

# Sonic Hedgehog Maintains Cellular and Neurochemical Homeostasis in the Adult Nigrostriatal Circuit

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## SUMMARY

Non cell-autonomous processes are thought to play critical roles in the cellular maintenance of the healthy and diseased brain but mechanistic details remain unclear. We report that the interruption of a non cell-autonomous mode of sonic hedgehog (Shh) signaling originating from dopaminergic neurons causes progressive, adult-onset degeneration of dopaminergic, cholinergic, and fast spiking GABAergic neurons of the mesostriatal circuit, imbalance of cholinergic and dopaminergic neurotransmission, and motor deficits reminiscent of Parkinson's disease. Variable Shh signaling results in graded inhibition of muscarinic autoreceptor- and glial cell line-derived neurotrophic factor (GDNF)-expression in the striatum. Reciprocally, graded signals that emanate from striatal cholinergic neurons and engage the canonical GDNF receptor Ret inhibit Shh expression in dopaminergic neurons. Thus, we discovered a mechanism for neuronal subtype specific and reciprocal communication that is essential for neurochemical and structural homeostasis in the nigrostriatal circuit. These results provide integrative insights into non cell-autonomous processes likely at play in neurodegenerative conditions such as Parkinson's disease.

## INTRODUCTION

Neuronal computations in the basal ganglia rely on correlated changes in the activity of mesencephalic dopamine (DA) neurons

and striatal acetylcholine (ACh) and fast-spiking (FS) GABAergic neurons, which result from reciprocal pre- and postsynaptic interactions (Threlfell et al., 2010). The concerted activity of DA neurons and ACh and FS interneurons gate glutamatergic input from the cerebral cortex and thalamus onto medium spiny projection neurons (MSN) allowing the translation of thought into action (Figure 1) (Bolam et al., 2006). These neuronal subtypes form cartridges of a repetitive mesostriatal circuit in which each of the numerous MSN contributes to only few units, but each of the many fewer DA, ACh, and FS neuron participates in several 100 units (Bolam et al., 2006). The phylogenetic conservation of circuit architecture (Reiner, 2010) suggests that the relative proportions of the constituent neurons of the mesostriatal circuit are important for proper circuit function. This view is supported by the pathophysiological finding that chorea, parkinsonism, and tics are associated with a loss of specific mesostriatal constituent neuronal subtypes such as MSN, DA, and FS neurons, respectively (DeLong and Wichmann, 2009).

The mechanisms maintaining cellular and neurochemical homeostasis in the mature mesostriatal system in the healthy brain are not fully elucidated, but signaling by neurotrophic factors has emerged as a likely process. For example, the glial cell line-derived neurotrophic factor (GDNF) protects catecholaminergic neurons from toxic insults, induces fiber outgrowth and is required for catecholaminergic neuron survival in the adult brain (Lin et al., 1993; Pascual et al., 2008). GDNF signaling can also act as a neuromodulator of dopaminergic signaling through the regulation of the quantal size of DA release (Pothos et al., 1998; Wang et al., 2001). Despite the implication of GDNF in DA neuron maintenance and function, which motivated several clinical trials of GDNF-based therapies in Parkinson's disease (PD) (Rangasamy et al., 2010), the regulation of GDNF expression in the healthy adult brain remains ill-defined.

Circumstantial evidence also implicates the secreted glycoprotein Sonic Hedgehog (Shh) in the maintenance of the

mesostriatal circuit because exogenously supplied Shh to the adult striatum increases the resilience of mesencephalic DA neurons to MPTP and 6-OHDA, neurotoxins used to model PD-related DA neuron loss in animals (Dass et al., 2005; Tsuboi and Shults, 2002). In these paradigms, the neuroprotective effect of Shh on mesencephalic DA neurons is comparable to that observed with striatal delivery of GDNF (Dass et al., 2002). In vitro, Shh synergizes with neural growth factor (NGF) in providing trophic support to basal forebrain-derived, postnatal ACh neurons (Reilly et al., 2002). Despite these observations, it is not clear whether there is a functionally relevant source of Shh that could act in the mature mesostriatal system and if so, which cell types would communicate by Shh signaling.

Here, we present evidence for reciprocal, trophic factor signaling between mesencephalic DA, striatal ACh, and FS neurons. We show that DA neurons utilize Shh to signal to ACh and FS interneurons in the striatum where it regulates extracellular ACh tone, expression of GDNF, and maintenance of these neurons. Conversely, Shh expression by DA neurons is repressed by signals that originate from ACh neurons and engage the canonical GDNF receptor Ret on DA neurons. The conditional ablation of Shh from DA neurons results in a progressive model of PD with face, construct and predictive validity. Thus, our results shed light onto aspects of the chemical neuroanatomy of the basal ganglia and may have far-reaching implications for the understanding of the physiopathology and the treatment of movement disorders such as PD.

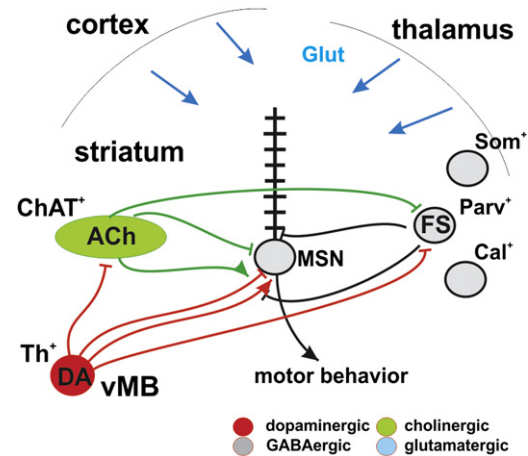
## RESULTS

### Shh Expression by DA Neurons Is Necessary for the Long-Term Maintenance of Mesencephalic DA Neurons

To examine whether Shh-mediated signaling occurs among neurons of the mesostriatal circuit, we first visualized expression of Shh in the adult brain using mice heterozygous for a conditional, gene expression tracer allele of Shh (*Shh-nLZ<sup>C/+</sup>*) (Figure S1A available online). We observed Shh expression by all tyrosine hydroxylase-positive ( $\text{Th}^+$ ) neurons in the substantia nigra pars compacta (SNpc) (Figures 2A and 2B), the ventral tegmental area (VTA) (Figure 2A), and the retrorubral field (RRF, data not shown) along the entire rostro-caudal axis of these nuclei at 3 months of age ( $100 \pm 0\%$ , 683 cells,  $n = 2$ ). We did not observe Shh expression by  $\text{Th}^+$  neurons of the diencephalon or olfactory bulb or by cells in the striatum (data not shown).

To determine whether Shh signaling within the mesostriatal circuit is of physiological relevance, we selectively ablated Shh expression from DA neurons mediated by Cre activity expressed from the DA transporter locus (*Dat-Cre*; all mouse strains used in this study are referenced in Supplemental Experimental Procedures). *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mutant animals were born alive and mobile with expected Mendelian frequency and no overt structural or motor signs at the end of postnatal development compared to *Shh-nLZ<sup>C/+</sup>/Dat-Cre* control littermates (Figures S1 and S2; Table S1; Supplemental Results A and B; for all comparative analyses herein littermates double heterozygous for *Shh-nLZ<sup>C/+</sup>* and *Dat-Cre* served as controls).

Unbiased stereological cell counting of  $\text{Th}^+$  and  $\text{Th}^-$  neurons in the SNpc and VTA revealed an adult-onset, progressive degen-

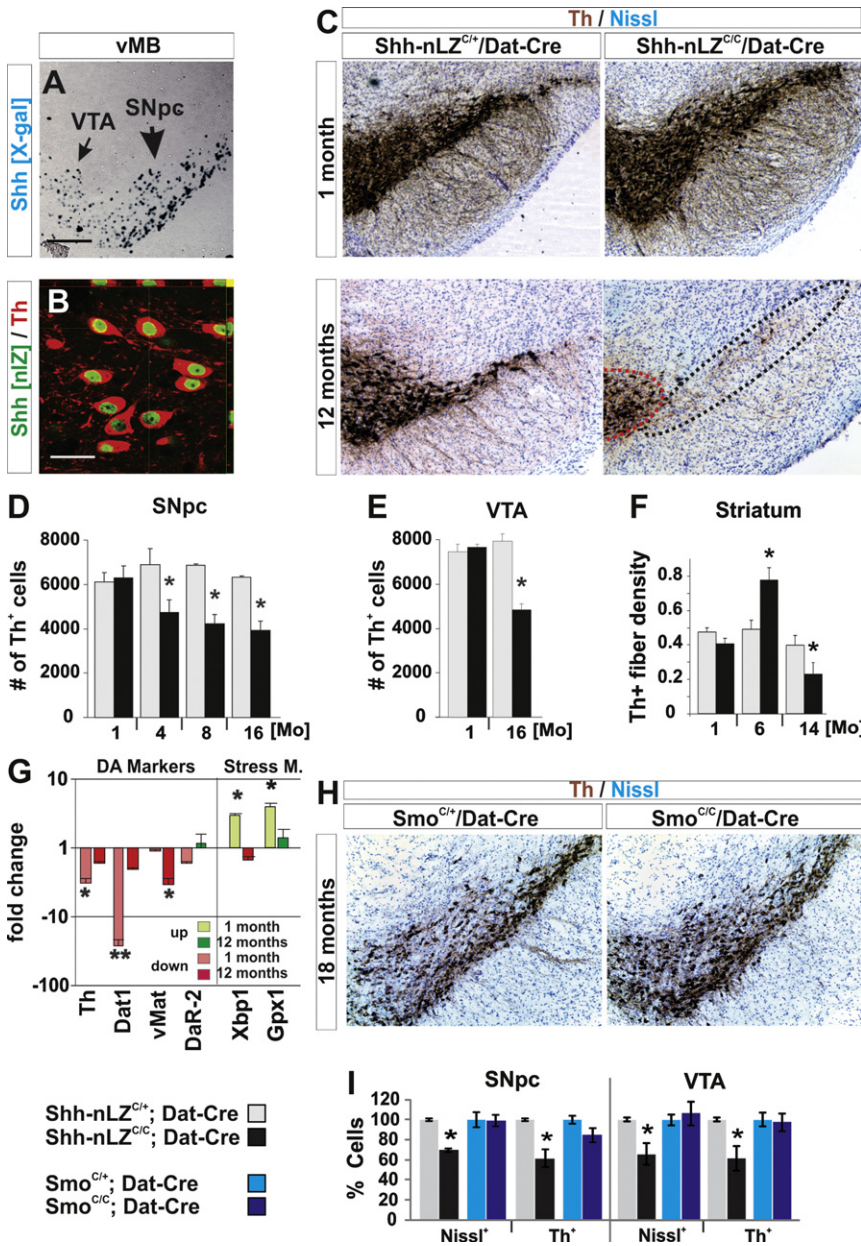


**Figure 1. Mesostriatal Circuitry**

Striatal interneurons and MSNs receive dopaminergic projections. ACh and FS interneurons project to striatal neuronal subtypes and dopaminergic projections. ACh, acetylcholine; Cal, calretinin; ChAT, choline-acetyl transferase; DA, dopamine; FS, “fast spiking”; GABA,  $\gamma$ -aminobutyric acid; Glut, glutamine; MSN; medium spiny projection neurons; Parv, parvalbumin; Som, somatostatin; Th, tyrosine hydroxylase; vMB, ventral midbrain.

eration of DA neurons that plateaus at  $\sim 40\%$  in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice compared to controls at 8 months of age (Figures 2C–2F and 2I). No change in the number of glutamate decarboxylase (GAD67)-positive cells was observed in the SN pars reticulata of *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice at 16 months of age (Figure S3A). Striatal  $\text{Th}^+$ -fiber density was normal at 1 month of age, increased at 8 months and decreased at 12 months of age in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice compared to controls (Figure 2F). Gene expression analysis of DA markers in the ventral midbrain (vMB) revealed a downregulation of Th, Dat, and DA receptor-2 (DaR2) at 5 weeks of age, which then returned to normal levels by 12 months in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* compared to controls (Figure 2G; all genes probed herein are listed in Table S2). The expression of the vesicular monoamine transporter-2 (vMat2) appeared normal at 5 weeks, but was diminished at 12 months. The activator of endoplasmic reticulum stress, Xbp1, and the antioxidant enzyme, glutathione-peroxidase-1 (Gpx1), were upregulated in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* animals at 5 weeks but not at 12 months of age (Figure 2G) indicative of the activation of physiological cell stress responses in the vMB in the absence of Shh expression in young adult mutant mice. In further support of a protracted dopaminergic cell syndrome in which neuronal degeneration is only the final step, we found progressive alterations in somato-dendritic and striatal DA content and deficits in amphetamine elicited DA release in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice (Supplemental Results C and Figures S3B–S3D).

Is the observed dopaminergic phenotype a cell autonomous effect of the interruption of Shh signaling? Shh can bind to several coreceptors which in turn facilitate the relief of repression of the serpentine transmembrane protein Smo by Ptc1 or Ptc2 (Izzi et al., 2011). To distinguish autocrine from paracrine Shh signaling, we analyzed the expression of the Shh coreceptors Ptc1 and Ptc2 and the phenotype of animals with a tissue



restricted ablation of *Smo* from DA neurons, which we produced using the same *Dat-Cre* allele with which we also achieved the tissue specific ablation of *Shh*. We did not find evidence for the expression of *Ptc1* in DA neurons utilizing a gene expression tracer mouse line (*Ptc1-nLZ*) or *Ptc2* by in situ hybridization, consistent with public gene expression data information (Gensat, <http://www.gensat.org>; data not shown). *Smo<sup>C/C</sup>/Dat-Cre* mutant animals were born alive and mobile with expected Mendelian frequency and no overt structural or motor signs through adulthood compared to *Smo<sup>C/+</sup>/Dat-Cre* control littermates (data not shown). Unbiased stereological cell counting of Th<sup>+</sup> and Th<sup>-</sup> neurons in the SNpc and VTA of 18-month-old *Smo<sup>C/C</sup>/Dat-Cre* mutants and *Smo<sup>C/+</sup>/Dat-Cre* littermate controls did not reveal DA neuron loss in the SNpc or VTA

whose cardinal cellular and functional aspects results from a non cell-autonomous mode of *Shh* signaling.

**DA Neuron-Expressed Shh Acts as a DA Neuron Protectant**

*Dat-Cre*-mediated ablation of the conditional *Shh* allele is ~80% complete in the SNpc at 2 months of age creating an experimentally induced heterogeneity among DA neurons in regard of *Shh* expression (Figure S2A). We therefore explored whether the progressive nature of the phenotype might be caused by slowly continuous *Cre*-mediated deletion of *Shh* alleles over time in adult *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mutant animals. In contrast to this prediction, however, we observed an increase in the relative numbers of *Shh* expressing DA neurons among all DA neurons

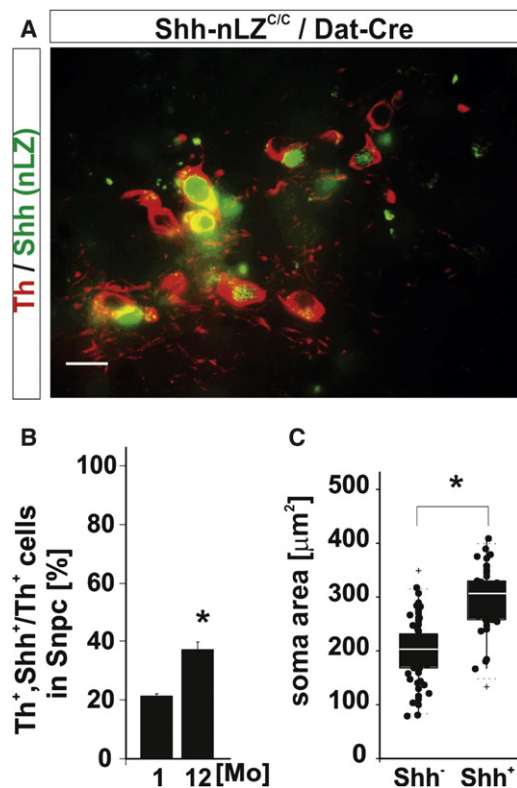
of the SNpc from ~20% at 2 months of age to ~37% at 12 months of age in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mutants (Figures 3A and 3B). We also observed that the soma size of Shh expressing DA neurons was larger than of DA neurons that lost Shh expression in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice at 12 months of age (Figure 3C). These observations demonstrate that (1) accumulation of Shh null alleles by continuous Cre activity is insignificant after 8 weeks of age, and (2) Shh-expressing DA neurons have a selective survival advantage over DA neurons in which Cre-mediated Shh ablation occurred. Thus, these results provide genetic evidence that Shh signaling originating specifically from DA neurons confers a competitive survival advantage among mesencephalic DA neurons in vivo.

### Dopamine Substitution and Cholinergic Antagonist Treatment Normalize Progressive Gait and Locomotion Disturbances in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* Mice

Next, we assessed the functional significance of the progressive structural and neurochemical alterations of the mesencephalic DA system in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice by longitudinal analyses of elicited and spontaneous motor performance.

Analysis of horizontal activity profiles of freely locomoting mice in an open field let us define successive phases of progressive changes in locomotion of *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice compared to control litter mates: locomotion activity was normal up to 6 weeks (phase I), reduced by ~35% at 2–5 months (phase II), increased by ~60% at 7–12 months (phase III), inconspicuous at 16 months (phase IV), but then rapidly deteriorating leading first to pelvic dragging, followed by partial hind limb paralysis and premature death at about 18 months (phase V; Figure 4A). The switch from relative hypoactivity to relative hyperactivity of *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice compared to control littermates manifested with high temporal specificity around 6 months of age (Figure 4B). The inversion in relative locomotion activity coincided with a switch from a 3-fold reduction to a 30% increase in striatal DA content (Figure S3C and Supplemental Results C) and a relative increase in the frequency of bouts of locomotion from phase II to phase III in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice compared to controls (data not shown).

We next investigated gait dynamics by ventral plane videography of *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice and controls of 3–16 months of age walking on a translucent treadmill. During phase III, the gait length coefficient of variability was increased ~30% at 10 months of age, and the time allotted for braking in each stride was shortened by 40% and the paw angle was increased by 50% at 11 months of age of *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice relative to controls (Figure 4C and Supplemental Results D; see Table S1 for all indices of gait analyzed). We then tested whether drugs efficacious in the management of PD would modify the locomotion abnormalities of *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice. We systemically injected L-DOPA (dopamine precursor) trihexylphenidate (THP) (muscarinic antagonist), or vehicle 30 min prior to the analysis of locomotion in 12-month-old *Shh-nLZ<sup>C/C</sup>/Dat-Cre* and control mice. The increased variability in stride length was normalized by L-DOPA and THP, brake stride ratios were normalized by THP but not L-DOPA, and alterations in paw angles were normalized by L-DOPA, but not THP (Figure 4D). Taken together, our behavioral studies revealed a dynamic and progressive loco-



**Figure 3. Shh Expression Confers a Survival Advantage to Dopaminergic Neurons**

(A) Shh expressing DA neurons in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice at 12 months of age.

(B) Incidence of Shh expression in the SNpc in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice at 1 and 12 months of age (\* $p < 0.05$ , unpaired t test,  $n = 12/\text{group}$ , 100 cells/subject; mean  $\pm$  SEM).

(C) Somatic area of Th<sup>+</sup>, Shh<sup>-</sup> and Th<sup>+</sup>, Shh<sup>+</sup> cells in the SNpc of *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice (\* $p < 0.05$ , single comparison by Mann-Whitney U test; box-whisker plots for median  $\pm$  95% confidence interval [CI] bar, 25–75 percentiles box,  $n = 4$ , 20 cells/subject).

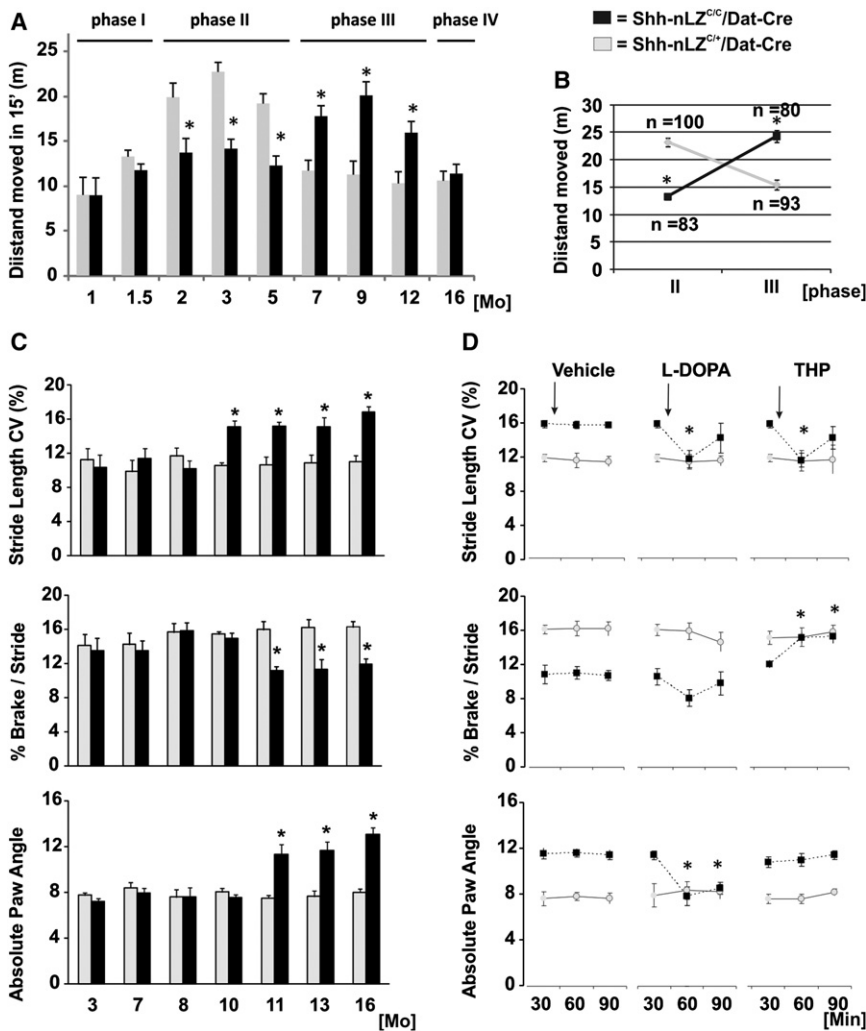
See also Figure S1 and S2.

motion phenotype whose pharmacological responsiveness suggests underlying alterations in the functional balance of dopaminergic and cholinergic neurotransmission.

### Ablation of Shh from DA Neurons Results in Progressive Cellular and Functional Corruption of the Adult Striatum

Similarly to BDNF, which supports survival of cortical-striatal neurons (Baquet et al., 2004), Shh can also be transported anterogradely through axons (Thérond, 2012). Because of the lack of evidence for an autocrine mechanism for Shh dependent support of DA neurons we therefore hypothesized that Shh signaling from dopaminergic projections to striatal targets might be of relevance to the maintenance of DA neurons.

We found that ~25% of all Ptc1<sup>+</sup> cells in the striatum are neurons (Figures 5A–5C) and that 6% of all striatal neurons coexpress Ptc1 (Figure 5F). Conversely, 100% of all ACh neurons and 98% of all FS interneurons express Ptc1 (Figures 5D–5F), consistent with the relative prevalence of ACh and FS neurons among all striatal neuronal subtypes (Bolam et al., 2006). Hence, our



**Figure 4. Abnormalities in Locomotion and Gait Dynamics in the Absence of Shh Expression by DA Neurons**

(A) Horizontal activity in an "Open Field" as a function of age (\* $p < 0.05$ , repeated-measures ANOVA followed by Tukey's post hoc test;  $n = 10$ /group; mean  $\pm$  SEM).

(B) High temporal predictability of transition from phase II to phase III ( $n = 83$ – $100$ ; 23 litters;  $p < 0.00001$ , phase  $\times$  genotype ANOVA; mean  $\pm$  SEM).

(C) Increase in coefficient of stride length variability (CV), reduction in brake/stride ratio, and increase in absolute paw angle during phase III in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* ( $n = 5$ ;  $p < 0.01$ ; ANOVA followed by Tukey's test; mean  $\pm$  SEM).

(D) Normalization of stride length CV, braking time and paw angle by L-DOPA (subcutaneously, 20 mg/kg) and/or THP (subcutaneously, 3 mg/kg;  $n = 8$ /genotype; \* $p < 0.05$ , genotype  $\times$  time ANOVA for repeated measures followed by Tukey's post hoc test; mean  $\pm$  SEM). See also Table S1.

expression data suggested that mesencephalic DA neurons could communicate by Shh signaling selectively with all ACh and FS neurons, and nonneuronal cells among their projection targets in the adult striatum.

In *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice compared to controls at 6 months of age, we observed a reduction in the number of ChAT<sup>+</sup> neurons in the striatum that was most pronounced in lateral/anterior aspects of the dorsal striatum (Figures 5G and S4A–S4C). ACh and FS interneurons make up together only ~6% of total striatal neurons (Figure 5F) and are locally projecting. These attributes make it impossible to distinguish neuronal loss from downregulation of phenotypic marker expression by the quantitation of the total number of neurons or the exploitation of specific projection patterns. However, the main striatal cell populations can be identified based on cell type specific perinuclear staining patterns that can be visualized by the DNA intercalating dye ToPro3 (Figures S5A–S5C). Based on this technique, we found a ~40% reduction in the numbers of ACh and FS interneurons but no change in the numbers of calretinin and somatostatin interneurons, MSN, and nonneuronal cells in 4-month-old *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice compared to controls (Figure 5H). ACh

and FS neurons as a group exhibit the largest nuclei and can therefore be distinguished from all other striatal cells by a nuclear circumference larger than 28  $\mu$ m (Figure S5C). This second approach, revealed a ~60% reduction of the numbers of striatal cells with nuclear circumference larger than 28  $\mu$ m in 12-month-old *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice compared to controls (Figure 5H). Stereological quantitation of ACh and FS neurons using ChAT and parvalbumin immunohistochemistry revealed an adult-onset, progressive reduction in the numbers of ChAT<sup>+</sup> and parvalbumin<sup>+</sup> cells, which plateaus at 8 months of age at ~50% and ~40%, respectively (Figures 5I and 5K). The kinetics of striatal ACh and mesencephalic DA neuron degeneration were correlated ( $R^2 = 0.98$ ;  $p < 0.006$ ; Figures 5I and 2D). Consistent with the activation of physiological cell stress response pathways in ACh and FS neurons prior to neurodegeneration, we found increased expression of the luminal endoplasmic reticulum (ER) protein BiP/Grp78 by large bodied cells in the striatum by mRNA in situ hybridization at 5 weeks of age which then becomes more pronounced at 12 months of age (Figures S6A–S6D). Thus, in aggregate, the analyses of perinuclear staining pattern, nuclear size, and cell type specific marker gene expression, demonstrate a cell type selective, adult-onset, progressive degeneration of ACh and FS neurons in the striatum in the absence of Shh expression by mesencephalic DA neurons that is correlated with the degeneration of DA neurons themselves.

We next examined steady state, extracellular ACh levels in the striatum by in vivo dialysis. Despite a mere 50% loss of ACh neurons, we found a ~6-fold, reduction in basal levels of extracellular ACh in 8-month-old *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice

compared to controls (Figure 5L). This observation suggests that surviving ACh neurons cannot functionally compensate for the reduction in their numbers in absence of Shh signaling from DA neurons.

To explore the molecular underpinnings of the inability of surviving ACh neurons to increase ACh production, we investigated the expression levels of candidate genes, which could inform about the neurophysiological status of the striatum before (at 5 weeks of age) and after (at 12 months of age) the onset of neurodegeneration. We found that the expression of striatal ChAT, vesicular acetylcholine transporter (vAChT), and GTPase regulator RGS4 were downregulated whereas the expression of striatal muscarinic autoreceptor M2 was upregulated in 5-week-old *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice compared to controls (Figure 5M(1)). The expression of ChAT, M2, and RGS4 was further distorted at 12 months of age whereas the expression of vAChT returned to normal levels (Figure 5M(1)). Acetylcholine esterase (AChE) expression was unaltered at 5 weeks but reduced at 12 months of age consistent with the observed reduction of striatal ACh levels in older animals (Figure 5M(1)). ACh tone in the striatum is in part regulated by muscarinic autoreceptors M2 and M4 whose functions in turn are negatively modulated by the GTPase accelerator RGS4 (Ding et al., 2006). Thus, the observed upregulation of M2 and downregulation of RGS4 gene expression indicate an enhancement of cholinergic autoreceptor function in surviving ACh neurons in the striatum of *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice consistent with the observed reduction in striatal cholinergic tone.

In contrast to the situation in ACh neurons, parvalbumin gene expression was strongly reduced at 5 weeks of age, but reached normal levels at 12 months suggesting a compensatory upregulation by surviving FS neurons (Figure 5M(2)). General GABAergic marker and DA receptor gene expression were not affected at 5 weeks but DA receptors D1–D4, DARP32, and *Gad1* were downregulated, while DA receptor interacting protein (D-IP) was upregulated at 12 months of age (Figure 5M(2)) suggesting that GABAergic neuronal subtypes in addition to FS neurons in the striatum become phenotypically involved subsequently to ACh and FS neurons.

In support of a direct control of gene expression by Shh signaling in the striatum, we found that the transcription factor *Gli3*, whose expression is inhibited by Shh signaling (Ulloa and Briscoe, 2007), is upregulated by the Smo antagonist cyclopamine (Chen et al., 2002) and downregulated by the Smo agonist “SAG” (Frank-Kamenetsky et al., 2002) when injected into the adult striatum of C57Bl/6 wt mice (Figure 5M(3)). M2 expression was also acutely and dose-dependently increased by cyclopamine and decreased by SAG injection into the striatum, indicating that Shh signaling impinges directly on the regulation of cholinergic tone in the healthy striatum (Figure 5M(3)).

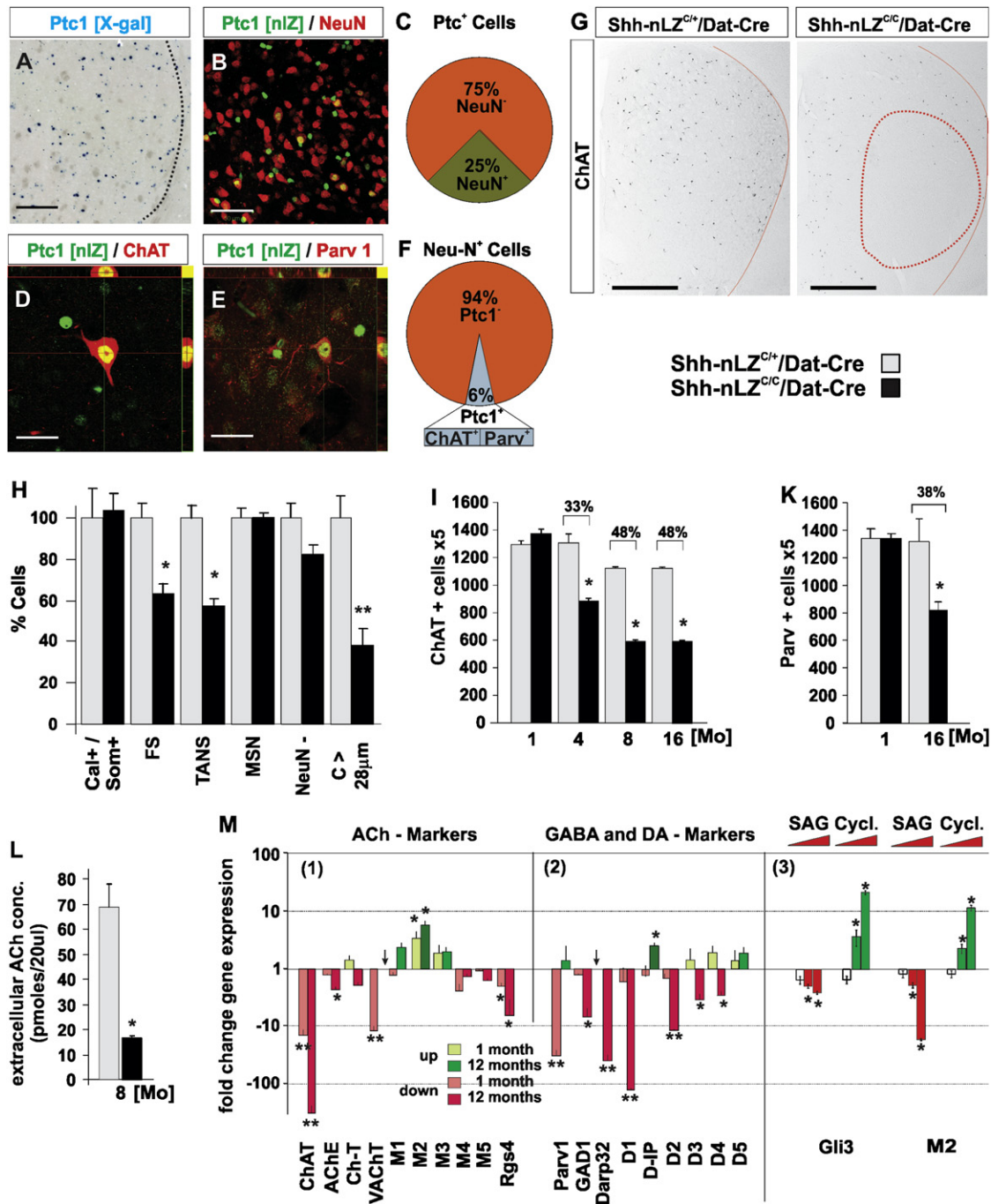
Thus, the absence of Shh signaling originating from DA neurons elicits a sequential structural and functional corruption of the striatum which begins with cell physiological alterations in ACh and FS neurons and culminates in a progressive, adult-onset degeneration of ACh and FS neurons without compensatory adaptations of surviving ACh neurons.

### DA Neuron-Derived Shh Maintains the Cellular Source of GDNF but Represses Its Transcription in the Striatum

GDNF is expressed by ACh and FS interneurons (Hidalgo-Figueroa et al., 2012). Consistent with the Shh-dependent maintenance of ACh and FS neurons, we found a progressive reduction in GDNF mRNA and protein expression, and an upregulation of the canonical receptor *Ret* and its coreceptor *Gfr $\alpha$ 1*, which bind all members of the GDNF family of ligands, in the striatum of *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice compared to controls (Figures 6A and 6B). The progressive reduction in striatal GDNF tissue content correlated with the progressive degeneration of ACh neurons in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice (Figures 6B and 2D;  $R^2 = 0.95$ ,  $p < 0.02$  for ACh neurons). We then utilized the cholinotoxin AF64 $\alpha$ , which causes reversible inhibition of ACh neurons at moderate concentrations and ACh neuron degeneration at higher concentration (Sandberg et al., 1984) to assess the relative contribution of ACh neurons over other sources to GDNF production in the striatum. We found that unilateral striatal injections of moderate concentrations of AF64 $\alpha$  led to a ~30% reduction in striatal GDNF protein content over vehicle injected controls 36 hr after toxin application in 4-month-old C57Bl/6 wt animals (Figure 6C). Together, these experiments demonstrate that Shh signaling originating from mesencephalic DA neurons contribute to the long-term maintenance of striatal GDNF production through trophic support of striatal ACh and FS neurons.

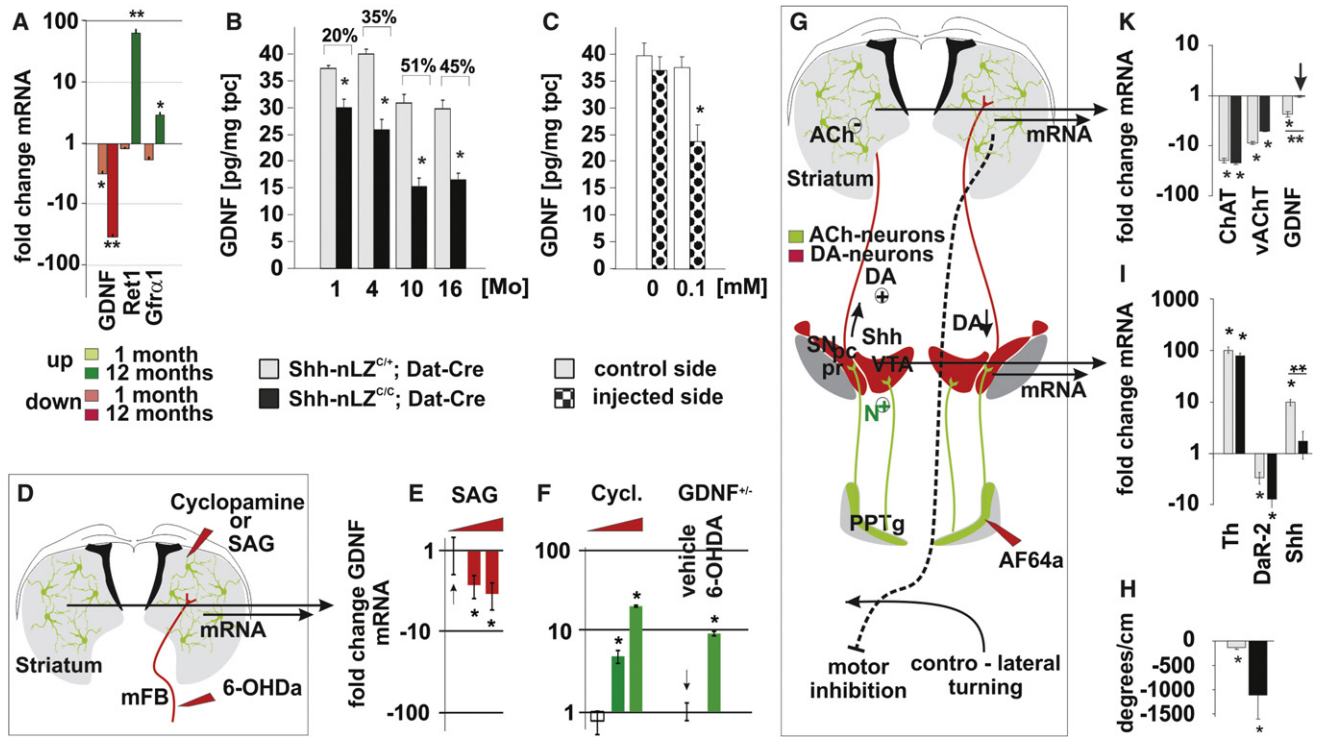
The analysis of the long-term effects of the chronic absence of Shh signaling from DA neurons does not provide information about whether Shh signaling plays a role in the transcriptional regulation of striatal GDNF expression in the absence of physiological cell stress and/or neurodegeneration. We therefore examined whether Shh signaling regulates striatal GDNF gene expression acutely by unilaterally injecting SAG or cyclopamine in 8-week-old C57/Bl6 male mice (Figure 6D). Comparative qRT-PCR analysis revealed a SAG specific reduction in GDNF mRNA and a dose-dependent, cyclopamine specific increase in GDNF mRNA 30 hr after injection (Figures 6E and 6F), demonstrating that GDNF expression in the adult striatum is dynamically regulated by Shh signaling. Consistent with the inhibition of GDNF expression by Shh signaling originating from DA neurons we observed an upregulation of GDNF in the striatum upon the interruption of the mesostriatal pathway by the unilateral injection of 6-OHDA into the medial forebrain bundle (mFB) of *GDNF-LZ* mice (Figure 6F). Together with the finding that systemic injections of the dopaminergic toxin MPTP results in the transient upregulation of striatal GDNF expression (Hidalgo-Figueroa et al., 2012), our results suggest that the relevant Shh signal for the regulation of GDNF expression in vivo could come from the vMB.

Guided by these results, we tested whether Shh produced specifically by DA neurons acutely regulates the expression of GDNF in the mesostriatal system in vivo. The pedunculopontine tegmental nucleus (PPTg) provides excitatory, nicotinic receptor mediated cholinergic input to mesencephalic DA neurons (Futami et al., 1995) (Figure 6G). Similar to previous observations upon the excitotoxic ablation of PPTg neurons (Dunbar et al., 1992), we found that unilateral injection of the cholinotoxin AF64 $\alpha$  into the PPTg of 2-month-old *Shh-nLZ<sup>C/C</sup>/Dat-Cre* or



**Figure 5. Neuronal Subtype Specific and Progressive Degeneration in the Striatum in Absence of Shh Signaling from Mesencephalic DA Neurons**

(A) X-Gal labeled coronal section of striatum of a *Ptc1-nLZ* mouse.  
 (B) Ptc1 expression in neuronal and nonneuronal cells of the striatum.  
 (C) Twenty-five ± 5.2% of Ptc1<sup>+</sup> striatal cells are neurons (n = 612 cells, 3 mice).  
 (D) Ptc1 expression in striatal ACh neurons.  
 (E) Ptc1 expression in striatal FS neurons.  
 (F) Six ± 2.2% of striatal neurons express Ptc1 (n = 420 cells, 3 mice); 100 ± 0% of ACh neuron express Ptc1 (n = 140 cells, 3 mice); 98 ± 2.0% of FS neurons express Ptc1 (n = 150 cells, 3 mice).  
 (G) Loss of ACh neurons is most pronounced in lateral areas of the dorsal striatum (circled in red; quantitation in Figure S4) in *Shh-nLZ<sup>Ct/C</sup>/Dat-Cre* mice.  
 (H) Quantification of striatal cell types by heterochromatin pattern (Figures S6A and S6B) and nuclear size (Figure S6C) in *Shh-nLZ<sup>Ct/C</sup>/Dat-Cre*. (\*p < 0.05, \*\*p < 0.001, t test (10 striatal slices/subject, n = 7 mice per genotype; mean ± SEM).



**Figure 6. Shh Signaling Inhibits GDNF Expression in the Striatum**

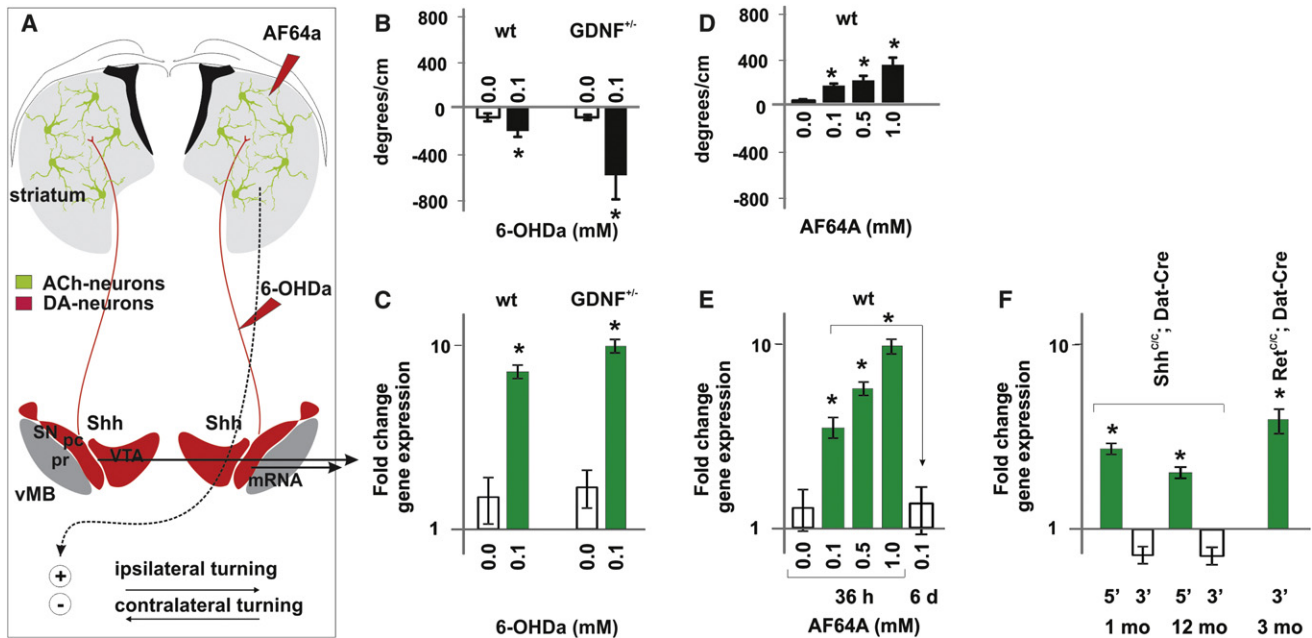
(A) Changes in GDNF, Ret, and Gfra1 gene expression in the striatum of 4-week-old and 12-month-old *Shh-nLZ<sup>Ci/Ci</sup>/Dat-Cre* mice (\**p* < 0.01, \*\**p* < 0.001, two tailed t test; *n* = 5/genotype; mean ± SEM).  
 (B) Quantification of striatal GDNF tissue content as a function of age in *Shh-nLZ<sup>Ci/Ci</sup>/Dat-Cre* mice (\**p* < 0.01, unpaired t test; *n* = 10–11/group; mean ± SEM).  
 (C) Injection of AF64α reduces GDNF tissue content. (37 ± 3%; \**p* < 0.05, unpaired t test; *n* = 12–14/group; mean ± SEM).  
 (D) Experimental design: comparative analysis of GDNF expression upon unilateral injection of SAG or cyclopamine into the striatum of *wt* mice, or 6-OHDA into the mFB of *GDNF-LZ<sup>-/-</sup>* mice.  
 (E) SAG decreases GDNF mRNA expression (\**p* < 0.05, ANOVA followed by Tukey’s post hoc Test; *n* = 5/group/drug concentration; mean ± SEM).  
 (F) Injection of cyclopamine into the striatum or 6-OHDA into the mFB increases GDNF mRNA expression (\**p* < 0.05, ANOVA followed by Tukey’s post hoc test; *n* = 5/group/drug concentration; the upregulation caused by cyclopamine was dose-dependent; mean ± SEM).  
 (G) Experimental design: comparative analysis of gene expression in the vMB and striatum upon unilateral injection of AF64α into the PPTg.  
 (H) Quantification of turning bias 36 hr postunilateral injection of AF64α into the PPTg in *Shh-nLZ<sup>Ci/Ci</sup>/Dat-Cre* mice (\**p* < 0.05, post hoc test after ANOVA; *n* = 5/genotype; mean ± SEM).  
 (I and K) Quantification of changes in gene expression in the vMB (I) or striatum (K) upon AF64α injection into the PPTg of *Shh-nLZ<sup>Ci/Ci</sup>/Dat-Cre* and control mice (\*\**p* < 0.001, AF64α × genotype ANOVA followed by Tukey’s post hoc test; *n* = 5/group/genotype; mean ± SEM).  
 See also Figures S1 and Table S2.

control mice elicited a contralateral turning bias consistent with reduced cholinergic stimulation of ipsilateral DA neurons (Figure 6H) (Lester et al., 2010). Comparative qRT-PCR analysis of dopaminergic markers between the ipsilateral and contralateral vMB revealed a transcriptional upregulation of Th and a downregulation of the DA autoreceptor DaR2 ipsilateral in both genotypes, suggesting an adaptive upregulation of mesencephalic

DA signaling upon AF64α injection into the PPTg (Figure 6I). AF64α injection into the PPTg also resulted in a ~10-fold upregulation of Shh transcription in the ipsilateral vMB compared to the contralateral control (Figure 6I). Thus, the comparative analysis of *Shh-nLZ<sup>Ci/Ci</sup>/Dat-Cre* mice, which are unable to express functional Shh, and of *Shh-nLZ<sup>Ci/+</sup>/Dat-Cre* control animals allows the distinction of Shh dependent and independent

(I) Progressive reduction in the numbers of ChAT<sup>+</sup> neurons in the striatum in *Shh-nLZ<sup>Ci/Ci</sup>/Dat-Cre* mice (*p* < 0.05, unpaired t test; *n* = 6–7/group, 12 sections per subject with a 5-section interval; mean ± SEM).  
 (K) Progressive reduction in the numbers of Parv<sup>+</sup> FS neurons in the striatum in *Shh-nLZ<sup>Ci/Ci</sup>/Dat-Cre* mice. (\**p* < 0.05, unpaired t test; *n* = 4/group, 12 sections per subject with a 5-section interval; mean ± SEM).  
 (L) Reduced extracellular ACh concentration in the striatum in 8 month old *Shh-nLZ<sup>Ci/Ci</sup>/Dat-Cre* mice (\*\**p* < 0.001, unpaired t test; *n* = 8/genotype, 4 samples/subject; mean ± SEM).  
 (M) Quantification of changes in gene expression in the striatum of 4-week-old and 12-month-old *Shh-nLZ<sup>Ci/Ci</sup>/Dat-Cre* mice (1 and 2) and after unilateral injection of increasing concentrations of SAG or cyclopamine (\**p* < 0.01, \*\**p* < 0.001, two tailed t test; *n* = 5/genotype or treatment; mean ± SEM).  
 See also Figure S5 and Table S2.





**Figure 7. Shh Expression by Mesencephalic DA Neurons Is Repressed by Signals Emanating from ACh Neurons**

(A) Experimental design: unilateral injection of AF64 $\alpha$  into the striatum or 6-OHDA in the mFB results in ipsi-lateral or contro-lateral, respectively, turning.  
 (B) Quantification of turning bias upon injection of 6-OHDA into the mFB of *wt* or *GDNF-LZ<sup>-/-</sup>* mice (\**p* < 0.01, AF64 $\alpha$  dose versus vehicle, ANOVA followed by Tukey's post hoc test; *n* = 5/dose; mean  $\pm$  SEM).  
 (C) Upregulation of Shh gene expression in the vMB bias upon injection of 6-OHDA into the mFB of *wt* or *GDNF-LZ<sup>-/-</sup>* mice, or AF64 $\alpha$  into *wt* mice (\**p* < 0.05, ANOVA followed by Tukey's post hoc test; *n* = 5/condition; mean  $\pm$  SEM).  
 (D) Quantification of turning bias upon injection of AF64 $\alpha$  into *wt* mice (\**p* < 0.01, AF64 $\alpha$  dose versus vehicle, ANOVA followed by Tukey's post hoc test; *n* = 5/dose; mean  $\pm$  SEM).  
 (E) Dose dependent upregulation of Shh gene expression in the vMB bias upon injection AF64 $\alpha$  into *wt* mice (\**p* < 0.05, ANOVA followed by Tukey's post hoc test; *n* = 5/condition; mean  $\pm$  SEM).  
 (F) Shh expression in the vMB is activated in *Shh-nLZ<sup>Cre</sup>/Dat-Cre* and *Ret<sup>Cre</sup>/Dat-Cre* mice (5': Shh exon 1 amplicon, 3': Shh exon 2,3 amplicon; *n* = 4–6/genotype and age, \**p* < 0.01; mean  $\pm$  SEM).  
 See also Figure S1 and Table S2.

regulation of gene expression in the experimentally undisturbed striatum that occurs in response to AF64 $\alpha$  injection into the PPTg.

Utilizing this experimental paradigm, we found that the expression of ChAT and vAChT in the ipsilateral striatum were downregulated to a similar extent, regardless of Shh expression by DA neurons compared to the contralateral striatum (Figure 6K). In contrast, we observed a ~4-fold downregulation of GDNF expression upon AF64 $\alpha$  injection into PPTg of control mice, i.e., mice that produce Shh in DA neurons, but not of *Shh-nLZ<sup>Cre</sup>/Dat-Cre* mutant animals (Figure 6K). These results provide genetic evidence that increased Shh signaling specifically originating from mesencephalic DA neurons results in the repression of GDNF transcription in the striatum.

**Cholinergic Neurons of the Striatum Regulate Shh Expression in Mesencephalic DA neurons**

The observed upregulation of Shh expression by DA neurons upon neurotoxic insult to the PPTg suggested that expression of Shh by DA neurons is not static but could be regulated by cell extrinsic signals. We therefore explored the dynamic range of Shh expression and whether the striatum in addition to the

PPTg might be a source of signals that could contribute to the regulation of Shh expression by DA neurons.

We first investigated whether the acute interruption of mesostriatal communication by unilateral injection of 6-OHDA into the mFB of C57BL/6 *wt* and *GDNF-LZ*-mice alters Shh expression by DA neurons. Thirty hours after toxin injection, we observed contralateral turning biases in *wt* and *GDNF-LZ* animals consistent with reduced DA signaling to the striatum (Figures 7A and 7B). In these animals, we found an upregulation of Shh expression in the vMB ipsilateral to the toxin injection. These results suggested that the striatum could be a source of signals that inhibit Shh expression in the vMB (Figure 7C).

Use of the cholinotoxin AF64 $\alpha$  afforded us to test next whether signals emanating from striatal ACh neurons could contribute to the repression of Shh transcription in DA neurons. Thirty hours postunilateral striatal injection of AF64 $\alpha$  into *wt* C57B/6 mice, we observed a dose-dependent ipsilateral turning bias consistent with a graded increase in striatal motor output due to a progressive, AF64 $\alpha$  induced, inhibition of cholinergic activity (Figures 7A and 7D) (Lester et al., 2010). In the ipsilateral vMB of these animals, we found an AF64 $\alpha$  dose-dependent, step-wise, upregulation of Shh transcription correlated with the

graded turning bias ( $R^2 = 0.79$ ,  $p < 0.001$ ; Figure 7E). The upregulation of Shh expression was transient and returned to control levels by 6 days posttoxin injection (Figure 7E). In addition to Shh expression, we also found AF64 $\alpha$  dose-dependent alterations in the expression of dopaminergic markers that resembled the distortions seen in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice (Supplemental Results D and Figure S3E) indicating that altered signaling by cholinergic neurons contributes to the dopaminergic cell syndrome that we observe in the absence of Shh signaling from DA neurons (Figure 2).

These findings suggested that the transcription of Shh in DA neurons is activated in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice. Because the design of the *Shh-nLZ<sup>C/C</sup>* allele leaves intact the promoter and most transcriptional enhancer regions of the native Shh locus after Cre mediated recombination we devised a qPCR based assay to test whether Shh expression was activated in DA neurons before and after ACh neuron degeneration in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice (for technical details see Supplemental Results A and Figure S1). We found an  $\sim 5$ -fold and  $\sim 4$ -fold increase in the transcription of the 5'-end of the Shh mRNA that can be expressed from the truncated Shh locus in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* at 4 weeks and 12 months of age, respectively. Thus, *Shh-nLZ<sup>C/C</sup>/Dat-Cre* animals cease to produce signals that otherwise inhibit Shh expression by mesencephalic DA neurons in the undisturbed brain (Figure 7F).

The identification of the receptor(s) on DA neurons that transmit the signals that impinge on the regulation of Shh expression can inform on the nature of the signals. We noted that the tissue specific ablation of the canonical receptor Ret, which can bind all members of the GDNF family of ligands, from DA neurons utilizing the same *Dat-Cre* allele also employed in the present study, resulted in alterations in dopaminergic marker gene expression, deficits in elicited DA release and late-onset, progressive DA neuron degeneration, (*Ret<sup>C/C</sup>/Dat-Cre* mice) (Kramer et al., 2007). We tested whether also the expression of Shh is altered in the vMB of *Ret<sup>C/C</sup>/Dat-Cre* mice. We found an  $\sim 6$ -fold upregulation of Shh expression in *Ret<sup>C/C</sup>/Dat-Cre* mice compared to litter controls at 3 months of age prior to observable neurodegeneration (Figure 7F). Taken together, our studies provide pharmacological and genetic evidence that ACh neurons of the striatum produce signals, which engage the canonical GDNF receptor Ret on DA neurons and repress the expression of Shh, and regulate the expression of multiple other genes in DA neurons.

## DISCUSSION

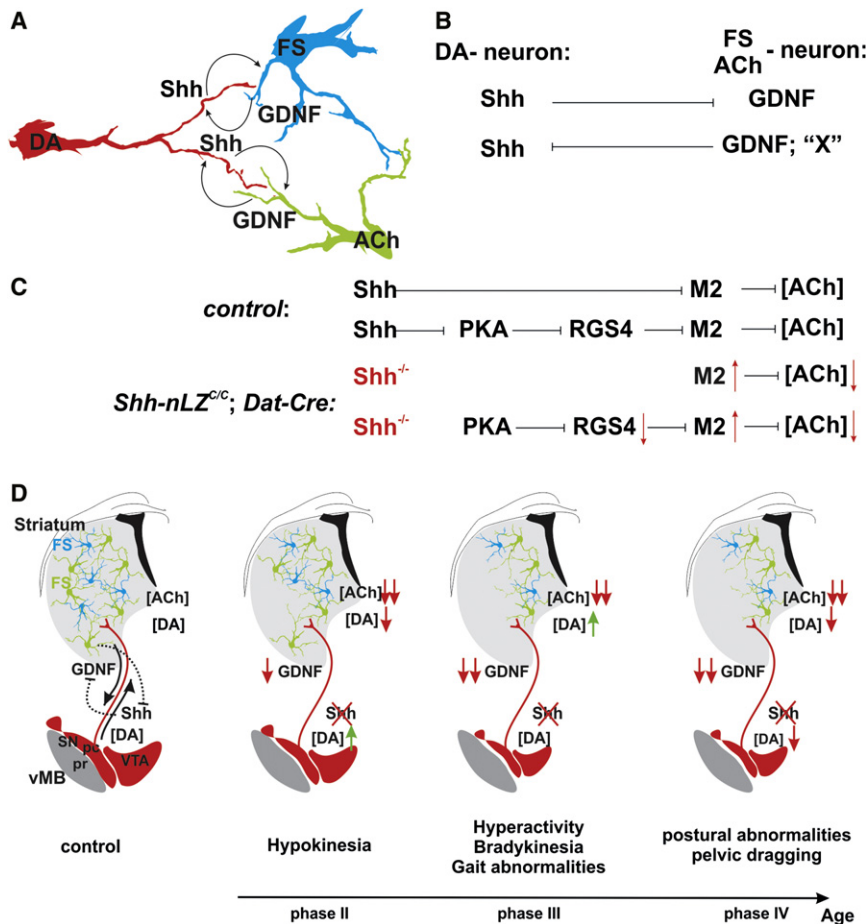
Our results reveal that mesencephalic DA neurons express Shh throughout life and demonstrate that DA neuron-produced Shh is necessary for the long-term structural and functional maintenance of mesencephalic DA neurons. Our studies, however, did not uncover any evidence for an autocrine mode of Shh signaling. Instead, we demonstrate that DA neuron-produced Shh regulates the availability of the dopaminotrophic factor GDNF and cholinergic physiology within the striatum in two distinct ways: First, DA neuron-produced Shh is required for the structural and functional maintenance of FS and ACh interneurons of the striatum, which are prominent sources of GDNF

in the adult basal ganglia. Second, signaling by DA neuron-produced Shh results in the transcriptional repression of GDNF and the regulation of expression of muscarinic autoreceptor signaling components. Taken together, our results reveal a means by which mesencephalic DA neurons communicate with a subset of their striatal neuronal targets and regulate the cellular and neurochemical homeostasis in the mesostriatal circuit in the adult brain. We further provide in vivo evidence that signals engaging the canonical GDNF receptor Ret expressed specifically on DA neurons and originating from the striatum inhibit the transcription of Shh in DA neurons. Our findings are consistent with the existence of a reciprocal trophic factor signaling loop between DA neurons on one side and ACh and FS neurons on the other side and reveal that the regulation of expression of these factors has rheostat properties.

### Reciprocal Trophic Factor Signaling between Mesencephalic DA, Striatal ACh, and FS Neurons

To resolve the mechanism of action of Shh signaling in the mesostriatal circuit required us to reconcile two sets of seemingly contradictory observations: (1) the apparent cell autonomous activity of Shh on DA neurons in the absence of evidence for autocrine signaling, and (2) the reciprocal inhibition of expression of Shh and GDNF in the mesostriatal circuit despite the finding that these factors are necessary for the trophic support of ACh and FS neurons, and DA neurons, respectively.

The inefficiency of Cre-mediated recombination of the Shh allele resulted in  $Shh^+$  and  $Shh^-$  DA neurons, which allowed us to investigate whether Shh expression by DA neurons confers a cell survival advantage. Our results reveal a  $\sim 2$ -fold enrichment of Shh-expressing DA neurons during phenotype progression in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice, demonstrating that mostly  $Shh^{-/-}$  DA neurons degenerate. Thus, our studies provide evidence for a neuroprotective function of DA neuron-expressed Shh on DA neurons in the adult mesencephalon and are consistent with findings that exogenously supplied Shh to the basal ganglia increases the resilience of mesencephalic DA neurons to neurotoxic insults (Dass et al., 2005; Tsuboi and Shults, 2002). Yet, it is unlikely that the degeneration of DA neurons in the absence of Shh expression by DA neurons results from the interruption of a cell autonomous effect of Shh because: (1) we cannot find evidence for the expression of the Shh coreceptors Ptc1 or Ptc2 on mesencephalic DA neurons, and (2) the *Dat-Cre* mediated tissue restricted ablation of the obligate necessary Shh signaling component Smo from DA neurons does not phenocopy the *Dat-Cre* mediated tissue restricted ablation of Shh from DA neurons. Instead, we find that DA neuron-produced Shh modulates gene expression, physiology, and survival of striatal ACh and FS neurons, which, in turn, express the dopaminotrophic factor GDNF (Figure 8A) (Hidalgo-Figueroa et al., 2012). Thus, the most parsimonious explanation for the apparent cell autonomous protection of DA neurons by Shh expression is the possibility that individual cartridges of mesostriatal circuits act as autonomous units. In this scenario, neuronal circuits in which DA neurons have escaped Cre-mediated recombination of the Shh alleles will continue to supply Shh to support ACh and FS neurons, and those ACh and FS neurons will continue to supply GDNF to support DA neuron survival. This model is



**Figure 8. Shh Signaling from Dopaminergic Neurons Controls Structural and Neurochemical Homeostasis in the Mesostratial Circuit**

(A) Shh and GDNF provide additional means of communication between DA, ACh, and FS neurons of the mesostratial circuit. (B) Rheostat properties of trophic factor signaling. (C) Potential signaling pathways by which Shh originating from mesencephalic DA neurons determines the set-point of extracellular ACh tone in the striatum. (Red arrows indicate direction of change observed in *Shh-nLZ<sup>Cre</sup>/Dat-Cre* mice.) (D) Absence of Shh signaling from DA neurons causes progressive structural and functional decay of the mesostratial circuit.

neurons to the striatum. Conversely, we find that the interruption of mesostratial communication by the neurotoxin 6-OHDA or striatal injection of the Shh antagonist cyclopamine leads to an upregulation of GDNF expression in the striatum, whereas striatal injection of the Shh agonist SAG or the pharmacological induced upregulation of endogenous Shh signaling specifically from mesencephalic DA neurons results in the inhibition of GDNF expression in the striatum. Thus, ACh neurons, which are trophically dependent on Shh from DA neurons, are a source of graded inhibitory signals for the transcription of Shh by DA neurons.

In a mirror arrangement, DA neurons

supported by the quantification of synaptic connectivity in the striatal microcircuit: although ACh, FS, and DA neurons elaborate widespread arborizations, each neuron only contributes to a few hundred of the estimated two million mesostratial circuits in the striatum (Bolam et al., 2006). Further support of a confinement of Shh action to the vicinity of Shh release sites comes from Loulier et al. (2005) who found strong expression in the adult striatum of the Hedgehog-interacting protein (Hhip), which inhibits Shh signaling by binding to secreted Shh, likely further limiting the poor diffusion of Shh once secreted (Ulloa and Briscoe, 2007). Thus, a given DA neuron might be able to signal via Shh to only a few ACh and FS neurons and receive trophic support from the same neurons resulting in the appearance of cell autonomy.

Trophic support of ACh and FS neurons by DA neuron-produced Shh on one side and of DA neurons by ACh and FS neuron produced GDNF on the other side could be provided in a static manner or be induced in response to physiological needs. We observe transcriptional activation of Shh loci in the vMB upon (1) injection of the dopaminergic neurotoxin 6-OHDA into the mFB, (2) induction of cholinergic dysfunction by injection of the cholinotoxin AF64α into the striatum, (3) genetic ablation of the canonical GDNF receptor Ret from DA neurons, and (4) genetic reduction of Shh signaling from DA

that are supported by GDNF modulate the expression of GDNF in the striatum by graded Shh expression (Figure 8B).

Although our data strongly implicates striatal GDNF in the regulation of Shh in DA neurons, our results do not exclude the possibility that other members of the GDNF family of ligands, all of which can signal via Ret and Gfra1, could contribute to the modulation of Shh expression in DA neurons.

The extent of relief of transcriptional inhibition of Shh expression by signals from ACh neurons is correlated with the degree of cholinergic dysfunction when averaged across DA neurons and has a dynamic range of 2- to 10-fold. This observation fits well with the established concentration-dependence and dynamic range of Shh signaling (Ulloa and Briscoe, 2007). Low levels of Shh signaling are necessary for tissue maintenance in the developing spinal cord. At higher concentrations Shh regulates in a concentration-dependent manner, gene expression mediated by either transcriptional repressor or activator forms of the Shh signaling components Gli-1, -2, and -3. At these expression levels, 1.8-fold alterations in the concentration of Shh results in distinct patterns of gene expression (Ulloa and Briscoe, 2007), suggesting that the dynamic range of 2- to 10-fold observed in our studies could result in several distinct physiological responses of ACh and FS neurons to Shh signaling.

Mutual trophic dependence combined with reciprocal inhibition of trophic factor expression must result in tight homeostatic control of Shh and GDNF expression and links the extent of Shh and GDNF signaling to the cell physiological status of DA, ACh, and FS neurons. We thus propose that attenuation of gene expression in response to physiological stress in DA, ACh, and FS neurons will result in a corresponding reduction in the repression of either Shh or GDNF, respectively, in such a way that cells in need will receive increased trophic factor support. After regaining intracellular homeostasis, reactivated gene expression will increase trophic factor production in those cells that had suffered from a cell physiological insult, and, in turn, will lead to a reduction in the expression of the corresponding trophic factor (Figure 8B).

These results also imply that trophic factor expression cannot be maintained chronically at levels that are beneficial for the survival of DA, ACh, and FS neurons once in distress. This reasoning points to multiple functions of GDNF and Shh signaling in the basal ganglia. Indeed GDNF signaling can regulate the quantal size of DA release of DA neurons (Pothos et al., 1998). Our studies reveal a corresponding function of Shh signaling on cholinergic neurotransmission extending the symmetry of Shh and GDNF signaling from trophic interactions to neuro-modulation within the nigro-striatal circuit.

#### Regulation of the Balance of Cholinergic and Dopaminergic Tone in the Striatum by Shh Signaling

Extracellular ACh tone in the striatum is variably regulated by DA neuron activity (Threlfell et al., 2010). However, dopaminergic activity does not exert its effects on ACh neurons exclusively through DA receptor signaling, but also through the regulation of the coupling of muscarinic autoreceptors to  $K^+$  and  $Ca^{2+}$  channels by altering the expression of “regulator of G-protein signaling” factors (RGS) (Ding et al., 2006). These findings raise the possibility that other signaling molecules other than dopamine produced by DA neurons are involved.

Our experiments show that Shh signaling originating from DA neurons impinges onto the regulation of the set-point of ACh in the striatum through the transcriptional regulation of M2 and possibly by engaging a pathway that involves the Shh-dependent inhibition of protein kinase A (PKA) (Ogden et al., 2008) which is an activator of RGS4 (Huang et al., 2007), whose inhibition increases the efficacy of muscarinic autoreceptor function (Ding et al., 2006) (Figure 8C).

Lowered cholinergic tone, a reduction of striatal GDNF levels due to progressive degeneration of ACh neurons, and a lack of functional adaptations by surviving ACh neurons should all influence the physiology of surviving DA neurons in *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice. Consistent with this expectation, we observe highly dynamic distortions in DA tissue content in both the vMB and the striatum. Hence, our results suggest that surviving DA neurons, but not surviving ACh neuron, are able to adapt their physiology dynamically in the face of progressive neurodegeneration and decreased ACh and GDNF/Ret signaling during early adulthood. However, by 10 months of age, we find the manifestation of discrete locomotion and gait disturbances, indicating that the progressive deterioration of the mesostriatal circuit surpasses the compensatory

capacity of DA neurons in aged *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice (Figure 8D).

#### Possible Implications for Understanding the Etiology of Diseases of the Basal Ganglia

Archetypes of basal ganglia models imply that an imbalance of cholinergic and dopaminergic signaling in the striatum is responsible for the hyper- and hypokinetic manifestations of progressive movement disorders such as PD (Obeso et al., 2010). Our work describes a mouse paradigm that recapitulates many of the key features of the progressive cellular, neurochemical, and functional pathologies observed in PD with apparent face-, construct-, and predictive-validity since the functional phenotype that is associated with progressive neuronal loss can be ameliorated with DA supplementation or a muscarinic antagonist also used in the management of PD. Yet, the resemblance of the phenotype of *Shh-nLZ<sup>C/C</sup>/Dat-Cre* mice with PD does not extend to the absolute direction of alterations in cholinergic tone.

In PD, ACh tone is increased (Wooten, 1990), while in our paradigm ACh tone is decreased in the absence of Shh signaling from DA neurons, which also must occur in PD. How can these findings be reconciled? Our data points to the possibility that Shh production is increased in still functioning DA neurons in response to the mounting pathophysiological cell stress in the basal ganglia of PD patients. This notion is supported by several in vivo experiments described herein: we demonstrate that the transcription of Shh in DA neurons is strongly upregulated upon (1) injection of 6-OHDA into the mFB, (2) induction of cholinergic dysfunction in the striatum, (3) induction of cholinergic dysfunction in the PPTg, and (4) the genetic ablation of part of the Shh locus which abrogates the production of functional Shh by DA neurons. Hence, our data are consistent with a scenario in which prior to reaching an advanced stage of PD, surviving DA neurons express elevated levels of Shh, which, in turn, cause an increase of ACh tone in the striatum mediated by a downregulation of muscarinic autoreceptor efficacy.

Our data suggest that the pharmacological manipulation of Shh signaling can be used to modulate cholinergic tone and reinforce the rationale for supporting growth factor signaling as a disease modifying therapeutic strategy in basal ganglia diseases. However, the uncovered negative feedback regulation of endogenous growth factor expression within the mesostriatal circuit predicts that exogenously supplied trophic factors could inhibit endogenous expression of the same factors possibly curtailing the therapeutic benefit of this approach. Instead, our results point to the possibility that undercutting the negative feedback regulation of endogenous growth factor expression could result in therapeutically effective increases of trophic factor signaling within the basal ganglia.

#### EXPERIMENTAL PROCEDURES

##### Mouse Strains

The *Shh-nLZ<sup>C</sup>* allele was generated by homologous recombination in ES cells. Additional construction details, mouse strains and genotyping procedures are described in Supplemental Experimental Procedures. All animal handling and procedures were approved by the Animal Care and Use Committee of Columbia University and performed in accordance with NIH guidelines.

**Immunohistochemistry**

Immunohistochemistry was performed on 16–100  $\mu\text{m}$  cryostat-cut sections using primary and secondary antibodies listed in [Supplemental Experimental Procedures](#). Images were acquired on a Zeiss LSM510 Meta confocal microscopes. Quantification of the size of populations of cells was estimated by the optical fractionator method described in [Supplemental Experimental Procedures](#).

**Quantification of GDNF Tissue Content**

Tissue levels of GDNF were measured by ELISA (GDNF Emax ImmunoAssay System; Promega, Madison, WI), according to the manufacturer's protocol.

**Quantification of Gene Expression**

Total RNA from striatum and lateral vMB containing the entire SN and VTA was isolated (RNeasy Mini Kit; QIAGEN) and reverse transcribed using oligo(dT) primers and the SuperScript First-Strand Synthesis System (Invitrogen), according to the manufacturers' protocols. Relative changes in gene expression were quantified by rtPCR using TaqMan gene expression assays (Applied Biosystems) with amplicons listed in [Table S2](#) and calculated by the  $\Delta\Delta\text{Ct}$  method.

**Neurochemical Analysis**

Determination of the concentration of dopamine and acetylcholine and neurotoxicological challenges were performed as described in [Supplemental Experimental Procedures](#).

**Locomotion Analysis**

Analysis of gait parameters by forced locomotion was performed by ventral plane videography (Digigait, Mouse Specifics, Inc., Boston, MA) Spontaneous motor activity was measured in an open field arena using automatic tracking at 6 Hz by an EthoVision 3.1 system (Noldus Information Technology, Leesburg, VA). Derivation of indices for turning bias is described in [Supplemental Experimental Procedures](#).

**Statistical Analysis**

The mean and SEM of values were calculated and the significance of all pairwise comparisons was determined by two-tailed distribution homoscedastic Student's *t* test and by ANOVA, including a repeated-measures factor when necessary. Follow up analysis between groups with multiple comparisons was by Tukey's post hoc test. Nonparametric data were analyzed by Mann-Whitney *U* test. For all box plots, the box includes data points between the 25<sup>th</sup> and 75<sup>th</sup> percentile of all values, with the line representing the median value. The lines and whiskers represent data between the ninth and ninety-first percentile and individual dots represent outlier points.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2012.05.018>.

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