 by using ImageJ to calculate the signal intensity contained in the double stained microphotographs obtained with confocal microscope using a × 60 objective as previously described [52]. Fluorescence intensity results are expressed as percentage of control (C57BL/6J mice).

Statistical Analysis

All values are given as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6. Statistical analysis was assessed by using two-way ANOVA or repeated measures, followed by the appropriate post hoc comparisons. A level of $p < 0.05$ was considered statistically significant.

Results

Knockdown of mGluR5 in D1R-Containing Neurons Ameliorates L-DOPA-Induced Dyskinesia

The aphakia mice lack nigrostriatal innervation in both hemispheres (Fig. 1(d, e)) and, therefore, display bilateral dyskinetic symptoms after L-DOPA treatment as shown previously [46, 48]. To evaluate the role of mGluR5 in direct pathway neurons in dyskinesia, we used the aphakia-mGluR5$^{KD\text{-}D1}$. Aphakia-mGluR5$^{KD\text{-}D1}$ and aphakia mice were chronically treated with L-DOPA (10 mg/kg) for 31 days, (Fig. 1(a)), and 3–4 paw dyskinesia was evaluated at regular intervals by a researcher blinded to treatment and genotype [46, 47]. L-DOPA increased 3–4 paw dyskinesia in aphakia mice, while in aphakia-mGluR5$^{KD\text{-}D1}$ mice, dyskinetic scores were significantly lower ($p < 0.001$) (Fig. 1(b)). Because nicotine attenuates and delays the onset of dyskinetic symptoms [34–36, 53], and also reduces brain mGluR5 [42], we wanted to address if nicotine's effect on dyskinesia is related to reduced mGluR5 expression on D1R-containing neurons. Chronic treatment with nicotine robustly and significantly reduced dyskinesia in aphakia mice ($p < 0.01$), similar to its previously published effects in unilateral 6-OHDA-lesioned mice and in MPTP-treated monkeys [38, 40, 54]. In aphakia-mGluR5$^{KD\text{-}D1}$ mice, nicotine treatment did not further reduce dyskinetic symptoms. In fact, the aphakia-mGluR5$^{KD\text{-}D1}$ mice showed slightly higher dyskinetic scores compared with aphakia mice treated with nicotine (Fig. 1(c)).

Knockdown of mGluR5 in D1R-Containing Neurons Does Not Affect L-DOPA's Therapeutic Efficacy

To check that the antidyskinetic effects of mGluR5 knockdown were not due to a reduction in the therapeutic effect of L-DOPA, we compared motor behavior in L-DOPA-treated aphakia and aphakia-mGluR5$^{KD\text{-}D1}$ mice. In this experiment, we used activity cages that measure spontaneous locomotor activity as reflected by sequential horizontal beam breaks, computing the total distance traveled during each trial. We found no difference between aphakia and aphakia-mGluR5$^{KD\text{-}D1}$ mice in horizontal movements or the distance traveled under basal conditions or after chronic L-DOPA treatment (Fig. 2(a, b)). Chronic nicotine treatment also had no effect in motor activity in the L-DOPA-treated or naïve aphakia or aphakia-mGluR5$^{KD\text{-}D1}$ mice (Fig. 2(a', b')).

We then challenged the mice for finer motor coordination and activity using the beam traversal and rotarod tests before and after L-DOPA treatment. The time to cross the beam was similar in aphakia and aphakia-mGluR5$^{KD\text{-}D1}$ mice after saline. Chronic L-DOPA significantly reduced the time

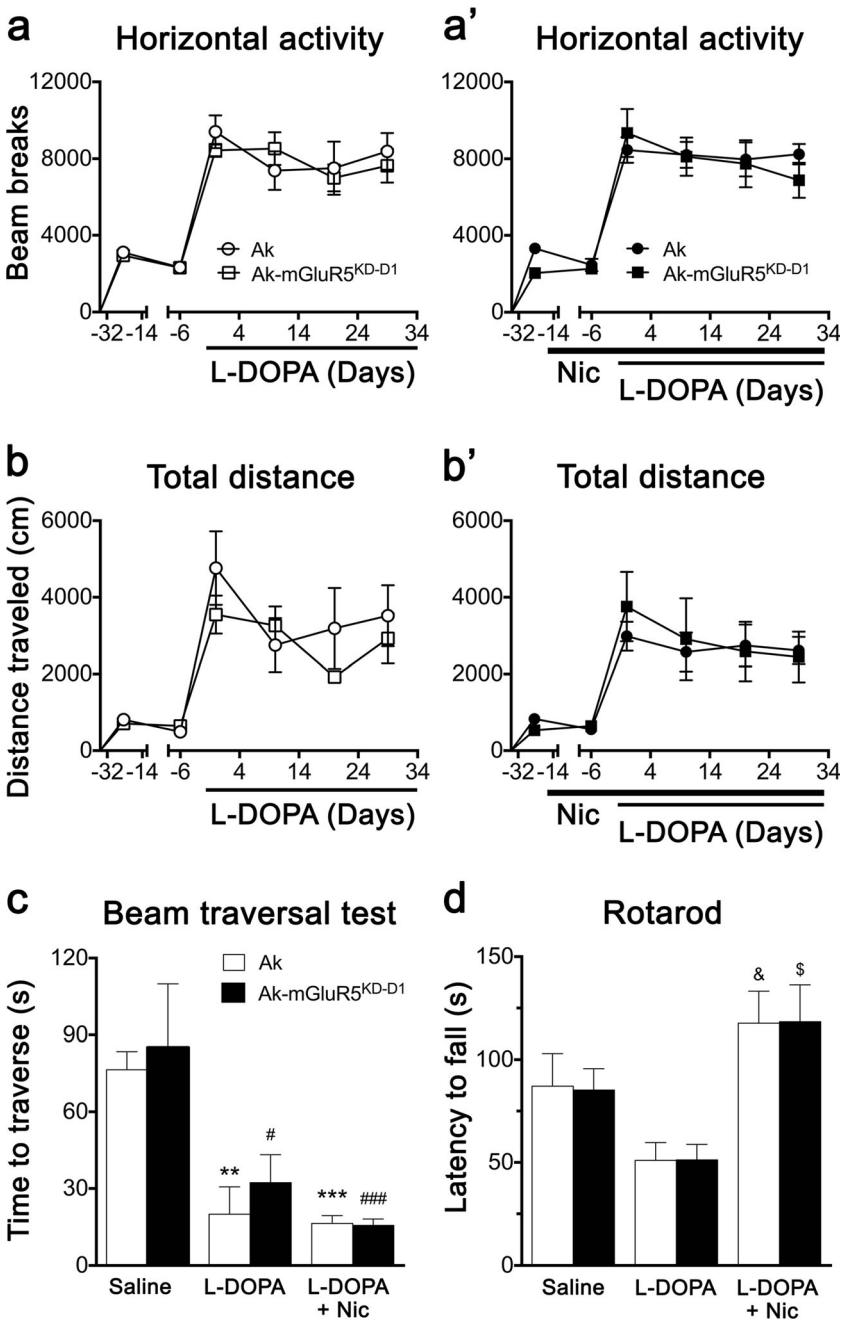


Fig. 2 Selective reduction of mGluR5 in D1R-containing neurons does not alter the therapeutic effect of L-DOPA. (a, a'). Horizontal locomotor activity and (b, b') total distance traveled were measured 30 min after L-DOPA administration for 30 min. Note that no differences were found between Ak and Ak-mGlur5^{KD-D1} neither in basal conditions nor with L-DOPA (a, b) or L-DOPA + nicotine (a', b'). Motor coordination and performance were measured in the beam traversal (c) and in the rotarod (d) tests at different time points: 5 weeks before any treatment (saline), or 45 min after L-DOPA administration (see timeline in Fig. 1(a)). Two-way ANOVA followed by Bonferroni's post hoc test showed significant differences for treatment [$F_{2,42} = 25.26$, $p < 0.001$ for (c); $F_{2,50} = 12.15$, $p = 0.0001$ for (*d*)]. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Ak saline, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. Ak-mGlur5^{KD-D1} saline, $^{\&}p < 0.05$ vs. Ak L-DOPA, $^{\$}p < 0.05$ vs. Ak-mGlur5^{KD-D1} L-DOPA. Data are expressed as mean ± SEM, $n = 6$–11 per group. Nic nicotine

to cross beam in both groups of mice ($p < 0.01$), with no significant difference between genotypes. Chronic nicotine treatment did not further reduce the time to cross the beam (Fig. 2(c)). In terms of motor coordination, dyskinesia reduced the latency to fall from the rod in both aphakia and aphakia-mGluR5^{KD-D1} mice (Fig. 2(d)). Interestingly, chronic treatment with nicotine in addition to L-DOPA significantly potentiated L-DOPA's motor coordination effect in both aphakia and aphakia-mGluR5^{KD-D1} mice ($p < 0.01$) (Fig. 2(d)).

Molecular Markers as Cue in the Effect of Nicotine in Aphakia Mice Lacking mGluR5 in D1R-Containing Neurons

We then investigated whether the decrease in L-DOPA-induced dyskinesia in aphakia-mGluR5^{KD-D1} mice is accompanied by a reduction of molecular markers of L-DOPA-induced dyskinesia—pERK, pAcH3, and FosB. As expected, L-DOPA induced the expression of molecular markers selectively in the completely denervated striatal areas in both hemispheres (e.g. see FosB

inset in Fig. 3(c)). Chronic L-DOPA treatment significantly increased the expression of FosB, pAcH3, and pERK in aphakia mice compared to the saline- and nicotine-treated aphakia animals ($p < 0.001$) (Fig. 3). Interestingly, in aphakia-mGluR5^{KD-D1} mice, the number of neurons immunoreactive for pERK was reduced by 66% ($p < 0.01$), pAcH3 activation was reduced by 33% ($p < 0.05$, $p < 0.01$, $p < 0.001$), and FosB expression by 39% ($p < 0.05$) (Fig. 3(a'–c')) compared to aphakia mice. Consistent with its effect on dyskinesia signs, chronic treatment with nicotine resulted in a significant decrease of pERK- and pAcH3-induced by L-DOPA in aphakia ($p < 0.01$) (Fig. 3(a', b')) but not in aphakia-mGluR5^{KD-D1} mice. However, nicotine did not alter L-DOPA-induced FosB expression in either aphakia or aphakia-mGluR5^{KD-D1} mice (Fig. 3(c, c')).

The Antidyskinetic Effect of the mGluR5 Negative Allosteric Modulator Is Primarily Mediated by mGluR5 on D1R-Containing Neurons

Because mGluR5 is similarly expressed in D1R- and D2R-containing striatal neurons, and in most interneurons [22], we then tested if the antidyskinetic effects of the negative

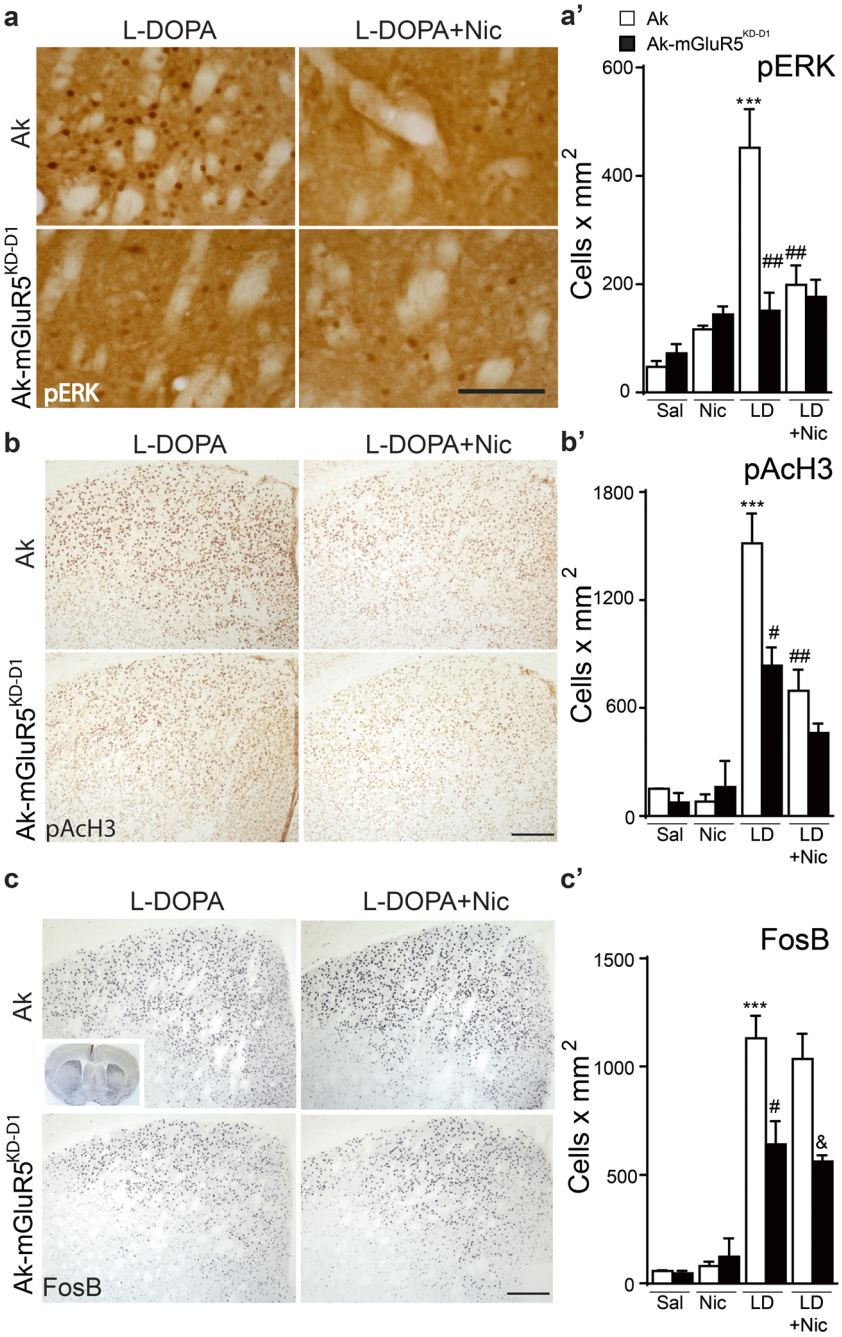


Fig. 3 Downregulation of mGluR5 in D1R neurons reduces L-DOPA-induced molecular markers of dyskinesia. (a, b, c) Microphotographs of striatal coronal sections from Ak and Ak-mGluR5^{KD-D1} mice sacrificed 1 h after the last L-DOPA injection, immunostained for pERK, pAcH3, and FosB. Scale bar = 100 μm, 200 μm. (a', b', c') Histograms represent the quantification of pERK, pAcH3, and FosB immunoreactive positive cells. Note that all molecular markers are expressed in the completely denervated striatal areas, see FosB image in (c). Two-way ANOVA followed by Bonferroni's post hoc test showed significant differences for genotype [$F_{1,26} = 4.49$, $p = 0.04$ for pERK; $F_{1,26} = 7.02$, $p = 0.01$ for FosB] and treatment [$F_{3,26} = 10.15$, $p = 0.0001$ for pERK; $F_{3,27} = 16.93$, $p = 0.0001$ for pAcH3; $F_{3,26} = 26.04$, $p = 0.0001$ for FosB]. $^{***}p < 0.001$ vs. Ak saline, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. Ak L-DOPA. $^{\&} < 0.05$ vs. Ak-mGluR5^{KD-D1} L-DOPA + nicotine. Data are expressed as mean ± SEM, $n = 5–8$ per group, except in saline- and nicotine-treated groups, $n = 3$. Nic, nicotine; LD, L-DOPA

allosteric modulators in aphakia-mGluR5^{KD-D1} mice were mediated by the mGluR5 expressed in D1R-containing neurons. Previous studies showed a marked antidyskinetic effect of the highly selective and efficacious mGluR5 negative allosteric modulator, PF470 [55]. To assess the specific contribution of mGluR5 on D1R-containing neurons, we compared the effect of PF470 on L-DOPA-induced dyskinesia in aphakia and aphakia-mGluR5^{KD-D1} mice. We treated mice with PF470 20 min before the last dose of L-DOPA (Fig. 4(a)). Our results showed that a single dose (1 mg/kg, i.p.) of PF470 decreased dyskinetic behavior in aphakia mice by about 80% ($p < 0.05$), as expected [56]. However, acute PF470 had no significant effect on dyskinetic behavior in aphakia-mGluR5^{KD-D1} mice (Fig. 4(b)), suggesting that the effect of the mGluR5 antagonists on dyskinesia is primarily due to its action on mGluR5 located in D1R-containing neurons. Similarly, PF470 did not further reduce the antidyskinetic effects of nicotine in aphakia or aphakia-mGluR5^{KD-D1} mice (Fig. 4(b)).

We next compared the effect of PF470 on expression of molecular markers of dyskinesia in aphakia and aphakia-

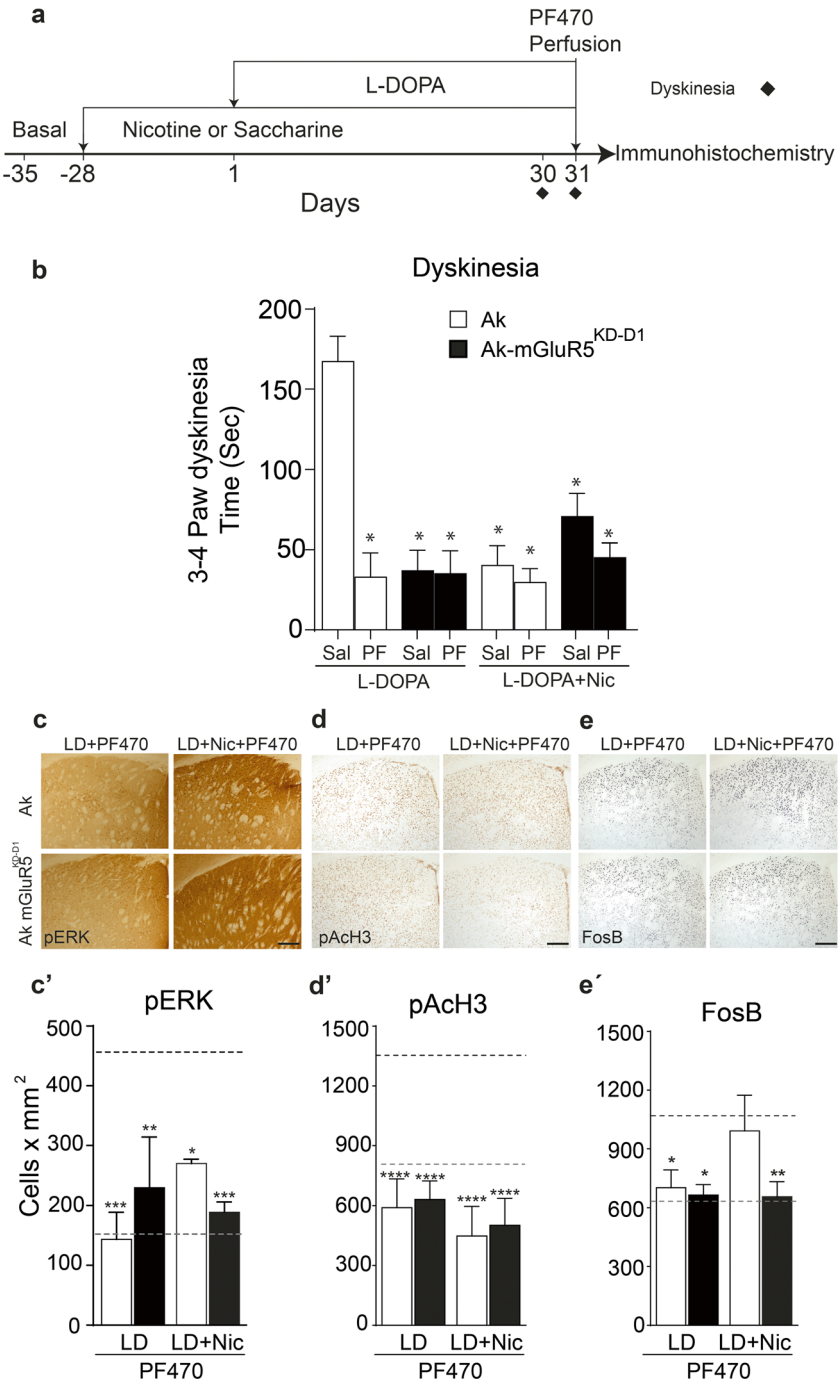


Fig. 4 The antidyskinetic effect of the mGluR5 negative allosteric modulator is mediated by mGluR5 on D1R-containing neurons. (a) Schematic view of the experimental timeline. (b) Acute administration of the mGluR5 negative allosteric modulator PF470 (PF) reduces already established L-DOPA-induced dyskinesia in Ak but not in Ak-mGluR5^{KD-D1} mice. One-way ANOVA followed by Bonferroni's post hoc test [$F_{7,71} = 15.23$, $p < 0.0001$]. *$p < 0.001$ vs. L-DOPA-treated Ak. (c–e') Immunohistochemical analysis illustrating that PF470 reduces L-DOPA-induced striatal pERK (c, c'), pAcH3 (d, d') and FosB (e, e') expression only in Ak mice. Black dotted line represents the expression levels induced by L-DOPA in Ak mice and the gray dotted line in Ak-mGluR5^{KD-D1} mice. Scale bar = 100 μm. Two-way ANOVA followed by Fisher's LSD post hoc test: genotype × treatment [$F_{2,19} = 6.58$, $p = 0.0067$ for pERK; $F_{2,19} = 8.65$, $p < 0.002$ for pAcH3; $F_{2,18} = 2.1$, $p = 0.15$ for FosB]. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. L-DOPA-treated Ak mice. Data are expressed as mean ± SEM. $n = 9$–14 per group in (b) and 3–4 per group in (c–e')

mGluR5^{KD-D1} mice. PF470's effect on molecular markers of dyskinesia mirrored its effects in the behavioral assays of dyskinesia. Thus, PF470 decreased pERK and pAcH3 in both aphakia and aphakia-mGluR5^{KD-D1} groups (Fig. 4(c, d')). In the mice chronically treated with nicotine, PF470 did not further alter pERK and pAcH3 in aphakia or aphakia-mGluR5^{KD-D1} mice. Although PF470 treatment decreased FosB expression induced by L-DOPA in aphakia and aphakia-mGluR5^{KD-D1} mice, as expected, in the nicotine-treated mice, this reduction occurred in the aphakia-mGluR5^{KD-D1} mice only (Fig. 4(e, e')).

mGluR5 Expression in D1R-Containing Neurons

Previous studies showed that mGluR5 is downregulated in mGluR5^{KD-D1} mice [15, 45]. Since aphakia mice express the tomato fluorescent protein in D1R-containing neurons, we were able to measure by immunofluorescence the expression of mGluR5. As expected, we found that saline-treated aphakia-mGluR5^{KD-D1} mice display lower expression of the mGluR5 in D1R-containing neurons compared to aphakia mice ($p < 0.01$) in the denervated striatum. This pattern of expression was similar in the nicotine-treated animals. Next, we asked whether L-DOPA is able to increase the expression of mGluR5 in striatal D1R-containing neurons in the aphakia-mGluR5^{KD-D1} mice, and second, if nicotine could further reduce mGluR5 in D1R-containing neurons. We found that L-DOPA increased the expression of mGluR5 in D1R-containing neurons in aphakia mice relative to saline-treated aphakia mice. This L-DOPA-induced increase in mGluR5 expression was prevented in aphakia-mGluR5^{KD-D1} animals ($p < 0.01$), although the expression was slightly higher compared to saline aphakia-mGluR5^{KD-D1} mice. Nicotine reduced mGluR5 fluorescence intensity in D1R-containing neurons in aphakia mice but not in aphakia-mGluR5^{KD-D1} (Fig. 5).

Discussion

The present study demonstrates that selective knockdown of mGluR5 in D1R-containing neurons decreases L-DOPA-induced dyskinesia in aphakia mice, an established genetic mouse model of PD [46–49]. We observed that aphakia-mGluR5^{KD-D1} mice, showed a robust decrease in dyskinesia but retained full therapeutic efficacy of L-DOPA compared to aphakia mice. Importantly, this reduction in behavioral indicators of dyskinesia was accompanied by reduced activation of the pERK/pAcH3/FosB signaling pathway, a known molecular cascade associated with L-DOPA-induced dyskinesia. Furthermore, administration of the highly selective mGluR5 negative allosteric modulator (PF470) reduced dyskinesia in aphakia but did not further modify dyskinetic behavior in apahakia-mGluR5^{KD-D1} mice, suggesting that the effect of PF470 on dyskinesia is primarily mediated by its action on mGluR5 in D1R-containing neurons. Given that nicotine has been shown to regulate glutamate neurotransmission, we also asked whether nicotine was able to further reduce dyskinesia in aphakia-mGluR5^{KD-D1} mice. Our results suggest that the effect of nicotine on dyskinesia is also mediated through mGluR5 in D1R direct pathway neurons. Chronic nicotine decreased dyskinesia in aphakia mice, but was unable to produce a further reduction of dyskinetic signs in aphakia-mGluR5^{KD-D1}. Interestingly, animals that received nicotine showed no reduction in the antiparkinsonian effect of L-DOPA and exhibited improvement in motor performance.

Previous research shows that striatal mGluR5 are increased when dyskinetic movements induced by L-DOPA appear [57]. In addition, it was demonstrated that there is a cross talk between D1R- and mGluR5-signaling pathways in direct pathway neurons that leads to ERK activation in the dopamine-depleted striatum [15], and it has been suggested that this modulation of the D1R signaling cascade mediates dyskinesia [8]. In our study, we found that aphakia-mGluR5^{KD-D1} mice display fewer dyskinesia than aphakia mice (Fig. 1), but still exhibit the full therapeutic efficacy of L-DOPA (Fig. 2). This is in line with previous findings showing that mGluR5 antagonists such as MPEP, MTEP, fenobam, and MRZ-8676 reduce dyskinesia in 6-OHDA-lesioned rats and MPTP-lesioned non-human primates [6, 58, 59].

Chronic L-DOPA treatment causes long-term modification of synaptic activity in dyskinesia. For instance, there is an increase in D1R-mediated response, leading to enhanced activation of ERK, histone 3, and FosB expression in the dopamine-denervated striatum [60–62]. The exact mechanisms underlying the reduction of dyskinesia in aphakia-mGluR5^{KD-D1} mice are not completely understood. One possible mechanism is that decreased levels of mGluR5 lead to a reduction in D1R signaling. A recent study demonstrated a cross talk between mGluR5 and D1R in the dopamine-depleted striatum by showing that mGluR5 modulates SKF38393-induced ERK activation [15]. Previous studies suggest at least two ways in which knocking down mGluR5 could affect D1R-dependent signaling pathway via ERK [15]: first, by decreasing diacylglycerol, which leads to lower activation of protein kinase C, which in turn reduces ERK signaling and second, by reducing intracellular calcium release, which reduces calcium calmodulin kinase II and ERK activation (Fig. 6) [63]. Here, we report that the reduced development of dyskinesia in aphakia-mGluR5^{KD-D1} mice is associated with a decrease in the molecular markers pERK, pAcH3, and FosB (Fig. 3). These results are in agreement with previous studies showing that mGluR5 antagonist reduced L-DOPA-induced ERK activation and FosB expression in the 6-OHDA rat model of dyskinesia [26, 64]. The reduced pERK, pAcH3 activation, and FosB expression induced by L-DOPA in aphakia-mGluR5^{KD-D1} mice suggests cooperation

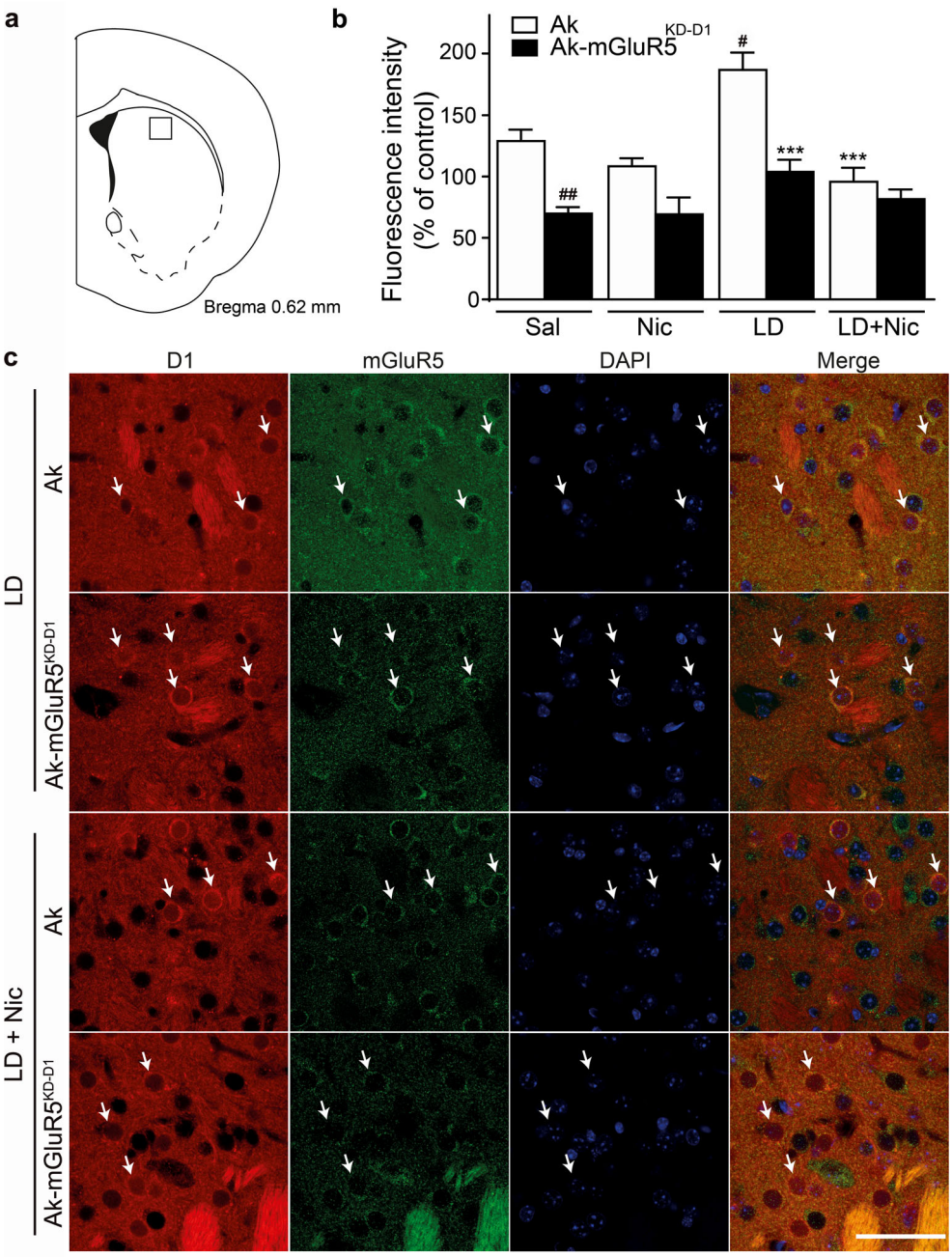
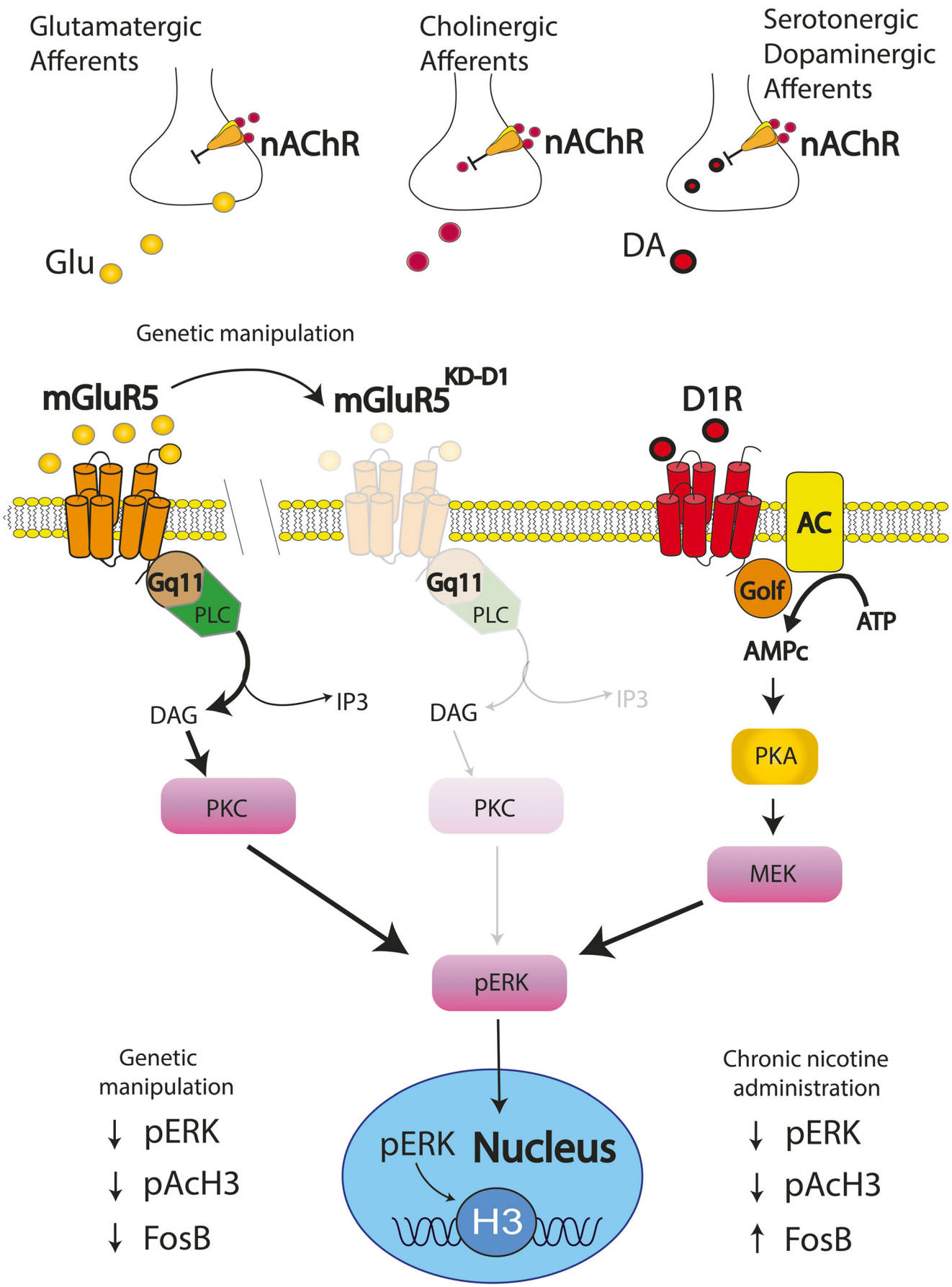


Fig. 5 Nicotine reduces mGluR5 expression in D1R-containing neurons. (a) Location of sampled areas is indicated by the black box in a schematic representation of a coronal section taken at 0.62 mm from bregma. (b) Graph represents mGluR5 expression in D1R-neurons expressed as the area under the curve (AUC) of fluorescence intensity. (c) Representative confocal images from L-DOPA-treated Ak and Ak-mGluR5^{KD-D1} mice without and with nicotine treatment illustrating D1R (red) and mGluR5 (green) coexpression. Nuclei are visualized via DAPI staining. Arrows indicate examples of dual D1R-positive and mGluR5-positive neurons. Scale bar = 100 μm. Two-way ANOVA followed by Bonferroni's post hoc test showed significant differences for genotype [$F_{1,46} = 43.9$, $p = 0.0001$] and treatment [$F_{3,46} = 13.2$, $p = 0.0001$]. $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ vs. Ak saline, $^{***}p < 0.001$ vs. Ak L-DOPA. $n = 4$–8 per group

between D1R and mGluR5 in the D1R-dependent signaling pathway in dyskinesia. Our finding that PF470 does not further modify dyskinesia in aphakia-mGluR5^{KD-D1} mice (Fig. 4) rules out a role of mGluR5 in non-D1R-containing neurons in the development of dyskinesia.

Nicotinic receptors have recently emerged as a potential therapeutic target to treat dyskinesia. nAChR are ionotropic receptors widely expressed in the central nervous system. The striatum expresses two nAChR implicated in regulating the release of different neurotransmitters: the most abundant is the *β2 (α4β2*, α6β2*), which is implicated in dopamine release, while α7 is preferentially located in glutamatergic terminals [3, 37, 53]. The location of nicotinic receptor in striatal projection neurons remains controversial. For instance, electrophysiological recordings using the puff technique revealed that 32% of striatal projection neurons respond to nicotine. In this study, we tested whether nicotine could modulate dyskinesia via mGluR5 using the aphakia-mGluR5^{KD-D1} mice. We found that nicotine robustly reduced L-DOPA-induced dyskinesia in aphakia mice, but was unable to modify the already reduced dyskinetic signs in the aphakia-mGluR5^{KD-D1} mice. These results suggest that nicotine effects and mGluR5 blockade function via a similar mechanism.


Glutamatergic Afferents
Cholinergic Afferents
Serotonergic Dopaminergic Afferents
nAChR
nAChR
nAChR
Glu
DA
Genetic manipulation
mGluR5
mGluR5 KD-D1
D1R
AC
Gq11
PLC
Gq11
PLC
Golf
ATP
AMPc
DAG
IP3
DAG
IP3
PKA
PKC
PKC
MEK
pERK
Genetic manipulation
↓ pERK
↓ pAcH3
↓ FosB
pERK Nucleus
H3
Chronic nicotine administration
↓ pERK
↓ pAcH3
↑ FosB

◀ **Fig. 6** Schematic diagram illustrating the molecular changes induced by L-DOPA and nicotine in aphakia and aphakia-mGluR5^{KD-D1} mice. Chronic nicotine treatment desensitizes presynaptic nAchR, decreasing L-DOPA-induced pERK and pAcH3 expression in dyskinetic Ak mice. mGluR5 genetic downregulation on D1R-containing neurons also reduces L-DOPA-induced pERK, pAcH3, and FosB expression in dyskinetic Ak mGluR5^{KD-D1} mice. Half-tones represent genetic manipulation in Ak mGluR5^{KD-D1} mice

The reduction of dyskinetic behavior in aphakia mice was accompanied with attenuation of the associated molecular markers pERK and pAcH3, but surprisingly not of FosB expression. A possible explanation for the unaltered FosB expression is that chronic nicotine treatment induces striatal FosB accumulation following dopamine receptor activation [16]. It is possible that in our nicotine-treated animals, FosB expression occurs, in part, independently of the ERK/pAcH3 pathway. However, future studies are needed to explore these possibilities.

The molecular mechanisms underlying the ability of nicotine to reduce dyskinesia in aphakia mice are not completely understood. There is evidence of pharmacological modulation of mGluR5 in the striatum by nicotine [18, 41, 42]. For instance, mGluR5 expression is downregulated in the caudate nucleus and putamen of smokers, suggesting an interaction between nicotine receptor and mGluR5 [42, 65]. This could be one of the reasons why we observed that nicotine did not further decrease the dyskinetic response to L-DOPA in aphakia-mGluR5^{KD-D1} mice. In fact, we found that nicotine treatment prevented the L-DOPA-induced mgluR5 expression in the denervated striatum (Fig. 5). Another possible explanation could involve the modulation of the nitrergic system by nicotine. Nicotine increases nitric oxide synthase expression in the striatum, which is involved in the development of dyskinesia [66–68]. We, and others, have shown that increasing nitric oxide signaling by administering a nitric oxide donor or a phosphodiesterase inhibitor decreases dyskinesia [47, 69, 70]. However, more studies are needed to clarify whether nicotine alleviates dyskinesia by modulating the nitrergic system. In addition, our results also showed that the antidyskinetic effect of nicotine is accompanied by a better performance of motor tasks, thus increasing the therapeutic effect of L-DOPA independently of mGluR5 in D1R-containing neurons.

In summary, the current study demonstrates the role of mGluR5 in D1R-containing neurons on dyskinesia. We found that the specific reduction of mGluR5 in direct pathway neurons reduces L-DOPA-induced dyskinesia in aphakia mice and the associated molecular markers. In support to this, we found that the antidyskinetic effect of a mGluR5 negative allosteric modulator is mainly due to the blockade of those receptors expressed in D1R-containing neurons. On the other hand, our results also indicate that the antidyskinetic effect of nicotine might be mediated by a downregulation of mGluR5 expression, because the combination of nicotine and reduction (or blockade) of mGluR5 did not have an added antidyskinetic effect. Further studies are needed to determine if combining subthreshold doses of these drugs might have an additive antidyskinetic effect. Our data suggest that therapies based on targeting mGluR5 (specifically expressed in D1R-containing neurons) or nicotine would be an efficient clinical approach to treat dyskinesia.

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Compliance with Ethical Standards

All experiments were conducted in accordance with the guidelines of the European Union Council Directive (86/609/EEC and 2003/65/CE). The protocol was approved by the Spanish Council ethics committee and the committee of ethics and animal experimentation of the Instituto Cajal (CSIC).

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