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The Zinc Finger Transcription Factor Sp9 Is Required for the Development of Striatopallidal Projection Neurons

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SUMMARY

Striatal medium-sized spiny neurons (MSNs), composed of striatonigral and striatopallidal neurons, are derived from the lateral ganglionic eminence (LGE). We find that the transcription factor Sp9 is expressed in LGE progenitors that generate nearly all striatal MSNs, and that Sp9 expression is maintained in postmitotic striatopallidal MSNs. *Sp9* null mice lose most striatopallidal MSNs due to decreased proliferation of striatopallidal MSN progenitors and increased *Bax*-dependent apoptosis, whilethe development of striatonigral neurons is largely

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, 7 figures, and 2 tables and can be found with this article online.

AUTHOR CONTRIBUTIONS

Q.Z. and Y.Z. performed all experiments and analysis except RNA *in situ* hybridization performed by C.W. L.A. helped perform and analyze ChIP-qPCR experiments. Z.X., Q.L., J.L., Z.L. and Y.Y. helped conduct experiments and analyze the data. M.H., Y.M., B.C., Z.-Q.X. and J.L.R. helped guide the project and analyzed results. Z.Y. designed experiments and analyzed results. J.L.R. and Z.Y. wrote the manuscript.

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unaffected. ChIP-qPCR provides evidence that Ascl1 directly binds the *Sp9* promoter. RNA-Seq and *in situ* hybridization reveal that Sp9 promotes expression of *Adora2a*, *P2ry1, Gpr6* and *Grik3* in the LGE and striatum. Thus, Sp9 is crucial for the generation, differentiation and survival of striatopallidal MSNs.

Keywords

Sp9; LGE; striatum; striatopallidal neuron; Drd2; Ascl1; *Adora2a*

INTRODUCTION

The striatum (caudate nucleus and putamen) is the largest component of the basal ganglia. The majority of striatal neurons (90–95%) are DARPP-32 expressing ($^+$) GABAergic medium-sized spiny neurons (MSNs). Two major subtypes of MSNs send outputs to other components of the basal ganglia: striatonigral (direct pathway) and striatopallidal (indirect pathway). Striatonigral MSNs express the dopamine D1 receptor (Drd1) and neuropeptide Tac1 (known as substance P), while striatopallidal MSNs express the dopamine D2 receptor (Drd2) and neuropeptide enkephalin (Enk) (Gerfen, 1992; Gerfen et al., 1990; Gerfen and Surmeier, 2011; Kawaguchi et al., 1990). About 5–10% of neurons in the striatum are aspiny interneurons; they are choline acetyltransferase $(ChAT)^+$, parvalbumin $(PV)^+$, somatostatin $(SST)^+$ and calretinin $(CR)^+$ (Kawaguchi et al., 1995).

Interneurons in the mouse striatum are known to mainly originate from the medial ganglionic eminence (MGE) (Marin et al., 2000; Xu et al., 2008). Recent studies provide evidence that human and monkey striatal interneurons are also derived from the MGE (Wang et al., 2014). The LGE, the primordium of the striatum, contains two distinct compartments: a dorsal part which is the source of most interneurons in the olfactory bulb (Stenman et al., 2003; Waclaw et al., 2006; Yun et al., 2001), and a ventral part which generates striatal MSNs (Anderson et al., 1997; Deacon et al., 1994; Olsson et al., 1998; Olsson et al., 1995; Stenman et al., 2003).

Translational profiles of striatonigral and striatopallidal MSNs have been defined using the fluorescent activated cell sorting (FACS) and translating ribosome affinity purification (TRAP) approaches. These studies identified more than 200 striatonigral-enriched genes and more than100 striatopallidal-enriched genes (Ena et al., 2013; Heiman et al., 2008; Lobo et al., 2006). Some transcriptional factors (TFs) that control striatal MSNs development are beginning to be identified. *Dlx1&2* are required for generating late-born MSNs (Anderson et al., 1997), whereas *Dlx6* controls striatal regional molecular properties (Wang et al., 2011). *Bcl11b* (also known as *Ctip2*) is critical for the differentiation of all MSNs (Arlotta et al., 2008). *Ebf1* is essential for the differentiation of striatonigral MSNs (Lobo et al., 2006; Lobo et al., 2008). The LIM homeobox gene *Isl1* is required for the specification of striatonigral cell identity and the correct development of the striatonigral pathway (Ehrman et al., 2013; Lu et al., 2014). Thus, we know some of the TFs involved in making the striatonigral MSNs, but little is known about the molecular mechanisms controlling the development of striatopallidal MSNs.

Here, we investigated the role of *Sp9* zinc finger TF in the development of MSNs. *Sp9* is widely expressed in the ganglionic eminences (GEs) and in all striatopallidal MSNs. We generated a *Sp9-Cre* knockin mouse and find that $Sp9^{+}$ progenitors give rise to most GABAergic neurons in the telencephalon, including cortical and olfactory bulb interneurons, striatal MSNs and striatal interneurons. In postmitotic MSNs, Sp9 is specifically expressed in all striatopallidal neurons, but not in striatonigral neurons. We also generated $Sp9^{LacZ/LacZ}$ null mutant mice, and observed a 97% reduction of striatopallidal MSNs but saw little change in striatonigral MSNs. BrdU pulse-labeling and BrdU birth-dating experiments indicated the neurogenesis ability of LGE progenitors, especially striatopallidal MSN progenitors, was compromised. Moreover, loss of *Sp9* function resulted in a lack of striatopallidal neuron differentiation and a *Bax*-dependent apoptosis in the striatum during postnatal life. Conditional inactivation of *Sp9* using *Drd2-Cre* transgenic mice further revealed the function of *Sp9* in regulating striatopallidal MSN survival, at least in part, was cell-autonomous. ChIP-qPCR experiments showed that Ascl1 was upstream of *Sp9* in the LGE and directly bound to its promoter. Finally, RNA-Seq and RNA *in situ* hybridization indicate that the prosurvival effect of Sp9 in striatopallidal MSNs may be through promoting the expression of three G-Protein Coupled Receptors (GPCRs), including adenosine A2a receptor (*Adora2a*), purinergic receptor P2Y, G-protein coupled 1 (*P2ry1*) and *Gpr6*, and glutamate ionotropic receptor kainate type subunit 3 (*Grik3*). Taken together, our studies uncover a crucial role for Sp9 in the generation, differentiation and survival of striatopallidal MSNs.

RESULTS

Sp9 Is Widely Expressed in the LGE, MGE and CGE

To systematically study the expression and function of the TF Sp9 in the telencephalon, we generated Sp9 polyclonal antibodies, and several *Sp9* mutant alleles. Based on the 'knockout-first' strategy (Skarnes et al., 2011; Testa et al., 2004), we generated a *Sp9* knockout-first null allele which in turn produced a *Sp9-LacZ* null allele ($Sp9^{LacZ/+}$) and a *Sp9* floxed allele ($Sp9^{Flox/+}$), following exposure to germline recombinases *Zp3-Cre* (de Vries et al., 2000) and *ACTB-Flpe* (Rodriguez et al., 2000), respectively (Figure S1A-D).

Sp9-LacZ expression was detected in the GEs at E10.5 (Figure 1A-D). Immunocytochemistry of Sp9 and *Sp9-LacZ* (β-galactosidase, β-gal) and *in situ Sp9* RNA hybridization demonstrated that *Sp9* RNA and Sp9 protein were widely expressed in the subventricular zone (SVZ) and mantle zone of the LGE, MGE and CGE at E13.5 (Figure 1E-H). A subset of $Sp9^{+}$ cells in the SVZ of GEs expressed the proneural protein Ascl1 and cell proliferation marker Ki67 (Figure 1I-K). This suggests that while Sp9 is not detectable in radial glia cells (primary stem/progenitor cells) in the ventricular zone (VZ), it is expressed in a subpopulation of dividing progenitors in the SVZ and postmitotic neurons in the mantle zone. The GEs generate neocortical interneurons, including in humans (Hansen et al., 2013; Ma et al., 2013). We observed that most migrating cortical interneurons expressed Sp9 at embryonic developmental stages (Figure 1E-H). Sp9 protein and *Sp9* RNA were not detected in $Sp9^{LacZ/LacZ}$ mutant mice (data not shown).

Sp9$^+$ Progenitors Generate Most Neocortical and Striatal GABAergic Neurons

To genetically identify the progenies of Sp9$^+$ progenitors, we generated *Sp9-Cre* knockin mice (Figure S1). Fate mapping at P30 using *Sp9-Cre; Rosa-YFP* mice showed that Sp9$^+$ progenitors generated > 96% of cortical interneurons (including PV$^+$, SST$^+$, CR$^+$, NPY$^+$ and VIP$^+$ subtypes; Figure S2A-M), and virtually all striatal interneurons (PV$^+$, SST$^+$, CR$^+$ and ChAT$^+$; Figure S2N-Q, S). Foxp1 is expressed by all postmitotic MSNs and is excluded from other striatal cell types (Arlotta et al., 2008; Tamura et al., 2004). All Foxp1$^+$ cells were GFP$^+$ (Figure S2R, S), demonstrating the *Sp9-Cre* is active during the development of both striatopallidal and striatonigral MSNs.

In the OB, the identities of GFP$^+$ cells were difficult to determine due to the high density of interneurons (Figure S2A). However, from the broad expression of GFP, we speculated that Sp9$^+$ progenitors also give rise to most (perhaps all) OB interneurons. We confirmed all of these results using *Sp9-Cre; Ai14* mouse lines (data not shown).

Sp9 Is Specifically Expressed in Drd2$^+$ Striatopallidal MSNs

Using *Drd2-EGFP* transgenic mice (Gong et al., 2003), we determine the specificity of Sp9's striatal expression (Figure S3). Sp9 was strongly expressed in the prenatal striatum; its expression continued into adulthood, albeit at a lower level (Figure S3A-L). Virtually all GFP$^+$ cells (Drd2$^+$ striatopallidal MSNs) in the *Drd2-EGFP* transgenic mouse striatum expressed Sp9 at E16.5, P0, P5, P17 and P35 (Figure S3A-L, O). In contrast, only about 7% of striatonigral MSNs in *Drd1-EGFP* mice expressed Sp9 at P5, and Sp9 was not detectable in Drd1-GFP$^+$ MSNs at P35 (Figure S3M). This result shows that Sp9 is expressed in both progenitors of striatopallidal and striatonigral MSNs, but in postmitotic MSNs, Sp9 expression becomes restricted to Drd2$^+$ striatopallidal MSNs. Next we assessed whether Sp9 regulated the development of MSNs.

Most Striatopallidal MSNs Are Lost in *Sp9$^{LacZ/LacZ}$* Mutant Mice

To investigate the function of Sp9 in the development of the striatum, we first analyzed *Sp9$^{LacZ/LacZ}$* mutant mice. While *Sp9$^{LacZ/+}$* mice (referred to as controls) developed normally and were fertile, *Sp9$^{LacZ/LacZ}$* mutant mice failed to thrive and developed general weakness from P7 onward (Figure 2A). These mice started to die at P14, and none survived beyond P22. The brain size and weight of the *Sp9* mutants were grossly reduced compared to controls at P20 (Figure 2B, C).

The most prominent phenotype in the *Sp9* mutant telencephalon was atrophy of the striatum; the volume of mutant striatum was reduced to 54% of controls at P9 (Figure 2D, E). Compared to *Sp9$^{LacZ/+}$; Drd2-EGFP* mice (Figure 2F-J), we observed loss 57% of Foxp1$^+$ cells and 97% of Foxp1$^+$/Drd2-GFP$^+$ cells in the striatum of *Sp9$^{LacZ/LacZ}$; Drd2-EGFP* mice at P9 (Figure 2K-O, X, Y). The remaining Foxp1$^+$/Drd2-GFP$^+$ cells were not evenly distributed in the mutant striatum. In the dorsal medial striatum, Foxp1$^+$/Drd2-GFP$^+$ cells were barely detectable (Figure 2M). In contrast, most remaining Foxp1$^+$/Drd2-GFP$^+$ cells were located in the lateral striatum (Figure 2M), a population of MSNs that are generated at early developmental stages (Anderson et al., 1997). *Drd2 in situ* hybridization confirmed the loss of the cell type with this molecular signature (Figure 2P, T). Furthermore the mutants

showed severe loss of Enk$^+$ cells, further evidence for a defect in striatopallidal MSNs (Figure 2R, V). A small population of Drd2-GFP$^+$ cells remained in the dorsal striatum of *Sp9* mutants; however, these Drd2-GFP$^+$ cells expressed ChAT, and thus were an interneuron subtype (Figure 2Q, U) (Durieux et al., 2009).

Striatal MSNs are roughly equally divided into striatopallidal and striatonigral neurons. Although Drd1$^+$ and Drd1-GFP$^+$ striatonigral neurons were not apparently affected (Figure 2S, W), the observation of loss 57% of striatal Foxp1$^+$ cells in *Sp9*$^{LacZ/LacZ}$ mutant striatum (Figure 2X) suggesting that striatonigral MSNs are slightly affected.

Sp9 Mutant LGE SVZ Has Reduced Proliferation

To test whether Sp9 regulates cell proliferation in the LGE, we performed a 30-min BrdU pulse-labeling at E13.5 and E15.5 (Figure 3A-H). At E13.5, the number of BrdU$^+$ cells in the *Sp9*$^{LacZ/LacZ}$ mutants was reduced in the SVZ, but not the VZ (Figure 3M). At E15.5, we observed a reduction in SVZ BrdU$^+$ progenitor cells and in SVZ Foxp1$^+$ postmitotic MSNs (Figure 3E-H, N).

Next, we performed a BrdU birth-dating analysis: an injection of BrdU was given at E12.5 and the number of BrdU$^+$ and BrdU$^+$/Foxp1$^+$ cells were counted in the striatum of P0 in *Sp9*$^{LacZ/LacZ}$ and wild type littermate controls (Figure 3I-L). The mutants had reduced BrdU$^+$ cells (996 ± 73 cells *vs.* 526 ± 62.5 cells per striatal section, p=0.0081) and BrdU$^+$/Foxp1$^+$ cells (911 ± 79.7 cells *vs.* 465 ± 52.7 cells per striatal section, p=0.0095; controls *vs.* mutants) (Figure 3O, P). The percentage of BrdU$^+$ and BrdU$^+$/Foxp1$^+$ cells was reduced in a similar ratio (47.2% *vs.* 49%). Thus, loss of *Sp9* function results in a loss of cycling progenitors (BrdU pulse-label data) in the LGE SVZ, which results in loss of striatal MSNs (BrdU birth-dating data).

Sp9 Specifically Regulates the Production of Striatopallidal MSNs

We have taken advantage of β-gal expression from the *Sp9*^{LacZ} mutant allele to follow Sp9$^+$ MSNs in the striatum of *Sp9* mutant mice. At P0, we found that both β-gal$^+$ (3638 ± 302.1 *vs.* 1223 ± 108.6, p=0.0016) and β-gal$^+$/Foxp1$^+$ (2866 ± 202.8 *vs.* 219 ± 10.8, p=0.0058) cells were greatly reduced in the *Sp9*$^{LacZ/LacZ}$ mutant striatum compared to *Sp9*$^{LacZ/+}$ control striatum (Fig. S4A-F). Because Sp9 is expressed in all striatopallidal MSNs and only in a few striatonigral MSNs, this provides evidence that loss of *Sp9* preferentially blocks production of striatopallidal MSNs.

To further assess the production of striatal MSNs at E16.5, we performed *in situ* RNA hybridization using ten striatal MSN markers. We used 5 markers for striatopallidal MSNs: *Drd2, Penk, Gpr6, Adora2a* and *Ptprm;* and 5 markers for striatonigral MSNs: *Drd1, Tac1, Ebf1, Pdyn* and *Chrm4* for striatonigral MSNs (Gerfen et al., 1990; Heiman et al., 2008; Lobo et al., 2006). All markers were detectable in *Sp9*$^{LacZ/+}$ controls, although *Gpr6* had low expression at this time point (Figure 4A-E, F-J). In contrast, in *Sp9*$^{LacZ/LacZ}$ mutant SVZ and striatum, striatopallidal MSN markers were either almost undetectable (*Gpr6* and *Adora2a*) or great downregulated (*Drd2, Penk,* and *Ptprm*) (Figure 4F'-J'). Our qRT-PCR analysis revealed similar results (Figure 4Q). On the other hand, striatonigral marker expression was modestly affected (Figure 4A'-E'). These results further support the

evidence from the BrdU birth-dating and Sp9-β-gal quantification data, that large population of mature striatopallidal MSNs are not produced in the absence of Sp9.

Next we compared GFP expression from the *Drd2-EGFP* allele in control vs. *Sp9* mutant E16.5 and P0 striatum (*$Sp9^{LacZ/+}$ vs $Sp9^{LacZ/LacZ}$).* The mutant had very few Foxp1+/Drd2-GFP^+ MSN cells in the SVZ and striatum (Figure 4K-P, R). Taken together, these results indicate that Sp9 specifically promotes the production of striatopallidal MSNs in the striatum.

Loss of *Sp9* Induces a *Bax*-dependent Apoptosis in the Postnatal Striatum

We next analyzed cell death in *$Sp9^{LacZ/LacZ}$* mutant postnatal striatum, as marked by Cleaved Caspase-3 expression. The number of Caspase-3^+ cells in the striatum of control mice increased from P0, reached a maximum at P3, and then declined (Figure 5A, C). The mutant had a robust increase in the number of Caspase-3^+ cells in the striatum at all postnatal stages analyzed (P0, P3, P5 and P7) (Figure 5B, C). Because most mature striatopallidal MSNs were not generated, because we did not observe Caspase-3^+ cells express Drd2-GFP, and because we did not observe a severe reduction of striatonigral MSNs, we inferred that most of the dying cells in *Sp9* mutant striatum were immature striatopallidal MSNs.

To determine whether *$Sp9^{LacZ/LacZ}$* striatopallidal MSNs apoptosis was Bcl-2-associated X protein (Bax)-dependent, we generated *$Sp9^{LacZ/LacZ}$; $Bax^{-/-}$* double mutants. We found that striatal cell death was nearly absent in the *$Sp9^{LacZ/LacZ}$; $Bax^{-/-}$* double mutants at P3 (data not shown). We analyzed the numbers of Foxp1^+ cells in the striatum of *$Sp9^{LacZ/+}$; $Bax^{-/+}$*, *$Sp9^{LacZ/LacZ}$; $Bax^{-/+}$* and *$Sp9^{LacZ/LacZ}$; $Bax^{-/-}$* mice at P15 (Figure S5A-I, P) and found that the number of Foxp1^+ cells in *$Sp9^{LacZ/LacZ}$; $Bax^{-/-}$* striatum was significantly larger than that in *$Sp9^{LacZ/LacZ}$; $Bax^{-/+}$* striatum (Figure S5P). Perhaps because this genetic manipulation didn't rescue Sp9-mediated cell proliferation in the LGE SVZ, the number of Foxp1^+ cells in *$Sp9^{LacZ/LacZ}$; $Bax^{-/-}$* striatum was still smaller than that of in *$Sp9^{LacZ/+}$; $Bax^{-/+}$* striatum (controls) (Figure S5P).

We noted that the “rescued” Foxp1^+ cells in *$Sp9^{LacZ/LacZ}$; $Bax^{-/-}$* mutant failed to fully mature into striatopallidal MSNs as they did not express Enk, Drd2 and Adora2a at P15 (Figure S5J-O, data not shown), suggesting that in addition to promoting LGE proliferation and survival, *Sp9* is essential for the differentiation and maturation of striatopallidal neurons.

Sp9 in Striatopallidal MSNs Is Required for their Survival

All striatopallidal MSNs in the striatum express Sp9 (Fig. S3). To directly investigate the requirement of Sp9 in postmitotic striatopallidal MSNs, we utilized a conditional knockout strategy. We generated *Sp9* conditional mouse mutants by breeding male *Drd2-Cre; $Sp9^{Flox/+}$* mice with female *$Sp9^{Flox/Flox}$* mice (Figure S1) with or without the *Rosa-YFP* Cre-reporter allele. The *Drd2-Cre* transgene expresses Cre specifically in immature and mature striatopallidal MSNs (Gong et al., 2007). The offspring of this cross, including *Drd2-Cre; $Sp9^{Flox/Flox}$* conditional mutants, developed normally and were fertile.

We quantified the number of Foxp1$^+$ and Foxp1$^+$/GFP$^+$ cells in the striatum of *Drd2-Cre; Sp9$^{Flox/Flox}$; Rosa-YFP* mice and *Drd2-Cre; Sp9$^{Flox/Flox}$; Rosa-YFP* triple transgenic mice at P30 (Figure 5D-F). The mutants had decreased numbers of Foxp1$^+$ and Foxp1$^+$/GFP$^+$ cells (Figure 5F). Next, we analyzed cell death in the striatum of these mice. Similar to *Sp9* constitutive mutants, the striatum of *Sp9* conditional mutants had increased numbers of Caspase-3$^+$ cells at P0, P3 and P5 (Figure 5G-I). Thus, the loss of Drd2$^+$ striatopallidal MSNs in *Sp9* conditional mutants was due to cell programmed death, demonstrating that Sp9 expression in postmitotic striatopallidal MSNs is necessary for their survival.

Loss of Striatopallidal MSNs Results in Hyperlocomotion

Activation of the striatonigral (direct) pathway promotes locomotion, whereas activation of the striatopallidal (indirect) pathway inhibits locomotion (Durieux et al., 2009; Durieux et al., 2012; Kravitz et al., 2010). Therefore, the loss of striatopallidal MSNs observed in the *Drd2-Cre; Sp9$^{Flox/Flox}$* conditional mutant mice could influence locomotive behaviors. To test this, we examined the locomotive activity of 2-month-old *Drd2-Cre; Sp9$^{Flox/Flox}$*, *Drd2-Cre; Sp9$^{Flox/+}$* (control) and *Sp9$^{Flox/+}$* (control) mice in an open filed. Compared to controls, *Drd2-Cre; Sp9$^{Flox/Flox}$* mice exhibited a significant increase in locomotion based on the total distance traveled (Figure 5J). However, on the basis of the ratio of the center to the total distance in the open field test, we did not observe any significant difference in anxiety-related behaviors (Figure 5K). In addition, *Drd2-Cre; Sp9$^{Flox/Flox}$* mice showed normal performance in a rotarod test (Figure 5L), indicating normal motor coordination and motor learning. Overall, these experiments indicate that loss of striatopallidal MSNs in *Drd2-Cre; Sp9$^{Flox/Flox}$* mice resulted in hyperlocomotion, but no measurable change in anxiety or motor coordination/learning.

Ascl1 Directly Binds *Sp9*'s Promoter and Enhances its Expression *in vivo*

Previous studies have shown that proneural factor Ascl1 is required for the production of striatal Drd2$^+$ MSNs at E18.5 (Casarosa et al., 1999). We confirmed this result using *in situ* RNA hybridization. Compared with *Ascl1$^{GFP/+}$* control mice, the expression level of *Drd2* in P0 *Ascl1$^{GFP/GFP}$* mutant mice was severely reduced (Figure S6E, F), whereas the expression of *Drd1* was less affected (Figure S6G, H). We also observed that the expression of Sp9 (both protein and RNA) was reduced in the ventral LGE SVZ of *Ascl1$^{GFP/GFP}$* mutants at E14.5 and E16.5 (Figure 6A-D, Figure S6A-D), suggesting that Ascl1 positively regulates Sp9 expression. This is consistent with previous reports (Long et al., 2009a; Long et al., 2009b).

Ascl1 promotes cell differentiation and proliferation in GEs (Castro et al., 2011). Thus, it is possible that Ascl1 controls striatopallidal MSN progenitor proliferation in the LGE SVZ through Sp9. To test this hypothesis, we informatically studied the putative promoter and enhancers near the *Sp9* locus. We found the consensus Ascl1 binding motif (E-box sites, CAGCTG or CACCTG) in the putative promoter, and in two enhancers (E953 and E245) (Castro et al., 2011; Pacary et al., 2011) (Figure 6E). To determine whether Ascl1 physically associates with these regulation regions, we carried out ChIP-qPCR using an Ascl1 rabbit polyclonal antibody and chromatin prepared from E13.5 LGE of wild type mice. The *Rnd3* E1 enhancer (Rnd3 E1) was used as a positive control as previous studies demonstrated that

Ascl1 promotes *Rnd3* expression in the embryonic brain by a direct regulation of *Rnd3* E1 (Pacary et al., 2011). We found that the *Rnd3* E1, *Sp9* putative promoter, E953 and E245 were all enriched in the immunoprecipitated genomic DNA over input (Figure 6F).

We performed a Dual-luciferase transcription activation assay using the P19 embryonal carcinoma cell line. Ascl1 activated transcription from *Sp9*'s putative promoter and to a lesser extent from *Sp9* E245 (Figure 6G), whereas it did not activate *Sp9* E953 (Figure 6G). Finally, we found that the Ascl1 binding motif (CAGCTG) contributed to regulation of the *Sp9* promoter, as deletion CAGCTG attenuated Ascl1-mediated activation (Figure 6H). Together, these results provide evidence that Ascl1 promotes Sp9 expression in the LGE SVZ, at least in part, through its direct binding to the promoter and possibly other regulatory elements within the *Sp9* locus (e.g. E245).

Sp9 Promotes *Adora2a* Expression in the Striatum

To investigate the cause of striatopallidal MSN apoptosis in *Drd2-Cre; Sp9*$^{Flox/Flox}$ conditional knockout mice, we performed RNA-Seq analysis. We compared gene expression profiles from the P0 littermate *Sp9*$^{Flox/Flox}$ control striatum (including SVZ) and conditional mutant striatum of *Drd2-Cre; Sp9*$^{Flox/Flox}$ mice and identified about 90 genes that were either significantly upregulated or downregulated in *Drd2-Cre; Sp9*$^{Flox/Flox}$ mice (Table S1) (GEO: GSE83373). Of these, we found that the expression of three GPCRs (*Adora2a, Gpr6* and *P2ry1*) was significantly decreased in *Sp9* conditional mutant striatum, especially *Adora2a* (p= 0.0000000134, Q-value= 0.00000115) (Table S2). Two of them (*Adora2a* and *Gpr6*) are markers for striatopallidal MSNs (Heiman et al., 2008; Lobo et al., 2006); the third one (*P2ry1)* is predominately expressed in striatopallidal MSNs during development. In addition, *Grik3* expression was also greatly reduced in *Sp9* conditional mutant striatum. As most striatopallidal MSNs have not yet died at P0, the majority of striatopallidal MSN-specific genes were not significantly downregulated (Table S2), and none of striatonigral MSN-specific genes were significantly downregulated (Table S2). We postulate that genes with an expression difference prior to cell death may lead to apoptosis.

To validate our RNA-Seq results, we performed RNA *in situ* hybridization on E16, E18 and P4 sections with a number of these genes (Figure 7 and Figure S7). First, we confirmed the absence of Sp9 in the mutant LGE mantle zone (striatum) at E16 (normal expression in the SVZ is present because the Cre eliminates Sp9 in the mantle zone). GFP$^+$ and Cre$^+$ cells were present in striatum as apoptosis had not begun (Figure S7AC'). The development of striatonigral MSNs appeared normal based on expression of their markers, *Drd1* and *Ebf1* (Figure S7D-E'). The expression of striatopallidal MSN markers, *Drd2*, *Penk* and *Ptprm*, were slightly reduced (Figure S7F, F', I-J'). Strikingly, *Adora2a* RNA striatal expression was almost undetectable (Figure S7G, G') and *Gpr6* expression was severely reduced (Figure S7H, H') (Table S2). Similar results were also observed in E18 striatum (Figure 7A-H'). In addition, we observed that the expression of *P2ry1* and *Grik3* were greatly reduced (Figure 7I-J'), consistent with our RNA-Seq data. At P4, we began to observe the loss of Drd2$^+$ striatopallidal MSNs in *Sp9* conditional striatum (Figure S7K, K'). However, based on our in situ RNA hybridization, *Adora2a* RNA expression remained almost undetectable (Figure S7L, L'), and *Gpr6*, *P2ry1* and *Grik3* expression were still severely reduced in *Sp9*

conditional striatum (Figure S7M-O'). *Adora2a* RNA striatal adult expression continued to remain very weak (data not shown). These data indicate that Sp9 is required to drive *Adora2a* expression in the striatopallidal MSNs. Furthermore, the *Sp9* conditional mutants had reduced expression of two other GPCRs, *Gpr6* and *P2ry1*, and glutamate ionotropic receptor, *Grik3*, in striatopallidal MSNs.

Adora2a, among its many functions, participates in protecting cells from neuronal death after brain insults (Chen et al., 2007; Rivera-Oliver and Diaz-Rios, 2014). We propose that the apoptotic cell death of striatopallidal MSNs in the *Sp9* constitutive and conditional mutant is likely in part a consequence of the loss of *Adora2a* expression, along with attenuated expression of *Gpr6*, *P2ry1* and *Grik3*.

DISCUSSION

Here we find that Sp9 has several critical roles in striatal MSN development. $Sp9^+$ LGE progenitors generate most MSNs, and Sp9 expression is maintained in postmitotic striatopallidal MSNs, but not striatonigral MSNs. In the absence of *Sp9,* striatal development was disrupted for three main reasons: 1) LGE SVZ cell proliferation was reduced; 2) striatopallidal MSN differentiation and maturation was arrested; 3) *Bax*-dependent apoptosis of these neurons. The later two processes largely did not affect striatonigral neurons. We suggest that Ascl1 drives *Sp9* expression in the SVZ through binding and activation of the *Sp9* promoter. Finally, striatopallidal MSN survival is mediated in part through Sp9 driving the expression of several GPCRs, especially *Adora2a*.

Sp9 Specifically Promotes Striatopallidal MSN Progenitor Cell Division in the LGE SVZ

Sp9 expression is detected in the SVZ but not the VZ (primary neural stem/progenitors) of the GEs. Sp9 is co-expressed with progenitor cell markers, Ascl1 and Ki67, particularly at the VZ-SVZ boundary (also known as SVZ1) (Petryniak et al., 2007) (Figure 1I-K). The reduced numbers of S phase cells labeled by BrdU pulse at E13.5 and E15.5 in the constitutive null mutant mice indicate that Sp9 promotes proliferation of the progenitors. This reduced proliferation leads to a reduced LGE neurogenesis, as revealed by our BrdU birth-dating analysis, based on significant reduction of $BrdU^+$ and $BrdU^+/Foxp1^+$ cells in the *Sp9* mutant striatum at P0 (Figure 3I-L, O, P). Thus, decreased progenitor proliferation contributes to reduced numbers of MSNs in the mutant mice. Although $Sp9^+$ progenitors in the LGE SVZ give rise to all striatal MSNs, Sp9 expression is restricted to striatopallidal MSN in the striatum, as only a few of striatonigral MSNs express Sp9. Using the *Sp9-LacZ* mutant allele, we found that there was a significant reduction of $\beta\text{-gal}^+$ cells in P0 *Sp9* mutant striatum compared to $Sp9^{LacZ/+}$ controls (Figure S4). This strongly suggests that the function of Sp9 in the LGE SVZ is mainly promoting striatopallidal MSN progenitor cell proliferation.

Regulation of *Sp9* Expression in the LGE by Ascl1

Ascl1 is a basic helix-loop-helix proneural TF that is expressed in the VZ and SVZ of the GEs. Ascl1 expression has been shown to promote both cell proliferation (Castro et al., 2011) and cell cycle exit (Castro et al., 2006; Farah et al., 2000; Nakada et al., 2004). In the

present study, we showed that Ascl1 is co-expressed with Sp9 in SVZ of the LGE (Figure 1I, J). In *Ascl1* mutant mice, the expression of *Sp9* is reduced (Figure S6A-D, Figure 6A-D). These data indicate that *Sp9* is genetically downstream of *Ascl1*. We also found that loss of striatopallidal MSNs was more severe than loss of striatonigral MSNs in *Ascl1* mutant striatum (Figure S6E-H). Thus, *Ascl1* mutants phenocopy *Sp9* mutants; both have reduced LGE cell proliferation and *Drd2* striatal expression (Casarosa et al., 1999; Castro et al., 2011). Ascl1 ChIP-chip analysis with promoter microarrays revealed that Ascl1 activates a large number of positive cell cycle regulators (Castro et al., 2011). We thus hypothesized that Ascl1 regulates development of striatopallidal MSNs in part through controlling *Sp9* expression, which specifically promotes striatopallidal MSN progenitor division. Indeed, our ChIP-qPCR experiments supported this idea by showing that Ascl1 directly binds to *Sp9*'s promoter.

Sp9 Controls the Survival of Striatopallidal MSNs Likely via Positively Regulating GPCR Expression

Sp9 expression and function starts from LGE progenitors and then becomes restricted to differentiating striatopallidal MSNs. Consistent with this expression pattern, reduction of cycling progenitors was observed in the *Sp9* constitutive mutant LGE SVZ and reduction of markers of striatopallidal MSNs was observed in the LGE mantle zone (striatum) of *Sp9* constitutive and conditional mutants. The *Sp9* constitutive mutant may result in the premature cell cycle exit of striatopallidal MSN; these neurons have a differentiation defect (fail to express markers of striatopallidal MSNs) and undergo *Bax*-dependent apoptosis largely in the early postnatal period. Indeed, we never observed striatal Caspase-3^+ cells that expressed Drd2-GFP. Conditional inactivation of *Sp9* in striatopallidal MSNs using *Drd2-Cre* transgenic mice largely phenocopied the striatal defects of *Sp9* constitutive mutants, showing that elimination of *Sp9* beginning at E13.5 in striatal postmitotic neurons leads to the failure of striatopallidal MSN differentiation followed by their apoptotic elimination.

Previous studies have shown that Ntrk2 (known as TrkB), a receptor for brain-derived neurotrophic factor (BDNF), is required for the development of striatum and survival of striatopallidal MSNs (Baydyuk et al., 2011; Li et al., 2012). However, we did not find altered RNA expression of *Ntrk2* in the embryonic striatum of *Sp9* mutants (Figure S7C, D). This suggests that programmed cell death occurring in the *Sp9* mutant striatum is not due to the lack of *Ntrk2*. By contrast, we observed that the expression of GPCRs in striatopallidal MSNs was downregulated in the absence of *Sp9* at E16.5, E18 and P4 striatum. These GPCRs are *Adora2a*, *Gpr6*, and *P2ry1*. *Adora2a* and *Gpr6* are only expressed in striatopallidal MSNs; *P2ry1* is mainly expressed in striatopallidal MSNs during development. We also noted that the expression of *Adora2a* remained barely detectable in *Sp9* constitutive and conditional mutants from E16 to P4. This demonstrated that the expression of *Adora2a* in striatopallidal MSNs was dependent on Sp9. Indeed, even in adults, although ~30% of striatopallidal MSNs remained in the *Sp9* conditional mutant striatum, very few expressed *Adora2a* (data not shown).

Adora2a mediates neuroprotection against brain injuries in various animal models of neurological disorders. Adora2a can either promote, or protect from, cell death, depending

on the nature of brain injuries (Chen et al., 2007; Rivera-Oliver and Diaz-Rios, 2014). We show that Sp9 is required for *Adora2a* expression in differentiating and mature striatopallidal MSNs. We propose that the nearly complete loss of *Adora2a*, combined with partial loss of *Gpr6*, *P2ry1* and *Grik3* in striatopallidal MSNs during development, contributed to the striatal apoptotic cell death in *Sp9* constitutive and conditional mutants.

Loss of Striatopallidal MSNs and Human Disease

Aberrant basal ganglia circuitry leads to locomotor dysfunction in humans. Many diseases, such as Huntington's disease (HD) and attention-deficit/hyperactivity disorder (ADHD), are associated with abnormal striatal MSNs (Russell, 2007; Vonsattel et al., 1985). A dramatic decrease in the expression of *Adora2a* in the striatum is evident at a very early stage of the HD (Glass et al., 2000). The preferential loss of striatopallidal MSNs in the early and middle phases of HD contributes to choreiform movements (Mitchell et al., 1999; Reiner et al., 1988). As *Drd2-Cre; Sp9*$^{Flox/Flox}$ mutants have hyperlocomotion, this further supports the idea that striatopallidal MSNs promote movement inhibition (Kravitz et al., 2010). Thus, *Drd2-Cre; Sp9*$^{Flox/Flox}$ mutants may serve as a model to study basal ganglia disorders caused by abnormal development and survival of striatopallidal MSNs.

EXPERIMENTAL PROCEDURES

Animals

All experiments were performed in accordance with institutional guidelines. We generated *Sp9*$^{LacZ/+}$ mice, *Sp9*$^{Flox/+}$ mice and *Sp9-Cre* knockin mice in this study. See supplemental experimental procedures for detailed methods. Other mouse strains used in this study are *Drd2-EGFP*, *Drd2-Cre*, *Drd1-EGFP* (Gong et al., 2007; Gong et al., 2003) (from MMRRC) and *Ascl1*$^{GFP/+}$ knockin knockout (Kim et al., 2007; Leung et al., 2007), *Bax*$^{-/+}$ (Knudson et al., 1995). We used 2 Cre-reporter mouse lines in this study: *Rosa-YFP* (Srinivas et al., 2001) and *Ai14* (Madisen et al., 2010). All lines in this study were maintained in a mixed genetic background of C57BL/6J, 129S6 and CD1.

RNA-Seq

The striatum (including SVZ) from P0 *Sp9* $^{Flox/Flox}$ littermate controls and *Drd2-Cre; Sp9* $^{Flox/Flox}$ conditional mutants were dissected (n=3, each group). Total RNA was purified with a Mini RNA Isolation Kit (Zymo). RNA-Seq was performed as recommended by the manufacturer (Illumina). Levels of gene expression were reported in FPKM (fragments per kilobase of exon per million fragments mapped) (Trapnell et al., 2012). A gene was considered to be expressed if it had an FPKM >1. For a gene to be called as differentially expressed, it required a *P*-value <0.05. Data from this experiment has been deposited in the GEO database (accession GSE83373).

Chromatin immunoprecipitation and qPCR

ChIP was performed on E13.5 LGE using Ascl1 rabbit polyclonal antibody (Cosmo Bio, SK-T01-003). Co-precipitated DNAs were purified with phenol-chloroform, and detected by qRT-PCR (Vogt et al., 2014). The Rnd3 E1 is the positive control (Pacary et al., 2011). See experimental procedures for detailed methods.

Luciferase assays

The DNA fragments of E953 and E245 enhancers of *Sp9* gene were created by PCR and subsequent cloned into pGL4.23 firefly luciferase vector (Promega) upstream (U) or downstream (D) of *Luc2* gene (e.g. pGL4.23-E953U or pGL4.23-E953D). The putative *Sp9* promoter was amplified by PCR and cloned into pGL4.10 promoterless firefly luciferase vector (Promega). Mouse embryonal carcinoma cell line P19 were grown in medium MEMα (Gibco 12571-063) supplemented with 10% fetal bovine serum (FBS, Gibco 10099-141). For luciferase assay, P19 cells transfections were performed in triplicate in 24-well plates by using Fugene HD transfection regent according to the manufacturer's protocol (Promega, E2311). Luciferase sparks were quantified by microplate luminometer (Turner BioSystems, Inc. ModulusTM Microplate Reader).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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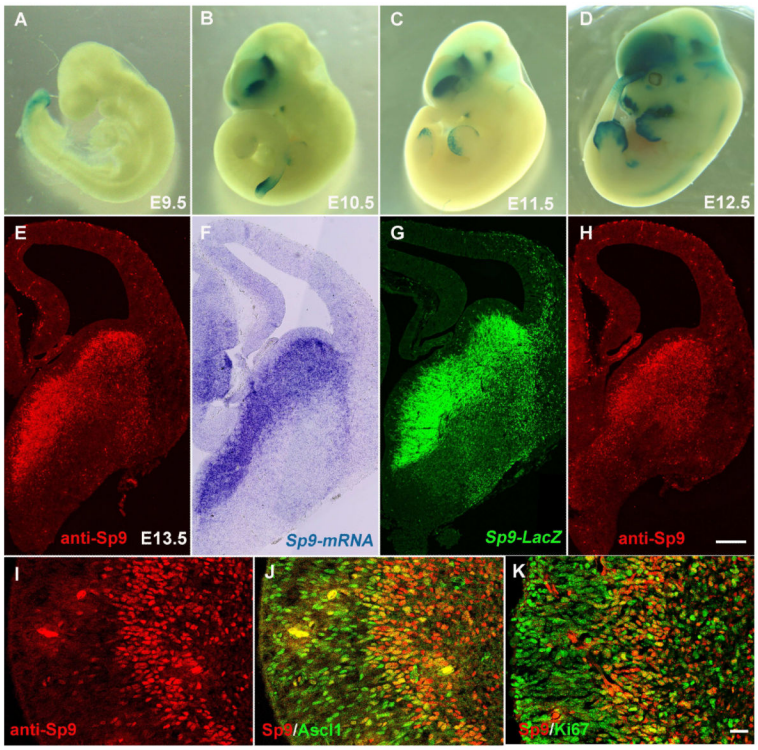

Figure 1. Sp9 Is Widely Expressed in the LGE, MGE and CGE

(A-D) Sp9 expression by whole mount β-gal staining on E9.5-E12.5 embryos of *Sp9*$^{LacZ/+}$ mice. (E-H) Sp9 protein, *Sp9* mRNA and *Sp9-LacZ* were widely expressed in the SVZ and mantle zone of E13.5 mouse LGE, MGE and CGE. (I-K) Sp9/Ascl1 and Sp9/Ki67 double-immunostained E13.5 mouse brain section showing Sp9^{+}/Ascl1^{+} and Sp9^{+}/Ki67^{+} cells in the SVZ of the LGE. Scale bars: 200 μm in H for E-H; 20 μm in K for I-K.

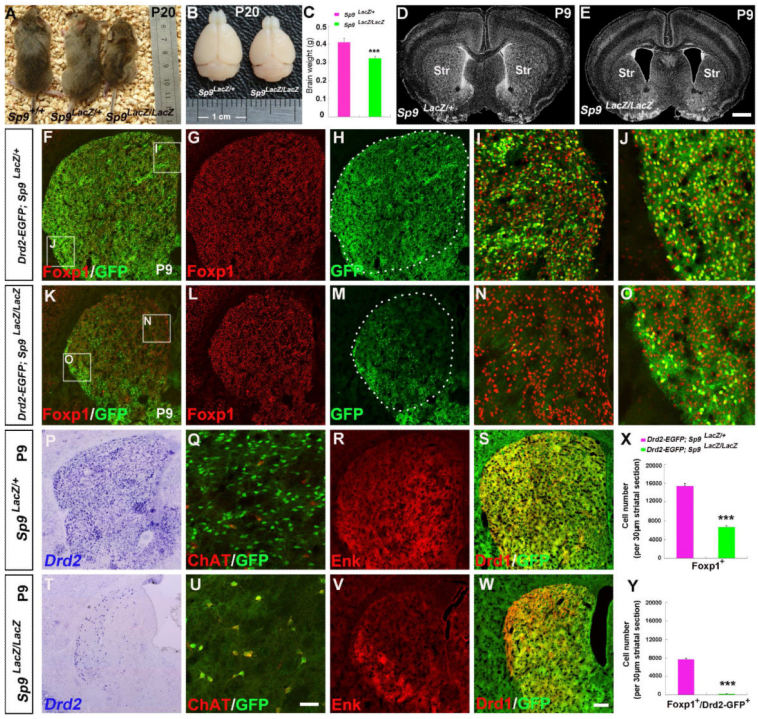

Figure 2. Striatopallidal MSNs Are Lost in Postnatal *Sp9*$^{LacZ/LacZ}$ Mutants

(A) *Sp9*$^{+/+}$, *Sp9*$^{LacZ/+}$ and *Sp9*$^{LacZ/LacZ}$ littermates were taken at P20 to compare their size.

(B) P20 images of *Sp9*$^{LacZ/+}$ and *Sp9*$^{LacZ/LacZ}$ brains.

(C) Brains were weighted at P20; the weight of *Sp9*$^{LacZ/LacZ}$ was significantly reduced compared to *Sp9*$^{LacZ/+}$ controls. Student's *t*-test, *** $p < 0.001$, n = 6, mean ± SEM.

(D, E) Telencephalon coronal section of *Sp9*$^{LacZ/+}$ control and *Sp9*$^{LacZ/LacZ}$ mutant counterstained with DAPI at P9. Note the reduction of striatal (Str) size in *Sp9*$^{LacZ/LacZ}$ mutants compared to *Sp9*$^{LacZ/+}$ controls. Scale bar: 1 mm.

(F-J) Foxp1/GFP double-immunostained striatal sections from *Drd2-EGFP; Sp9*$^{LacZ/+}$ P9 mice.

(K-O) Foxp1/GFP double-immunostained striatal sections from *Drd2-EGFP; Sp9*$^{LacZ/LacZ}$ mutant mice at P9 showed severe reduction of GFP^{+} striatopallidal MSNs. Note that almost all GFP^{+} striatopallidal MSNs were eliminated in the dorsal medial striatum.

(P, T) Severe reduction of *Drd2* RNA in *Sp9*$^{LacZ/LacZ}$ mouse striatum at P9 shown by *in situ* hybridization.

(Q, U) In the dorsal striatum of *Drd2-EGFP; Sp9*$^{LacZ/+}$ control mice (Q), less than 3% of Drd2-GFP^{+} cells expressed ChAT, whereas in the dorsal striatum of *Drd2-EGFP; Sp9*$^{LacZ/LacZ}$ mutant mice (U), most of the remaining Drd2-GFP^{+} cells express ChAT (>67%), suggesting they are ChAT^{+} interneurons rather than striatopallidal MSNs.

(R, V) Severe reduction of Enk^{+} cells in *Sp9*$^{LacZ/LacZ}$ mice compared to *Sp9*$^{LacZ/+}$ mice at P9 shown by immunostaining.

(S, W) Drd1/GFP double-immunostained striatal sections from P9 controls (*Drd1-EGFP; Sp9*$^{LacZ/+}$) and mutants (*Drd1-EGFP; Sp9*$^{LacZ/LacZ}$). Expression of Drd1 and Drd1-GFP were not obviously reduced in *Sp9* mutant striatum compared to controls. Scale bars: 200 μm in W for F-H, K-M, P, R-T, V, W; 50 μm in U for I, J, N, O, Q, U.

(X, Y) Approximately 57% of Foxp1^{+} and 97% Foxp1^{+}/Drd2-GFP^{+} cells were reduced in the P9 striatum of mutant (*Drd2-EGFP; Sp9$^{LacZ/LacZ}$*) compared to control (*Drd2-EGFP; Sp9$^{LacZ/+}$*). Student's *t*-test, *** $p < 0.001$, n = 3, mean ± SEM.

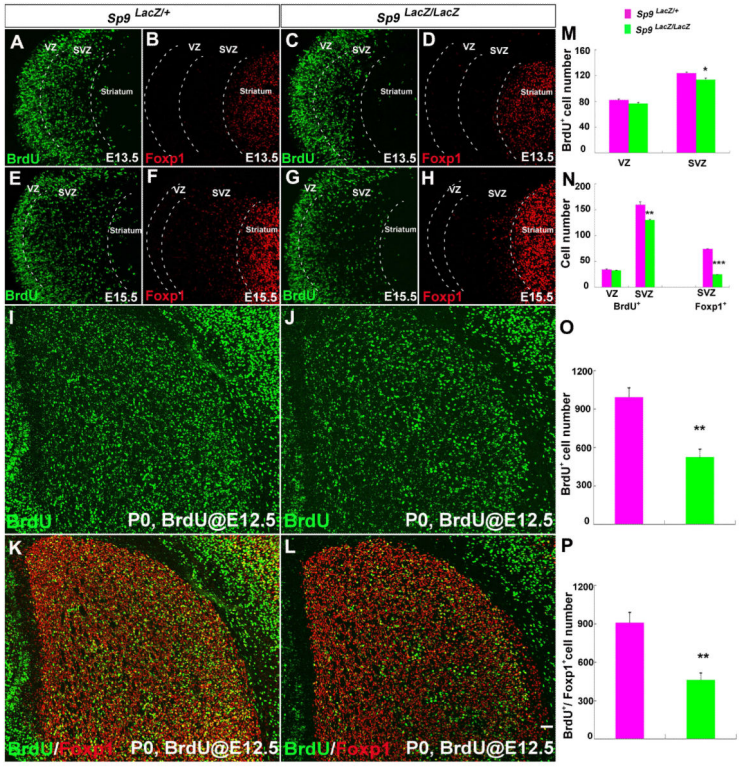

Figure 3. *Sp9* Mutants: Reduction of Proliferation in the LGE SVZ, and Reduction of MSN Production in the Striatum

(A-H) Immunostaining of BrdU and Foxp1 of LGE sections at E13.5 and E15.5, 30 min after a BrdU pulse.

(M, N) Quantification showing $Sp9^{LacZ/LacZ}$ mutants had reduced $BrdU^+$ and $Foxp1^+$cells in the SVZ, but not in the VZ. Student's *t*-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, n = 3, mean ± SEM.

(I-L) Immunostaining of BrdU and Foxp1 of striatal sections at P0 after a BrdU injection at E12.5. Scale bars: 50 μm in L for A-H, I-L.

(O, P) Quantification showing *Sp9* mutants had reduced $BrdU^+$ and $BrdU^+/Foxp1^+$cells in the striatum. Student's *t*-test, ** $p < 0.01$, n = 3, mean ± SEM.

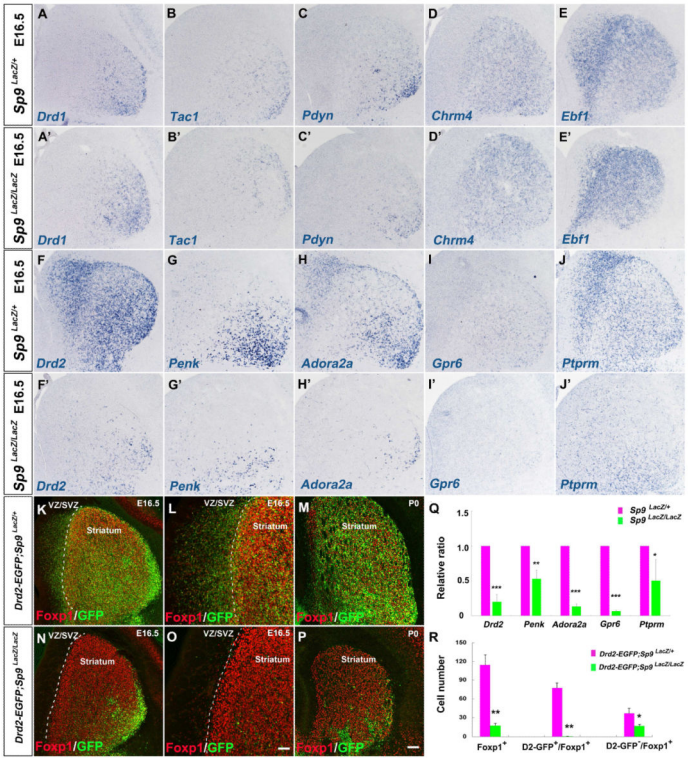

Figure 4. Most Mature Striatopallidal MSNs Are not Generated in *Sp9* Mutants

(A-E') *In situ* RNA hybridization of striatonigral MSN markers on E16.5 brain sections showing the development of striatonigral MSN were less affected.

(F-J') RNA expression of striatopallidal markers was significantly reduced. Note complete loss of *Adora2a* RNA in the *Sp9* mutant LGE SVZ and mantle zone (striatum) except a few cells in the lateral striatum (H').

(K-P) Most Drd2-EGFP$^+$ cells were lost in the SVZ and striatum at E16.5 and P0. Scale bars: 100 μm in P for A-K, M, N, P; 50 μm in O for L, O.

(Q, R) Validation of striatopallidal MSN loss in the E16.5 LGE by q-PCR (Q) and quantification of Foxp1$^+$, Foxp1$^+$/Drd2-GFP$^+$ and Foxp1$^+$, but Drd2-GFP$^-$ (negative) cells in the LGE SVZ at E16.5 (R). Student's *t*-test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, n = 3, mean ± SEM.

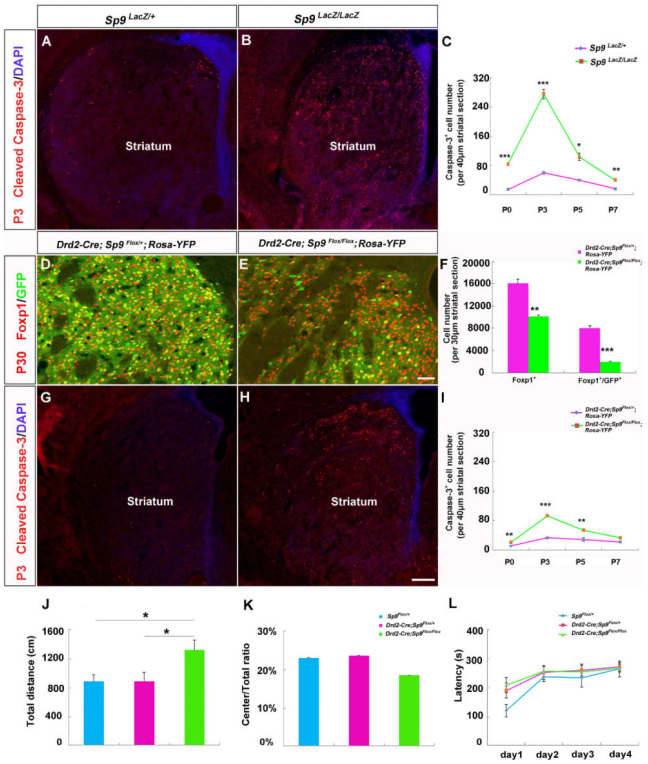

Figure 5. Programmed Cell Death Eliminates Striatopallidal MSNs in Postnatal *Sp9* Constitutive and Conditional Mutants

(A, B) Significant increase in expression of Cleaved Caspase-3 in *Sp9* mutant striatum compared to controls at P3.

(C) Temporal profile of numbers of Caspase-3$^+$ cells in postnatal striatum in *Sp9* mutant and control mice. One-way ANOVA followed Tukey-Kramer post hoc test, * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, n = 3, mean ± SEM.

(D-F) Foxp1/GFP double-immunostaining revealed a significant decrease in numbers of Foxp1$^+$, and Foxp1$^+$/GFP$^+$ cells in the striatum of *Drd2-Cre; Sp9 $^{Flox/Flox}$; Rosa-YFP* conditional mutants compared to *Drd2-Cre; Sp9 $^{Flox/+}$; Rosa-YFP* (controls) at P30. Student's *t*-test, ** $p < 0.01$; *** $p < 0.001$, n = 3, mean ± SEM. Scale bar: 50 μm.

(G-I) Caspase-3$^+$ cells in controls and *Sp9* conditional mutants at P3 showing a significant increase in numbers of Caspase-3$^+$ cells in the striatum of *Sp9* conditional mutants compared to controls at P0, P3 and P5. One-way ANOVA followed Tukey-Kramer post hoc test, ** $p < 0.01$; *** $p < 0.001$, n = 3, mean ± SEM. Scale bars: 100 μm in H for A, B, G, H.

(J, K) In the open field test, adult *Drd2-Cre; Sp9$^{Flox/Flox}$* mice exhibited increased locomotor activity compared to *Sp9$^{Flox/+}$* or *Drd2-Cre; Sp9$^{Flox/+}$* littermate controls (J, measured by the total distance), but they exhibited similar anxiety related behavior (K, measured by the center distance/total distance ratio). One-way ANOVA followed Tukey-Kramer post hoc test, * $p < 0.05$, mean ± SEM.

(L) In the rotarod test, adult *Drd2-Cre; Sp9$^{Flox/Flox}$* mice and littermate control mice displayed similar motor coordination and learning ability in the latency to fall during three training days (day1,2,3) and one trial day (day 4).

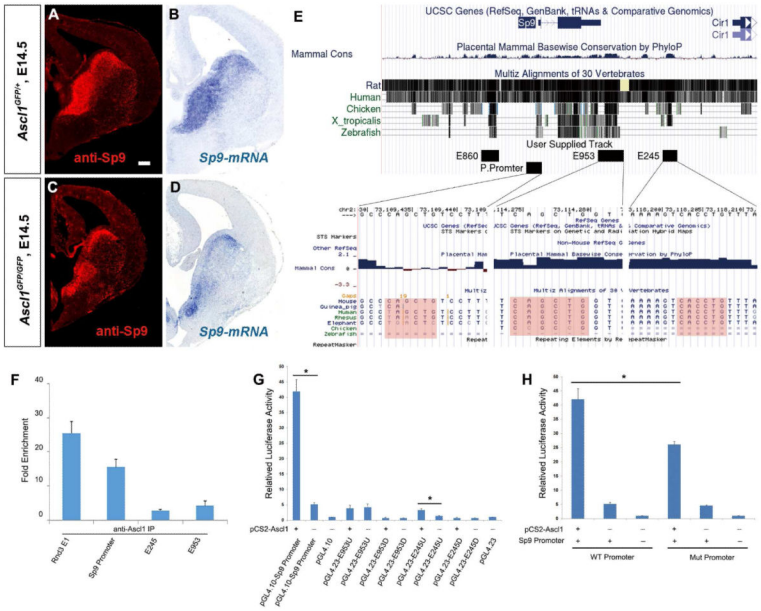

Figure 6. Ascl1 Directly Regulates the *Sp9* Expression in the LGE

(A-D) Sp9 expression in the LGE was reduced in *Ascl1*$^{GFP/GFP}$ mutant mice compared to *Ascl1*$^{GFP/+}$ control mice at E14.5. Scale bar: 200 μm.

(E) Evolutionarily conserved noncoding elements in the *Sp9* locus (putative promoter, enhancer E953 and E245) contain the consensus Ascl1-binding motif (E-box sites, CAGCTG or CACCTG).

(F) Ascl1 ChIP-qPCR demonstrated Ascl1 binding in chromatin from wild type E13.5 LGE to the *Sp9* putative promoter, E953 and E245. The *Rnd3* E1 is a positive control.

(G) Ascl1 activated transcription from the *Sp9* putative promoter and E245 in Dual-luciferase assay within P19 cells. Student's *t*-test, * $p < 0.05$, n = 3 individuals, mean ± SEM.

(H) Deletion of the Ascl1 binding motif reduced activation of the *Sp9* putative promoter by Ascl1. Student's *t*-test, * $p < 0.05$, n = 3 individuals, mean ± SEM.

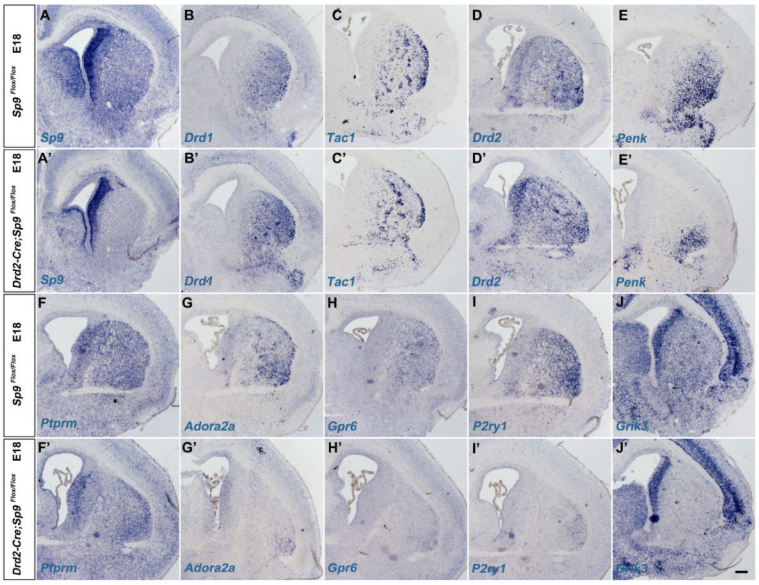

Figure 7. Sp9 Promotes the Expression of *GPCRs* in Striatopallidal MSNs

(A, A') *Sp9* RNA expression in the striatum was greatly reduced in *Drd2-Cre; Sp9* $^{Flox/Flox}$ conditional mutants compared to *Sp9* $^{Flox/Flox}$ controls at E18, whereas its expression in the SVZ appeared normal. (B-C') *Drd1* and *Tac1* RNA expression appeared normal. (DG') Striatal expression of *Drd2*, *Penk* and *Ptprm* were slightly reduced, whereas expression of *Adora2a* was nearly undetectable except in the lateral striatum. (H-J') Expression of *Gpr6*, *P2ry1* and *Grik3* were greatly reduced in the striatum. Scale bar: 200 μm in J' for A-J'.