Published in final edited form as:

Brain Res. 2013 January 23; 1491: 236–250. doi:10.1016/j.brainres.2012.11.010.

Serotonin hyperinnervation and upregulated 5-HT_{2A} receptor expression and motor-stimulating function in nigrostriatal dopamine-deficient Pitx3 mutant mice

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Abstract

The striatum receives serotonin (5-hydroxytryptamine, 5-HT) innervation and expresses 5-HT_{2A} receptors (5-HT_{2ARs}) and other 5-HT receptors, raising the possibility that the striatal 5-HT system may undergo adaptive changes after chronic severe dopamine (DA) loss and contribute to the function and dysfunction of the striatum. Here we show that in transcription factor Pitx3 gene mutant mice with a selective, severe DA loss in the dorsal striatum mimicking the DA denervation in late Parkinson's disease (PD), both the 5-HT innervation and the 5-HT_{2AR} mRNA expression were increased in the dorsal striatum. Functionally, while having no detectable motor effect in wild type mice, the 5-HT_{2R} agonist 2,5-dimethoxy-4-iodoamphetamine increased both the baseline and L-dopa-induced normal ambulatory and dyskinetic movements in Pitx3 mutant mice, whereas the selective 5-HT_{2AR} blocker volinsanerin had the opposite effects. These results demonstrate that Pitx3 mutant mice are a convenient and valid mouse model to study the compensatory 5-HT upregulation following the loss of the nigrostriatal DA projection and that the upregulated 5-HT_{2AR} function in the DA deficient dorsal striatum may enhance both normal and dyskinetic movements.

Keywords

L-3,4-dihydroxyphenylalanine (L-dopa); 5-HT_{2A} receptor; basal ganglia; dopamine; dyskinesia; Parkinson's disease; qRT-PCR; striatum

1. Introduction

The striatum is critical to movement control (Albin et al. 1989; DeLong 1990). In addition to the dense DA innervation, it receives a modest 5-HT innervation that provides the endogenous agonist for 5-HT receptors (Soghomonian et al., 1987; Steinbusch, 1981; Van Bockstaele et al., 1996). Histochemical studies in animal brains and postmortem human

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brains show that 5-HT₂ARs are a main 5-HT receptor type expressed in the striatum (Hall et al., 2000; Hoyer et al., 1986; López-Giménez et al., 1999; Mengod et al., 1997; Pazos et al., 1985) and in the medium spiny neurons (MSNs) (Cornea-Hebert et al., 1999; Laprade et al., 1996; Li et al., 2004; Rodríguez et al., 1999). Electrophysiological studies indicate that activation of 5-HT₂ receptors, likely 5-HT₂ARs, may increase MSN activity by inhibiting a background potassium conductance (North and Uchimura, 1989). Therefore, changes in 5-HT innervation and 5-HT₂AR expression may contribute to the function and dysfunction of the striatum and consequently movement control.

In Parkinson's disease (PD), the massive DA innervation to the striatum is severely lost, particularly in the dorsal striatum (Hornykiewicz, 2001), leading to potential homeostatic compensatory changes in other neurotransmitter systems (Cenci and Konradi, 2010; Gerfen et al., 1990; Greene, 2012). Toxin lesions of the nigrostriatal DA system during neonatal period or adulthood can induce 5-HT hyperinnervation in the striatum (Brown and Gerfen, 2006; Gaspar et al., 1993; Kostrzewa et al., 1998; Maeda et al., 2003; Rozas et al., 1998; Zeng et al., 2010; Zhou et al., 1991). Postmortem studies indicate that in late stage PD brains, the 5-HT innervation in the striatum may be decreased (Kish et al., 2008; Raisman et al., 1986), potentially depriving MSNs a compensatory response. Reported changes in 5-HT₂AR expression in the striatum in animal PD models are more variable, while data in human PD patients are not available. In rodents, lesions of the nigrostriatal DA system were suggested to increase 5-HT₂AR gene expression (Basura and Walker, 1999; Numan et al., 1995; Zhang et al., 2007), although contradicting findings have also been reported (Huot et al., 2011a; Li et al., 2010). Studies in non-human primate PD models indicated that toxin lesions of the DA system did not increase 5-HT₂AR expression in the striatum until the appearance of L-dopa-induced dyskinesia (Huot et al., 2012; Riahi et al., 2011). In rodents, it was reported that toxin lesions of the nigrostriatal DA system increased 5-HT₂AR expression selectively in the direct pathway DA D1 receptor-expressing medium spiny neurons (D1-MSNs) (Laprade et al., 1996), indicating a potential importance of 5-HT₂ARs in promoting movements due to the established motor-promoting role of D1-MSNs (Bateup et al., 2010; Kravitz et al., 2010). To provide a convenient mouse model for the study of 5-HT compensatory responses after DA loss, we set out to characterize the potential changes in 5-HT innervation and 5-HT₂AR expression in the striatum in transcription factor Pitx3 gene mutant mice that have a selective, severe and consistent DA deficiency in the dorsal striatum and produce robust and consistent L-dopa motor responses (Ding et al., 2007; Smits et al., 2006). We also hypothesized that in the dorsal striatum in Pitx3 mutant mice, the 5-HT₂AR gene expression and function may be increased to compensate for the lost DA excitation, a homeostatic response seeking to maintain normal motor activity. Additionally, since the basal ganglia motor circuit is in a dyskinesia-prone state after chronic severe DA loss, the 5-HT₂AR-induced excitation may also increase dyskinetic movements.

2. Results

2.1. Selective dopamine denervation in the dorsal striatum in PitxHomo mice

We first verified our locally bred Pitx3 mutant mice according to the PCR-based procedure provided by The Jackson Laboratory (see section 5.1 in Materials and Methods). As shown in Fig. 1A, our genotyping procedure clearly identified Pitx3 wild type (PitxWT) mice, Pitx3 heterozygous mice and Pitx3 homozygous mutant (PitxHomo) mice. PitxHomo mice were also identified by their being aphakia. Next, we screened the DA innervation in the striatum to further ensure that our PitxHomo mice have a DA denervation profile in the striatum similar to that reported in the literature (Beeler et al., 2009; Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003). Using tyrosine hydroxylase (TH) immunoreactivity as a marker for DA axons, we observed a gradient loss of DA innervation with a virtually complete DA fiber loss in the dorsal striatum, a substantial DA fiber loss in

the middle striatum, and only a moderate DA fiber loss in the ventral striatum (Fig. 1B–C). To quantify the density of DA axons, we estimated the area fraction ratio of the total area of the DA axon terminals to the area of the region of interest in the coronal sections cut around Bregma 1.1 mm (Paxinos and Franklin, 2001). As shown in Fig. 1D, in 4 PitxWT mice, the ratio was similar, around 65 % in the different striatal subregions. The ratio was < 1 because of the gaps between DA axon terminals and also the areas occupied by cortical axon bundles and striatal cell somata. In 4 PitxHomo mice, the ratio was vastly different in the different striatal subregions: only 0.42 ± 0.03 % in the dorsal striatum, but 6.78 ± 0.30 % and 26.51 ± 1.82 % in the middle and ventral striatum, respectively. The corresponding DA fiber loss was 99%, 90 % and 59 % in these 3 striatal subregions. Thus, we concluded that in our locally bred PitxHomo mice, the DA innervation was severely reduced in the dorsal striatum whereas the ventral striatum retained a substantial amount of residual DA axons, consistent with the published results in young adult PitxHomo mice (van den Munckhof et al., 2003, 2006).

2.2. 5-HT hyperinnervation in the striatum of PitxHomo mice

We next investigated the potential compensatory 5-HT hyperinnervation in the DA-deficient striatum in PitxHomo mice. We labeled 5-HT axons by immunostaining serotonin transporter protein (SERT) that is selectively expressed in 5-HT neurons in adult animals (Nielsen et al., 2006; Verney et al., 2002; Zhou et al., 1998). As shown in FIG. 2A,B, compared with PitxWT mice, we observed a substantially increased 5-HT innervation in the dorsal striatum in PitxHomo mice. This increase was also significant in the middle striatum but minimal in the ventral striatum. To quantify these changes, we calculated the ratio of the area covered by 5-HT axon terminals to the total area of the region of interest in the coronal sections cut around Bregma 1.1 mm (Paxinos and Franklin, 2001). As illustrated in Fig. 2C, in 5 PitxWT mice, the area fraction ratio was 2.32 ± 0.14 %, 3.21 ± 0.19 %, and 6.04 ± 0.35 % in the dorsal, middle, and ventral striatum, respectively. This pattern is consistent with the established gradient 5-HT innervation in striatal subregions (Soghomonian et al., 1987). In 5 PitxHomo mice, the area fraction ratio was 7.13 ± 0.36 %, 6.96 ± 0.38 %, and 6.93 ± 0.39 % in the dorsal, middle, and ventral striatum, respectively. These results indicate a 3- and 2-fold increase in 5-HT axons in the dorsal and middle striatum in PitxHomo mice.

2.3. Increased 5-HT_{2A}R gene expression in the DA-deficient dorsal striatum in PitxHomo mice

The substantial 5-HT hyperinnervation described in the preceding section indicates an increased endogenous 5-HT release in the dorsal striatum in PitxHomo mice. Now, we ask this question: is the expression of 5-HT_{2A}Rs, a main 5-HT receptor type in the striatum (Brown and Gerfen, 2006; Huot et al., 2011a; Pazos et al., 1985), also increased in PitxHomo mice? 5-HT_{2A}Rs are expressed in the medium spiny neurons in the striatum (Cornea-Hebert et al., 1999; Rodriguez et al., 1999). We reasoned that in the severely DA-denervated dorsal striatum in PitxHomo mice, 5-HT_{2A}R expression may be increased to compensate for the DA loss and may undergo further changes during L-dopa treatment. To test this idea, we set out to determine the 5-HT_{2A}R mRNA levels in the striatum of PitxHomo mice. As a first approximation, we initially compared the optical density of the gel signal of conventional RT-PCR amplicon for 5-HT_{2A}R mRNA in the dorsal striatum in drug-naïve PitxHomo mice and drug-naïve PitxWT mice. Using β -actin mRNA as the internal control, we found that the gel signal for 5-HT_{2A}R mRNA was consistently stronger in PitxHomo mice than in WT mice (Fig. 3A). The optical density of the 5-HT_{2A}R mRNA gel signal in the dorsal striatum in 6 PitxHomo mice was 2.7 ± 0.2 fold of that in 6 PitxWT mice (Fig. 3B), indicating an increased 5-HT_{2A}R mRNA expression in the dorsal striatum of the DA deficient PitxHomo mice.

Next, we used quantitative RT-PCR (qRT-PCR) to more accurately compare the 5-HT_{2A}R mRNA levels in PitxHomo mice and PitxWT mice. As illustrated in Fig. 4A, the 5-HT_{2A}R mRNA level in the DA-denervated dorsal striatum in saline-injected or baseline PitxHomo mice was 2.6 ± 0.1 -fold of that in the dorsal striatum of saline-injected or baseline PitxWT mice (Fig. 4A), consistent with our conventional qualitative RT-PCR results (Fig. 3). In contrast, 5-HT_{2A}R mRNA levels in the ventral striatum were not different between PitxHomo mice and PitxWT mice (Fig. 4B), indicating that the increase in 5-HT_{2A}R gene expression was specific to the DA-denervated dorsal striatum in PitxHomo mice.

We also examined whether L-dopa treatment affects 5-HT_{2A}R gene expression in the dorsal striatum in PitxHomo mice. We gave one group of PitxHomo mice 2 injections/day of 4 mg/kg L-dopa plus 5 mg/kg peripheral dopa decarboxylase inhibitor benserazide and another group of age-matched PitxHomo mice 5 mg/kg benserazide to control for potential benserazide effects. Age-matched PitxWT mice received 2 saline injections per day to control for any potential natural changes in the 5-HT_{2A}R mRNA level during the 20-day period of experimentation. The dose of 4 mg/kg L-dopa was chosen because it consistently stimulated motor activity (described in the next section). Four mg/kg L-dopa is within the clinical L-dopa dose range (Standaert and Young, 2006). Under these conditions, on day 1 of treatment, after 1 injection of L-dopa, the 5-HT_{2A}R mRNA level in the dorsal striatum in the PitxHomo mice was reduced to 2.2 ± 0.2 -fold of that in PitxWT from 2.6 ± 0.1 -fold before L-dopa treatment ($n = 6$ mice for each group, mice killed 35–40 min after injection) (Fig. 4A). Further decrease was observed with continued L-dopa treatment, reaching the lowest level on day 4 after 7 injections of L-dopa, with the 5-HT_{2A}R mRNA level in PitxHomo mice decreasing to 1.7 ± 0.1 -fold of that in PitxWT. The statistical significance of the L-dopa treatment effect was detected by one-way ANOVA combined with post hoc Newman-Keuls test, indicating a partial normalization of 5-HT_{2A}R mRNA expression (Fig. 4A). However, as the L-dopa treatment continued, the level of 5-HT_{2A}R mRNA returned to the pre-treatment level on days 10, 14 and 20 (Fig. 4A) and remained there on day 40 (not shown). In these same PitxHomo mice, the 5-HT_{2A}R mRNA level in the ventral striatum was not different from that in PitxWT mice, before and during the twice a day 4 mg/kg L-dopa treatment (Fig. 4B).

In control experiments, neither benserazide (5 mg/kg) (Fig. 4C) nor saline injection affected the 5-HT_{2A}R mRNA level in the dorsal striatum in PitxHomo mice. Additionally, no significant difference was detected in the 5-HT_{2A}R mRNA level in the dorsal striatum between PitxHomo mice and PitxWT mice before and during 4 mg/kg L-dopa treatment (Fig. 5). Finally, mRNA levels for dopamine D₁ and D₂ receptors at baseline and during the same 4 mg/kg L-dopa treatment were also similar in the dorsal striatum between PitxWT and PitxHomo mice (Fig. 5). Taken together, the data presented in the preceding sections indicate a substantially upregulated 5-HT_{2A}R gene expression selectively in the dorsal striatum in PitxHomo mice that was only partially normalized during the first week of twice a day 4 mg/kg L-dopa treatment. In the following sections, we will examine the potential motor effects of the increased HT_{2A}R gene expression and 5-HT hyperinnervation in the dorsal striatum in PitxHomo mice.

2.4. 5-HT_{2A} agonism increases and 5-HT_{2A} antagonism decreases normal movements and dyskinesia in PitxHomo mice

Enhanced 5-HT_{2A}R excitation may help trigger spike output in direct pathway MSNs that may increase both the baseline and L-dopa-induced motor activity including dyskinesia. The 5-HT hyperinnervation is likely to produce an increased endogenous 5-HT level that may induce detectable motor effects. To test these possibilities, we investigated the motor effects of the 5-HT_{2A} agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) and the selective 5-HT_{2A}R antagonist volinanserin (VOL) (Bishop et al., 2004; Kehne et al., 1996; Knight et al., 2004).

The speed of horizontal movements was used as a measure for normal movements. Horizontal movements are the most common natural motor activity in rodents and are equivalent to walking in humans (Kuoppamäki et al., 2007; Fox and Lang, 2008; Stockwell et al., 2008; Taylor et al., 2010; Moretti et al., 2011). The horizontal movement speed was calculated by dividing the horizontal travel distance by the time the mouse was in horizontal positions, recorded by a locomotor monitor, thus excluding the interference from the time used for rearing and dyskinesia. The dyskinetic movements were video-recorded and then identified and quantified by off-line manual analysis. Based on our pilot experiments and the doses used in published studies (Bishop et al., 2004; Taylor et al., 2006), we used 1 mg/kg DOI and 0.2 mg/kg VOL that elicited motor effects in PitxHomo mice but were without detectable effect in PitxWT mice, indicating specific effects via 5-HT₂ or 5-HT_{2A} receptors, assuming that non-specific effects are similar in PitxWT and PitxHomo mice. Also, during the first several days of L-dopa treatment in the DA-deficient PitxHomo mice, the motor effects of L-dopa increased with each injection due to DA sensitization mechanisms (Nadjar et al., 2009; Schwarting and Huston, 1996), leading to a non-normal distribution of motor activity (Fig. 6). Thus, the motor activity data during the first week were analyzed separately.

In PitxWT mice, IP injection of DOI at 1 mg/kg (twice a day, behavioral monitoring during the morning injection only) did not affect the speed of normal ambulatory horizontal movements or induced any dyskinetic movement (Table 1). In contrast, in PitxHomo mice, DOI (1 mg/kg, IP injection) increased the speed of normal horizontal movements by 23.1% on average during days 8–20 of the treatment (Table 1). Simultaneously, DOI also increased the total duration of the spontaneous dyskinetic events by 19.2% in PitxHomo mice (Table 1). These DOI effects were prevented when 0.2 mg/kg VOL, a highly selective 5-HT_{2A} antagonist, was injected 15 min before DOI. These results indicate that DOI had detectable motor-stimulating effects in PitxHomo mice, likely by activating the upregulated 5-HT_{2A}Rs.

Next, we tested whether the motor effects of DOI and L-dopa were additive, based on the fact that both D1Rs and 5-HT₂Rs can increase D1-MSN activity that in turn promotes movements. We found that while without any apparent effect in PitxWT mice, injection of 4 mg/kg L-dopa with 5 mg/kg benserazide increased the speed of normal horizontal movements by 120% in PitxHomo mice, (Fig. 6A, Table 1). In a separate group of PitxHomo mice, L-dopa injection (4 mg/kg together with 5 mg/kg benserazide) increased the total duration of dyskinetic movements by 1650% (Fig. 6B, Table 1). This large percentage increase in dyskinetic duration was due to the small baseline dyskinetic duration. In another 2 separate groups of PitxHomo mice, combined injection of DOI (1 mg/kg) and L-dopa (4 mg/kg plus 5 mg/kg benserazide) induced a 24% faster speed of normal horizontal movements and a 25% longer dyskinesia duration than L-dopa alone (Fig. 6A, B, Table 1). These DOI effects were prevented when 0.2 mg/kg VOL was injected 15 min before DOI, indicating that these DOI effects were mediated by 5-HT_{2A}Rs. Additionally, combined VOL and L-dopa injection in 2 separate groups of PitxHomo mice induced a 17.9% slower speed of normal horizontal movements and a 18.4% shorter dyskinesia duration than L-dopa alone (Fig. 6A, B, Table 1). When injected alone, VOL decreased the speed of normal horizontal movements by 16.3% and the duration of dyskinetic movements by 15.4% in PitxHomo mice (Table 1). Combined injection of L-dopa with DOI or VOL was without overt effect in PitxWT mice (Table 1).

Finally, we noticed that the motor effects of DOI and VOL were smaller during treatment days 2–4 (Fig. 6A, B). We extracted DOI and VOL effects during days 2–4 and during days 8–20 by subtracting the L-dopa effects. The average DOI effect on normal movement speed was 2.78 ± 0.05 cm/10 s during days 2–4 and 3.80 ± 0.08 cm/10 s during days 8–20 ($p < 0.01$,

unpaired t-test), and the average VOL effect was -2.25 ± 0.09 cm/10 s during days 2–4 and -2.82 ± 0.07 cm/10 s during days 8–20 ($p < 0.01$, unpaired t-test). These results indicate a smaller 5-HT_{2A}R-derived motor-stimulating effect during L-dopa treatment days 2–4, consistent with our qPCR data showing a partial and transient normalization of 5-HT_{2A}R mRNA expression on L-dopa treatment days 2 and 4.

3. Discussion

Our main findings are: (1) both the 5-HT innervation and 5-HT_{2A}R mRNA expression were substantially increased in the severely DA-deficient dorsal striatum in PitxHomo mice, demonstrating that these mice are a convenient mouse model to study the compensatory 5-HT upregulation after DA loss; (2) the upregulated 5-HT_{2A}R function contributed to the baseline and also L-dopa-induced motor activity in these mice. In the following sections, we will discuss our main findings and also the technical limitations and concerns.

3.1. Chronic DA loss causes 5-HT hyperinnervation and increased 5-HT_{2A}R gene expression in the dorsal striatum in PitxHomo mice

In the brain, the transcription factor Pitx3 gene is selectively expressed in midbrain DA neurons and its null mutation causes the death of nigral DA neurons shortly after their birth (Smits et al., 2006). Our immunohistochemical data demonstrated that in our locally produced PitxHomo mice, there was a virtually complete (99%) DA loss in the dorsal striatum and the severe (90%) DA loss in the middle striatum, consistent with literature reports on Pitx3 gene mutant mice (Nunes et al., 2003; van den Munckhof et al., 2003; Smidt et al., 2004). These severe DA losses lead to a 3-fold and 2-fold increase in 5-HT innervation in the dorsal and middle striatum, respectively. In the ventral striatum in these mice, the DA loss was about 58% and the 5-HT innervation was increased only slightly without reaching statistical significance. These observations are consistent with the reported 5-HT hyperinnervation and increased 5-HT release after toxin-induced lesion (>80%) of the nigrostriatal DA system (Basura and Walker, 2000; Brown and Gerfen, 2006; Gaspar et al., 1993; Kostrzewa et al., 1998; Zeng et al., 2010) and also with the increased SERT-binding sites in the dorsal striatum in PitxHomo mice (Smits et al., 2008). Thus, our present study provides unequivocal evidence that 5-HT hyperinnervation occurs in the PitxHomo mice where the DA innervation in the dorsal striatum is depleted genetically.

In addition to the robust 5-HT hyperinnervation, our qRT-PCR results clearly show that the 5-HT_{2A}R mRNA level in the dorsal striatum in PitxHomo mice was 2.6-fold of that in age-matched PitxWT mice. In contrast, the 5-HT_{2A}R mRNA level in the ventral striatum was not different between PitxHomo mice and PitxWT mice. Taken together, these results indicate a specific compensatory increase in 5-HT_{2A}R gene expression in the severely DA-denervated dorsal striatum in PitxHomo mice. Our results are consistent with published reports that showed that toxin-induced DA denervation, particularly inflicted during neonatal period, upregulated the 5-HT_{2A}R gene expression and functionality (Basura and Walker, 1999; Bishop et al., 2004; Brown and Gerfen, 2006; Zhang et al., 2007). The molecular mechanisms underlying the upregulation of 5-HT_{2A}R gene expression are not clear. However, loss of the massive DA innervation in the striatum is known to alter gene expression (El Atifi-Borel et al., 2009; Gerfen et al., 1990; Konradi et al., 2004; Meurers et al., 2009; Scholz et al., 2008; Valastro et al., 2008; Yacoubian et al., 2008). Therefore, an increase in 5-HT_{2A}R gene expression may be a direct compensatory response and also an indirect consequence of multiple DA loss-induced molecular changes (Cenci, 2010; Greene, 2012; Grünblatt et al., 2011). We did not, however, detect any significant change in 5-HT_{2C}R mRNA in the striatum of PitxHomo mice. We speculate that this is because 5-HT_{2C}Rs may be mainly expressed in non-MSNs in the striatum (Bonsi et al., 2007) that may not undergo major compensatory changes. Certainly, receptor functionality may be

altered without changes in mRNA levels. DA D1 and D2 receptors clearly fit this scenario, because even though their mRNA levels were not changed, their functionality was increased, as reflected in supersensitive D1 and D2 receptors and also supersensitive L-dopa effects (Gerfen, 2010; Hwang et al., 2005; Nadjar et al., 2009; van den Munckhof et al., 2006).

Additionally, our data indicate that L-dopa treatment (2 injections/day of 4 mg/kg L-dopa) induced a partial normalization of 5-HT_{2A}R gene expression during the first week of the treatment. With continued L-dopa treatment, 5-HT_{2A}R mRNA returned to and remained at the pre-treatment high level. This partial normalization of 5-HT_{2A}R gene expression was temporally associated with a smaller motor effects of 5-HT_{2A}R ligands during the first week, indicating a likely causative relation. The mechanisms underlying the transient nature of L-dopa's normalizing effect were not investigated. We speculate that a key factor may be that 2 injections/day of 4 mg/kg L-dopa can neither supply enough DA nor mimic the rich dynamics of the physiologically released DA that is controlled by the extremely large number of DA axon terminals, DA neuron firing and transporter-mediated uptake (Rice et al., 2011). Therefore, during the first few day of L-dopa treatment, the chronically DA-deprived DA signaling system in the dorsal striatum was partially normalized even with small amounts of DA. But soon, the DA signaling system detected the inadequacy of the exogenously supplied DA, and consequently abnormalities in gene expression returned. This speculation is consistent with the fact that even with the best possible dopaminergic therapy, the structure and function of the basal ganglia continue to deteriorate in PD patients (Katzenschlager et al., 2008; Olanow et al., 2009), indicating that the exogenous DA does not match the intensity and dynamics of the DA axon-supplied DA.

Additionally, the robust and consistent 5-HT hyperinnervation and upregulated 5-HT_{2A}R gene expression in the striatum in PitxHomo mice demonstrate that these mice are a convenient mouse model to further study the compensatory 5-HT responses. Compared with toxin-lesioned mice, PitxHomo mice are advantageous because their DA loss is nature-made and consistent among individual mice that avoids the time-consuming toxin injection surgery that often leads to variable DA lesions (Francardo et al., 2011; Putterman et al., 2007; Schwarting and Huston, 1996; Stanic et al., 2003; Thiele et al., 2011).

3.2. Increased 5-HT innervation and 5-HT_{2A}R expression enhance both normal and dyskinetic movements

5-HT hyperinnervation can convert more L-dopa to DA, besides releasing more 5-HT, that in turn may stimulate MSNs (Basura and Walker, 2000; Gil et al., 2010; Yamada et al., 2007). Increased 5-HT_{2A}R expression may increase D1-MSN activity (Laprade et al., 1996; North and Uchimura, 1989). Indeed, we observed that while having no detectable effect in PitxWT mice, the selective 5-HT_{2A}R antagonist VOL reduced basal and L-dopa-induced normal and dyskinetic motor activity in PitxHomo mice. These results indicate an enhanced tonic endogenous 5-HT_{2A}R activity, likely due to the increased 5-HT innervation and 5-HT_{2A}R expression in PitxHomo mice, particularly the increased 5-HT_{2A} expression may occur selectively in D1-MSNs (Laprade et al., 1996). We also found that 5-HT₂ class agonist DOI increased normal and dyskinetic motor activity in PitxHomo mice, while having no detectable motor effect in PitxWT mice. Furthermore, when co-administered, DOI and L-dopa increased horizontal normal movement speed and dyskinesia duration more strongly than L-dopa alone, suggesting that the upregulated 5-HT_{2A}R functionality may stimulate, additively with D1Rs, the D1-MSNs, leading to increased motor activity. These results are consistent with those of Bishop and Walker (2003), Bishop et al. (2004) and Taylor et al. (2006) who showed enhanced 5-HT_{2A}R-mediated motor activity and a potential 5-HT_{2A}R and D1R synergy in DA toxin-lesioned rats. Our results and

interpretation are also consistent with Laprade et al. (1996) who showed that 5-HT2AR expression following DA depletion was upregulated selectively in D1-MSNs.

We also made this novel observation that has not been reported in animal PD models: in PitxHomo mice, the effects of DOI or VOL (injected alone or with L-dopa) on normal and dyskinetic movements were always in the same direction. In other words, DOI increased both normal and dyskinetic movements while VOL decreased both normal and dyskinetic movements. We speculate that the positive correlation indicates that anti-parkinsonian effect and dyskinetic effect may share, at least partially, common pharmacological and signaling mechanisms, as suggested by clinical data (Nutt et al., 2010). Particularly, 5-HT2ARs and D1Rs may act together to increase D1-MSN output and increase motor activity non-selectively. These shared mechanisms may underlie the clinical observation that 5-HT2AR antagonism reduced dyskinesia and also worsened normal motor activity (Huot et al., 2011a,b).

We argue that DA loss-induced 5-HT hyperinnervation and 5-HT2AR expression upregulation are clinically relevant for the following reasons. First, postmortem examination indicates that 5-HT innervation in the striatum is decreased in late stage PD (Kish et al., 2008). The decreased 5-HT innervation may deprive the D1-MSNs a source of excitation, compounding the deterioration in basal ganglia function caused by DA loss. Second, the DA loss-induced 5-HT hyperinnervation and 5-HT2AR expression upregulation demonstrate a remarkable compensatory capability of the 5-HT system. We speculate that if a similar compensatory response is induced in the PD brain, via a novel therapeutic intervention, then the increased 5-HT axons in the striatum can release more 5-HT and convert more L-dopa to DA, thus leading to increased motor activity. Additionally, 5-HT2AR agonism may be an adjunct therapy for PD with moderate dyskinesia, because even though 5-HT2AR agonism may increase dyskinesia, PD patients prefer anti-PD effect with moderate dyskinesia over parkinsonism without dyskinesia (Hung et al., 2010).

3.4. Concerns on the suitability of studying PD-related motor activity in PitxHomo mice and other technical limitations

Since our experimental questions are related to PD and PitxHomo mice are clearly not a perfect PD model, several concerns need to be addressed. The first concern is that since the DA loss occurs early in PitxHomo mice, there may be developmental abnormalities that may confound data interpretation. While we can not rule out potential developmental abnormalities, the L-dopa-induced motor responses (increased normal and dyskinetic movements) can be fully accounted for by DA denervation supersensitivity as reported in rodent toxin PD models, PD patients and accidentally MPTP-poisoned patients (Cenci, 2010; Langston, 1985; Nadjar et al., 2009; Schwarting and Huston, 1996). Clearly, DA supersensitivity is certain to occur after severe DA loss regardless of the age of the animal.

The second concern is that PitxHomo mice do not have overt locomotor deficit or a face validity for PD such that these mice are not suitable for this study. We argue that our experimental questions on the consequences of severe DA loss match PitxHomo mice. These mice have a pre-made, consistent and severe DA loss in the dorsal striatum, mimicking a key aspect of PD pathology and thus possessing a pathological validity. But it is not a perfect match. In late stage PD brain that readily generates dyskinesia, the DA loss is often 95 to 99% in the putamen, the part of the striatum most closely related to motor control (Hornykiewicz, 2001). In PitxHomo mice, the DA loss is 99% in the dorsal striatum, but the middle part of the striatum retains ~ 10% DA. The middle striatum has motor function (Voorn et al., 2004). In an unpublished study, we have documented that when we further decrease the DA level in the striatum in PitxHomo mice by a small dose of tyrosine hydroxylase inhibitor that had no behavioral effect in PitxWT mice, PitxHomo mice became

clearly hypokinetic. This indicates that the residual DA in the middle striatum fragilely maintains the locomotor function while the severely DA deficient dorsal striatum together with the upper middle striatum may contribute critically to the generation of supersensitive DA responses including normal and dyskinetic movements.

The third concern is about the dyskinetic nature of the abnormal involuntary paw movements accompanied by involuntary vertical trunk movements in PitxHomo mice. Clinically, a precise definition for dyskinesias in human patients is lacking, but they are commonly described as abnormal (not seen under normal condition) and purposeless and involuntary (unwanted) movements (Fahn, 2005). The L-dopa-induced abnormal movements in PitxHomo mice were never seen in PitxWT mice. These movements were also apparently purposeless and involuntary, although this conclusion is impossible to confirm in mice. Therefore, the L-dopa-induced abnormal movements reported here are likely to be dyskinetic movements. Ding et al. (2007) has also answered this question positively after an extensive comparison between the L-dopa-induced abnormal movements in PitxHomo mice and the established dyskinetic movements in rodents and primates.

The fourth concern is that in addition to the robust L-dopa-induced dyskinesia, we also observed spontaneous, although infrequent, dyskinetic events. These events were also observed by Ding et al. (2007). The underlying mechanisms are not known. We speculate that these spontaneous dyskinetic events were produced by spontaneous vesicular DA release in the upper middle striatum where there is a 5-10% residual DA and the D1Rs are supersensitive such that when an occasional, large amplitude spontaneous DA release occurs, dyskinesia may be triggered. In the striatum of normal animals, spontaneous DA release can be triggered by action potentials generated in the axon terminals or cell body (Zhou et al., 2001, 2005). Similar spontaneous vesicular DA release is likely to occur in the striatum in PitxHomo mice.

Finally, in our study, DOI and VOL were injected systematically. Consequently, 5-HT₂/5-HT_{2A} receptors in multiple brain areas in PitxHomo mice might have contributed to our observed motor effects. We argue, however, that the observed effects of systemically injected DOI and VOL were likely due to the upregulated 5-HT₂ARs and 5-HT hyperinnervation in the dorsal striatum, because the same DOI and VOL treatments induced no detectable motor effect in PitxWT mice.

4. Conclusions

Our present study demonstrates that PitxHomo mice are a convenient mouse model to study the mechanisms and functional consequences of the compensatory 5-HT hyperinnervation and 5-HT₂AR expression upregulation following DA depletion in the striatum. We also found that in this mouse model, the 5-HT hyperinnervation and the enhanced 5-HT₂AR function promotes both normal and dyskinetic movements.

5. Materials and methods

5.1. Animals

Two breeding pairs of heterozygous Pitx^{+/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME), resulting in a small colony of homozygous Pitx^{-/-} (PitxHomo), heterozygous Pitx^{+/-}, and wild-type Pitx^{+/+} (PitxWT) mice. The genotypes were determined by PCR-based genotyping to identify WT, homozygotes, and heterozygotes (Fig. 1A). The genotyping primers were TTCTACCGAGGAAAGCTGGA and TGCTTTGCTGGACATGGTAG. PitxHomo mice are also aphakia and thus clearly identified. PitxHomo mice are viable and fertile. Experiments were performed in PitxHomo

and PitxWT mice. Mice had free access to food and water. All procedures were approved by The Institutional Animal Care and Use Committee of The University of Tennessee Health Science Center in Memphis, Tennessee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

5.2. Treatment regimen

L-3,4-dihydroxyphenylalanine methyl ester hydrochloride (L-dopa), benserazide hydrochloride and ($\pm$)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) were purchased from Sigma-Aldrich (St. Louis, MO). Selective 5-HT_{2A} receptor antagonist volinanserin (VOL) [(R)-(+)-a-(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidine-methanol, also known as MDL100907] was provided by the Drug Supply Program of the National Institute of Mental Health. These drugs were delivered to the mice via intraperitoneal (IP) injection. Behavioral tests were performed between 9 AM and 1 PM. DOI was dissolved in saline. VOL was first dissolved in dimethyl sulfoxide (DMSO) at high concentrations, then diluted in a solvent vehicle mix of Tween20 and Poly ethylene glycol 400 (PEG) to minimize the noxious stimulation of DMSO. The injected solution contained only 0.1% (0.02 μ L) or less DMSO that did not, when injected alone, induce any detectable response in motor activity.

Only male mice were used in this study. At the beginning of experiments, the mice (PitxHomo mice and PitxWT) were 2 months old. In the experiments to determine the effect of L-dopa on 5-HT_{2A} mRNA level, mice received either saline injection (0.02 mL), benserazide injection (5 mg/kg in 0.02 mL) or mixed injection (0.02 mL) of L-dopa (4 mg/kg) and benserazide (5 mg/kg). The injection was twice a day. Some of the mice were randomly chosen and sacrificed around 35–40 min after injection during the course of treatment to harvest brain tissues; other mice were used for behavioral tests.

5.3. Monitoring and quantification of motor activity

Ambulatory normal horizontal motor activity was monitored and quantified by an Activity Monitor system (Med Associates). In PitxHomo mice, dyskinetic events consisted mainly of characteristic stereotypic abnormal paw movements accompanied by stereotypic vertical trunk movements. These dyskinetic movements were identical to the dyskinetic events described and video-recorded in the literature (Ding et al., 2007). As used by Ding et al. (2007), dyskinesia duration provides a direct description of the dyskinesia in PitxHomo mice with bilateral DA loss, whereas the dyskinesia rating scale is suitable for unilateral DA-lesioned PD models (Lundblad et al. 2005). The duration of dyskinetic events was manually counted according to the method of Ding et al. (2007). We placed individual mice in home cages and video-recorded their motor activity in home cages before and after injection of L-dopa or other ligands. A 10-min video 30 min after L-dopa injection was analyzed off-line. When necessary, the videos were viewed at slow motion or frame by frame. The duration of individual dyskinetic events including one-paw, two-paw and three-paw dyskinetic movements was manually counted and added together to produce the total dyskinetic duration.

5.4. Immunohistochemistry

Immunohistochemistry followed published procedures (Zhou et al., 2009). Under deep anesthesia, drug naïve mice were intracardially perfused with a cold phosphate buffered saline (PBS) and then 4% paraformaldehyde in a phosphate buffer (PB). The brains were fixed in the same fixative at 4 °C overnight. Coronal brain sections (50 μ m in thickness) were cut on a Leica VT1200S vibratome. Free-floating sections were incubated with 2% fat-free milk, 1% bovine serum albumin (BSA), and 0.4% Triton X-100 in the PBS for 1 h at room temperature to block nonspecific binding and permeate the cell membrane,

respectively. After thorough rinsing, the free-floating sections were incubated for 48 h at 4°C with the primary antibodies (see below) and then rinsed in the PBS, followed by incubating with the secondary antibodies (see below) for 3 h at room temperature in the dark. Both the primary and secondary antigen-antibody reactions occurred in the PBS containing 3% normal donkey serum, 1% BSA, and 0.2% Triton X-100. The two primary antibodies were a polyclonal TH antibody raised in sheep (Millipore; diluted at 1:1000) and a SERT antibody raised in goat (Santa Cruz Biotechnology; diluted at 1:800). The secondary antibodies were: (1) donkey anti-sheep IgG antibody, conjugated with green Alexa Fluor 488 (diluted at 1:200; Invitrogen), used for labeling TH, and (2) donkey anti-goat IgG antibody, conjugated with red Alexa Fluor 568 (diluted at 1:200), used for labeling SERT. Fluorescence images were acquired on a Zeiss 710 confocal laser scanning microscope, using identical excitation laser power, pinhole and gain for 2 groups of mice. Images were analyzed and quantified by ImageJ (<http://rsbweb.nih.gov/ij/>). Specifically, the Analyze/Measure function of ImageJ was used to estimate the fractional area, that is, the area covered by immunostained DA or 5-HT axon terminals relative to the total area, providing a measure on the density of these axon terminals.

5.5. RNA isolation from live coronal striatal slices

To extract RNA from live striatal neurons, we used the tissue cutting method used for electrophysiology (Ding et al., 2011a,b; Zhou et al., 2008, 2009). Briefly, under isoflurane anesthesia, mice were decapitated and their brains were quickly dissected out, and immersed in the ice-cold oxygenated cutting solution for 2.0 min. The high sucrose cutting solution contained (in mM): 220 sucrose, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂, 20 D-glucose, with pH maintained at 7.4 by continuously bubbling with 95% O₂ and 5% CO₂. Coronal brain slices (400 µm thickness) containing the striatum were cut in the ice-cold oxygenated high-sucrose cutting solution using the Leica VT1200S vibratome. Middle striatal slices were used whereas anterior and posterior striatal sections were discarded. Under visual control, a punch of 1 mm diameter was made in the dorsal and ventral striatum in both sides of the slice, yielding 6 dorsal punches and 6 ventral punches from each mouse. The dorsal striatal punch was made immediately below the corpus callosum and the ventral striatal punch was made immediately below and lateral to the anterior commissure (see the inset of Fig. 1C1). The middle striatum was not punched because there is no landmark and difficult to locate in live tissue sections.

Total RNA in these striatal punches was extracted using a RNA isolation kit (RNAeasy mini kit for lipid tissue, Qiagen), followed by treatment with RNase-free DNase I to remove genomic DNA contamination. Total RNA content in our samples was around 80 ng/µL, as estimated by a Nanodrop spectrophotometer system. To minimize potential degradation, the total RNA samples were immediately reverse transcribed to make cDNA. Reverse transcription was carried out by using a high capacity cDNA reverse transcription kit (Applied Biosystems) following manufacturer's protocol and also the procedures used in our previous studies (Ding et al. 2011a,b; Ding and Zhou, 2011; Zhou et al., 2008, 2009). cDNA samples were stored at -20°C for a few days before PCR amplification.

5.6. Conventional qualitative RT-PCR and gel electrophoresis

The synthesized cDNAs were amplified using a hot-start Platinum PCR SuperMix (Invitrogen) as detailed in our previous studies (Ding et al. 2011a,b; Ding and Zhou, 2011; Zhou et al., 2008, 2009). Briefly, 10 µl of the cDNA sample was amplified for 40 cycles in the presence of the specific primer pairs for 5-HT_{2A}R and β-actin cDNAs (see Table 2 for primer pair sequences). The thermal cycling protocol was 5 min at 95 °C for the initial denaturation, then 40 cycles of 30 s at 94 °C to denature, 30 s at 55 °C to anneal, and 50 s at 72 °C to extend, followed by a final extension step at 72°C for 5 min.

The amplicons from PCR amplification were separated by 1.5% agarose gel electrophoresis, visualized by Gelgreen under UV light and photographed on an AlphaImager gel imaging and analysis system (Alpha Innotech). The positive bands were then cut out and the DNA fragments were extracted using a Qiagen extraction kit. The extraction products were sequenced at the Molecular Resource Center of The University of Tennessee Health Science Center in Memphis, Tennessee and positively identified with the published 5-HT2A sequence.

5.7. Quantitative real-time PCR (qRT-PCR)

Levels of mRNAs for 5-HT2AR and 5-HT2CR and β -actin (as internal control) were analyzed by using a Roche Light Cycler 480 quantitative real-time PCR system and the Universal Probe Library (UPL) probes and the associated primers (Roche Applied Science, Indianapolis, IN). The sequences for these primers are listed in Table 3. In our qPCR protocol, the final concentration of each qRT-PCR primer was 0.1 μ M, the concentration of each probe was 0.1 μ M, and the reaction volume was 10 μ l. The cycling conditions were 95°C for 5 min for the initial denaturation, followed by 45 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 20 s. Using β -actin mRNA as the internal control, mRNAs for 5-HT2AR and 5-HT2CR were quantified employing the comparative crossing point (C_p) method in the form of $2^{-[\Delta C_p]}$ (Ding et al., 2011a,b; Luu-The et al., 2005). The C_p values of the real-time fluorescence intensity curve were calculated using the second derivative method (Luu-The et al., 2005). The calculation was performed by the built-in software on the Light Cycler 480. For 5-HT2AR mRNA level in the striatum in PitxWT mice, $[\Delta C_p]_{C_p,5HT2A,PitxWT} = C_{p,5HT2A,PitxWT} - C_{p,\beta-actin,PitxWT}$. For 5-HT2AR mRNA level in the striatum in PitxHomo mice, $[\Delta C_p]_{C_p,5HT2A,PitxHomo} = C_{p,5HT2A,PitxHomo} - C_{p,\beta-actin,PitxHomo}$.

4.8. Statistics

Descriptive statistics was used to obtain mean and standard error (SE). The unpaired t-test was used to make comparisons between PitxWT and PitxHomo mice and between different groups of PitxHomo mice. Two-way ANOVA was used to determine the potential genotype X treatment interaction. One-way ANOVA combined with post hoc Newman-Keuls test was used to compare the effects of different treatments in PitxHomo mice. Use of these different tests was noted in the text and figure legends. $p < 0.05$ was considered statistically significant. Calculation was performed using Origin (OriginLab, Northampton, MA) and StatMost (Dataxiom Software, Los Angeles, CA).

Acknowledgments

This work was supported by The National Institutes of Health (NIH) grants R01NS058850 and R03NS076960. The authors thank Dr. Hao Chen for advice on statistics.

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The dorsal striatum in Pitx3 mutant mice has a pre-made and severe dopamine loss

Both 5-HT innervation and 5-HT_{2A}R gene expression are increased in the dorsal striatum in Pitx3 mutant mice

Pitx3 mutant mice are a convenient mouse model to study homeostatic 5-HT responses

The upregulated 5-HT_{2A}R function enhances both normal and dyskinetic Movements

Fig. 1. Profound DA loss in the dorsal striatum in PitxHomo mice. DA axons were labeled by tyrosine hydroxylase (TH) immunostain. Images were 2 μ thick single confocal optical sections. **(A)** Genotyping of Pitx mice. PCR-based genotyping was used to identify Pitx WT, Pitx homozygotes (Homo), and Pitx heterozygotes (Hetero). **(B)** Typical intense DA axons in dorsal **(B1)**, middle **(B2)**, and ventral **(B3)** striatum in PitxWT mice. **(C)** DA axons in dorsal **(C1)**, middle **(C2)**, and ventral **(C3)** striatum in PitxHomo mice. Note that the dorsal striatum has very few DA axons. The inset in **C1** is a Nissl-stained coronal section showing our working demarcation of the dorsal, middle and ventral striatum used for the convenience

of describing our results. Based on our knowledge, a precise demarcation of these regions does not exist. #, anterior commissure. (**D**) DA axon density was estimated by calculating the ratio of the total of DA axon terminals to the area of the region of interest in the coronal sections from 4 PitxWT mice and 4 PitxHomo mice. All sections were from the anterior part of the striatum around Bregma 1.1 mm (Paxinos and Franklin, 2001). Bars are the mean of the 4 mice. Dots are the means of the values obtained in each of the 4 mice in each group. **, $p < 0.01$, unpaired t-test.

Fig. 2. 5-HT hyperinnervation in the dorsal striatum in PitxHomo mice. DA axons were labeled by 5-HT transporter (SERT) immunostain. Images were 2 μ thick single confocal optical sections. (**A: A1, A2, A3**) Typical 5-HT axons in dorsal, middle and ventral striatum in PitxWT mice. (**B: B1, B2, B3**) Typical 5-HT axons in dorsal, middle and ventral striatum in PitxHomo mice. (**C**) 5-HT axon density was estimated by calculating the ratio of the total area of 5-HT axon terminals to the area of the region of interest in the coronal sections in 5 PitxWT mice and 5 PitxHomo mice. All sections were from the anterior part of the striatum around Bregma 1.1 mm (Paxinos and Franklin, 2001). Bars are the mean of the 5 mice. Dots

are the means of the values obtained in each of the 5 mice in each group. **, $p < 0.01$, unpaired t-test. The 5-HT innervation in the ventral striatum was slightly higher in PitxHomo mice than in PitxWT mice, but $p = 0.2$, t-test.

Fig. 3.

Conventional PCR indicates an increased 5-HT2AR mRNA level in the dorsal striatum in PitxHomo mice. **(A)** While the PCR amplicon gel signal for β -actin (399 bp) is similar for the dorsal striatum in PitxWT and PitxHomo mice, the PCR amplicon gel signal for 5-HT2AR (266 bp) is clearly stronger in the dorsal striatum in the PitxHomo mice than in PitxWT mice. **(B)** Optical density of the gel signal for 5-HT2AR mRNA in the dorsal striatum in 6 pairs of 2-month old PitxHomo mice and PitxWT mice. The beta actin-normalized 5-HT2AR mRNA in PitxHomo mice was normalized to the beta actin-normalized 5-HT2AR mRNA in PitxWT mice. Bars are the respective means of the 2 groups of mice. Dots are the means of the normalized values in individual mice. **, $p < 0.01$, unpaired t-test.

A Changes in 5-HT_{2A}R mRNA level in dorsal striatum in PitxHomo mice (Normalized to age-matched saline-treated WT)

B L-dopa had no effect on 5-HT_{2A}R mRNA level in ventral striatum (PitxHomo normalized to PitxWT)

C Benserazide had no effect in dorsal striatum (PitxHomo normalized to PitxHomo)

Fig. 4. qRT-PCR determination of 5-HT_{2A}R mRNA levels in the striatum in PitxHomo mice before and during L-dopa treatment. (A) 5-HT_{2A}R mRNA levels were normalized to age-matched saline-treated PitxWT mice. The respective baseline 5-HT_{2A}R mRNA levels in PitxWT and PitxHomo mice that received no injection were identical to these in PitxWT and PitxHomo mice that received saline injection. L-dopa injection was twice a day at 4 mg/kg plus 5 mg/kg benserazide. On day 1, the mice were sacrificed after the 1st injection; on day 2, after the 3rd injection; and so on. 5-HT_{2A}R mRNA levels in the dorsal striatum in PitxHomo mice was elevated before L-dopa treatment, partially normalized during the first

week, and returned to the pre-treatment level on day 10. Unpaired t-test indicated a higher 5-HT2AR mRNA level at baseline or during saline treatment in the dorsal striatum PitxHomo mice than that in PitxWT mice. One-way ANOVA detected a statistically significant difference in the 5-HT2AR mRNA levels on these different days ($F_{(7,40)} = 5.32$, $p < 0.01$), and post hoc Newman-Keuls test detected a L-dopa treatment effect on days 2 and 4 comparing with day 0. *, $p < 0.05$, **, $p < 0.01$. The 5-HT2AR mRNA level in non-injected PitxHomo mice was identical to that in saline-injected PitxHomo mice. **(B)** In these same mice (i.e. the tissue samples were from the same mice used in **A**), 5-HT2AR mRNA levels in the ventral striatum were not different between PitxHomo mice and PitxWT mice at baseline or during the same L-dopa treatment regimen. $p > 0.05$ for unpaired t-test for comparing baseline 5-HT2AR mRNA levels in PitxWT and PitxHomo mice and one-way ANOVA ($F_{(4,25)} = 0.122$) for comparing L-dopa treatment effect over the days. **(C)** Benserazide at 5 mg/kg did not affect 5-HT2AR mRNA level in the dorsal striatum in PitxHomo mice when compared to saline-treated, age-matched PitxHomo mice. $p > 0.05$, one-way ANOVA ($F_{(3,20)} = 0.636$). In **A**, **B** and **C**, each group had 6 mice for each test day.

Fig. 5.

mRNA levels of D1R, D2R and 5-HT2CR were not affected in the dorsal striatum in PitxHomo. mRNA samples were from the same tissues used for 5-HT2AR mRNA and qPCR was performed on the same plates for 5-HT2ARs. Values were normalized to age-matched PitxWT mice. No significant difference was detected before or during the 20-day 4 mg/kg L-dopa treatment. Six to 9 mice in each group. $p > 0.05$ for both unpaired t-test for comparing baseline mRNA levels in PitxWT and PitxHomo mice and one-way ANOVA ($F_{(6,35-39)} = 1.42-1.54$) for comparing L-dopa effects over the days.

Fig. 6.

Upregulated 5-HT₂ARs stimulate motor activity in PitxHomo mice. Motor activity was automatically recorded. Day 0 was the day before the first injection. Mice received 1 IP injection in the morning and another injection in the afternoon. Locomotor tests were performed in the morning everyday for the first 6 days and then every other day. **(A)** 5-HT₂AR agonist DOI (1 mg/kg) increased and 5-HT₂AR antagonist VOL (0.2 mg/kg) decreased the normal horizontal movement speed between 31–40 min after IP injection of 4 mg/kg L-dopa with 5 mg/kg benserazide. Ten mice for each group. **(B)** Effects of up-regulated 5-HT₂AR function on total dyskinesia duration in PitxHomo mice. The period of 31–40 min after injection was analyzed for dyskinetic events. The durations of individual dyskinetic events (one-paw, two-paw and three-paw) were manually counted and added together to produce the total dyskinetic duration. Note the basal dyskinesia that occurred with control vehicle injection or without any injection (not shown). Ten mice in each group. In both **A** and **B**, *, $p < 0.05$, one-way ANOVA [$F_{(2,27)} = 9-14$ for different days] and post-hoc Newman-Keuls test on measurements in individual days. ##, $p < 0.01$ for one-way ANOVA [$F_{(2,18)} = 4396$ for **A** and $F_{(2,9)} = 410$ for **B**] and post-hoc Newman-Keuls on averaged daily measurements during days 8–20; data from days 1–8 were excluded due to the non-normal distribution. Note that (1) the VOL effects on individual treatment days (L-dopa vs. L-dopa + VOL comparison) did not reach statistical significance ($p > 0.05$, post-hoc Newman-Keuls test), indicating a modest tonic 5-HT₂AR activity, (2) DOI effects on individual treatment days 2–5 did not reach statistical significance, indicating a relatively weaker 5-HT₂AR activity during these days. Additional behavioral effects are listed in Table 1.

Table 1

Normal horizontal and dyskinetic movements under different conditions

	Horizontal speed, cm/10s PitxWT	Horizontal speed, cm/10s PitxHomo	Dyskinesia duration PitxWT, s	Dyskinesia duration PitxHomo, s
Vehicle (Treatment A) *	6.67±0.05	6.55±0.04	0	9.86±0.04
DOI (Treatment B)	6.44±0.04 B vs. A: p>0.05	8.06±0.05 B vs. A: ↑23.1% P<0.01	0	11.75±0.05 B vs. A: ↑19.2% P<0.01
VOL (Treatment C)	6.38±0.04 C vs. A: p>0.05	5.48±0.03 C vs. A: ↓16.3% P<0.05	0	8.34±0.04 C vs. A: ↓15.4% P<0.05
L-dopa (Treatment D)	6.52±0.04 D vs. A: p>0.05	15.72±0.05 D vs. A: ↑120% P<0.01	0	162.65±3.55 D vs. A: ↑1650% P<0.01
DOI+L-dopa (Treatment E)	6.62±0.04 E vs. A: p>0.05	19.51±0.06 E vs. D: ↑24.1% P<0.01	0	202.41±5.30 E vs. D: ↑24.5% P<0.01
VOL+L-dopa (Treatment F)	6.42±0.04 F vs. A: p>0.05	12.90±0.05 F vs. D: ↓17.9% P<0.01	0	132.79±2.24 F vs. D: ↓18.4% P<0.01

values are mean±SE of the daily averages during treatment days 8 through 20. Two-way ANOVA indicates a significant genotype X treatment effects and interaction for normal horizontal speed (genotype $F(1,72)=37335$, $p=0$; treatment $F(5,72)=8464$, $p=0$; interaction $F(5,72)=8298$, $p=0$). Separate one-way ANOVA detected no significant treatment effect for PitxWT mice ($F(5,36)=1.32$, $p=0.21$) but significant treatment effect for PitxHomo mice ($F(5,36)=14788$, $p=0$). Post hoc Newman-Keuls test results were listed in the table. The first 8 days were excluded from this table due to the time-dependent L-dopa sensitization. Five to 10 mice in each group.

* the solvent vehicle is described in detail in section 5.2 of the text.

Table 2

Regular PCR primer pairs.

mRNA (accession No.)		Start position	Primer (5' to 3')
Htr2a (NM_172812.2)	F	1641	atagccgcttcaactccaga
	R	1906	gtcactcacacaagg
β -actin (NM_007393.3)	F	819	tcatactattggcaacgagc
	R	1217	aacagtccgcctagaagcac

Table 3

qRT-PCR primer pairs and Universal Probe Library (UPL) probes.

mRNA (accession No.)		Start position	Primer (5' to 3')	UPL probe No.
Drd1a(NM_010076.3)	F	1676	tctggtttacctgatccctca	82
	R	1717	gcctcctccctcttcaggt	
Drd2 (NM_010077.2)	F	888	tgaacagcgaggagaatgg	17
	R	938	ctggtgcttgacagcatctc	
Htr2a (NM_172812.2)	F	1431	tgatgtcacttgccatagctg	3
	R	1518	agagcttgctgggcaaag	
Htr2c (NM_008312.4)	F	669	tgcttaaaactgaagcaataatgg	104
	R	722	aggccaattaggtgcacaag	
β -actin (NM_007393.3)	F	1009	tgacaggatgcagaaggaga	106
	R	1066	cgctcaggaggagcaatg	