Activation of the Sonic Hedgehog Effector Smoothened Counteracts L-Dopa Induced Dyskinesia by Restoring Cholinergic Interneuron Function

Lauren Malave
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Activation of the Sonic Hedgehog effector Smoothened counteracts L-Dopa induced dyskinesia by restoring cholinergic interneuron function

By: Lauren Malave

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Activation of the Sonic Hedgehog effector Smoothened counteracts L-Dopa induced dyskinesia by restoring cholinergic interneuron function

By: Lauren Malave

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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CITY UNIVERSITY OF NEW YORK
Abstract

Activation of the Sonic Hedgehog effector Smoothened counteracts L-Dopa induced dyskinesia by restoring cholinergic interneuron function

By: Lauren Malave

Advisor: Andreas Kottmann

Many types of neurons act as multimodal signaling centers. Yet, we have only limited insight into the regulation and functional consequences of neuronal co-transmission. For example, dopamine (DA) neurons, whose degeneration causes motor deficits characteristic of Parkinson’s Diseases (PD), communicate with all their targets by DA but only a selective subset of their targets using GABA, Glutamate, and the secreted cell signaling protein Sonic Hedgehog (Shh). It is unknown whether Levo-dopamine (L-Dopa) induced dyskinesia (LIDs), a severely debilitating side effect of DA supplementation in PD, might appear because DA neuron targets are exposed to high DA- but low Shh- signaling in medicated patients. Here I show that restoring the balance of DA and Shh signaling attenuates LID formation and acute expression in mouse and macaque models of PD. Cholinergic neurons are responsive to Shh signaling via stimulation of the Shh effector GPCR smoothened. Using conditional KO mice of pre or postsynaptic Shh we show that reduced signaling in cholinergic neurons is sufficient and necessary for LID formation. Conversely, selective expression of a constitutive active form of Smo (SmoM2) in cholinergic neurons is sufficient to render the sensitized aphakia model resistant to LID. The relative degree of imbalance of DA and Shh signaling rather than their absolute strength determines the severity of LID and highlights the bidirectional effect both factors have on LIDs. Activation of Smo reduces MAP-kinase pathway signaling, a physiological marker of LID, selectively in CINs of the dorsolateral but not dorsomedial striatum and enhances the neuronal
activity marker p-rpS6^{240/244} through activation of Smo on CINs. Additionally, semi-chronic, pulsatile optogenetic stimulation of DA neurons results in LID-like behaviors that can be attenuated by Smo activation. Together, my data reveal that balanced Shh and DA signaling is a critical modulator of cholinergic physiology and provide an unexpected link between LID and DA neuron degeneration. Furthermore, since pulsatile L-Dopa dosing might induce a perversion in the DA neuron provided teaching signal resulting in acquisition and selection of un-purposeful, abnormal motor programs seen in LIDs, then the novel animal models described here could be a starting point for in vivo analysis to assess the function and importance of neuronal co-transmission of Shh signaling.
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“I am a great believer in luck. The harder I work the more of it I seem to have.”

Coleman Cox

“Learn to fail or fail to learn.”

Thomas Edison

“Am I getting closer? Will I ever get there? Does it even matter?”

Kevin Parker

Apocalypse Dreams

Dedication

I dedicate my thesis and attribute my hard work to my parents, Linda and Barry Malave. They fostered an environment in which my genetics could succeed.
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1. Chapter 1: Introduction

1.1 Basal ganglia circuitry

*Components of the basal ganglia*

In order to execute learned motor behavior, the brain coordinates a complex, heterogeneous network of nuclei known as the basal ganglia (Alexander and Crutcher 1990; Boraud et al. 2018; Graybiel 1998; Graybiel et al. 1994). This network is composed of the striatum, subthalamic nucleus (STN), globus pallidus interna (GPi), globus pallidus externa (GPe), substantia nigra par reticulata (SNr), and substantia nigra pars compacta (SNpc). The major input processing area of this circuit that is involved in movement command and action selection is the striatum. The striatum receives information both internally, from nuclei within the basal ganglia, and externally, from the cortex and thalamus, in a feedback loop manner to control movement (Albin et al. 1989; Alexander et al. 1986; Parent and Hazrati 1995). One of the main innervations of the striatum comes from dopaminergic neurons of the midbrain SNpc.

Dopamine (DA) is a key player in neuromodulation with its tonic and phasic firing abilities providing a reward prediction error signal in reinforcement learning (Apicella et al. 1992; Bayer and Glimcher 2005; Fiorillo et al. 2003; Schultz 1992). In the adult brain, 75% of dopaminergic input comes from the midbrain (Wallen and Perlmann 2003). A lateral subset of these midbrain dopaminergic projections from the SNpc mainly target the dorsal striatum and make up the mesostriatal, or commonly termed nigrostriatal pathway (Haber et al. 2000; Ikemoto 2007; Lammel et al. 2008). Alternatively, there is the mesolimbic pathway that consists of medial midbrain dopaminergic neurons from the ventral tegmental area (VTA) and projects to the ventral striatum (Morales and Margolis 2017). Although both areas target the striatum, their functions are very different. The nigrostriatal pathway is involved in learned movement control,
while, the mesolimbic pathway is involved in emotion and mood driven behaviors (Lammel et al. 2008; Morales and Margolis 2017; Poulin et al. 2014). These differences are reflected in the motor impairment disorder Parkinson’s Disease (PD), which leads to specific degeneration of the SNpc DA neurons while VTA DA neurons remain intact.

Furthermore, within the dorsal striatum there are different compartments that fulfill functionally diverse roles in motor command (Graybiel 2008). Although SNpc dopaminergic neurons have arborous projections throughout, the dorsomedial striatum (DMS) encodes for early skill acquisition and goal-directed behavior, while the dorsolateral striatum (DLS) encodes for advanced skill acquisition and habitual behavior (Chatham et al. 2014; Dickinson and Weiskrantz 1985; McGeorge and Faull 1989; Perrin and Venance 2019; Redgrave et al. 1999). During procedural learning, there is a shift in activity from the DMS to the DLS when the skill is learned (Yin et al. 2004; Yin et al. 2005). Differences between the tasks are reflected by distinct firing patterns of striatal neurons, receptor density gradients, and alterations in cortico-striatal plasticity via NMDA/AMPA receptor ratios between the DLS and DMS (Chuhma et al. 2014; Yin et al. 2009). Interestingly, the DLS is the homologous area in humans for the greatest DA neuron loss in PD (Antonini et al. 1995; Pavese et al. 2011).

The SNpc dopaminergic input to the striatum acts in tandem with glutamatergic input from cortical and thalamic nuclei to translate thought into context appropriate action (Daw et al. 2005; Hunnicutt et al. 2016; Maia and Frank 2011; Parent and Hazrati 1995). Cortical and thalamic information streams are integrated onto striatal neurons where DA modulates their synapse strength (Wickens et al. 2003). Cortical glutamatergic input of the striatum come from pyramidal projection neurons and are critical for reinforcement learning (Alexander et al. 1986; Graybiel and Grafton 2015). As mentioned above, DA acts as a neural substrate that translates
prediction error by encoding differences between the outcome of an action and the expected result. This allows strengthening of specific synapses based on prior experience and contributes to reinforcement learning, which streamlines performance (Bi and Poo 1998; Schultz 1992; Schultz 2006; Schultz 2013; Schultz et al. 1997). The mechanism of DA signaling relies on a triple coincidence when stimulation of glutamate, postsynaptic depolarization, and binding of DA occur at the same time to elicit spine growth and plasticity (Yagishita et al. 2014). The disorganization of this process is observed in PD as akinesia, a symptom that involves slow movement, and is caused by a constant negative reward predication error as a result of the loss of DA, and therefore, the loss of this plasticity (Beeler et al. 2012).

**Striatal neurons within the basal ganglia**

Our capacity to execute context appropriate and well-coordinated movements requires the dynamic selection and suppression of possible actions. The multifaceted cell types within the striatum process and integrate incoming signals to produce these controlled movements (Silberberg and Bolam 2015). A majority of the neuronal population within the striatum is the projection neurons, making up 95%, and they are anatomically termed medium spiny neurons (SPN; Figure 1.1). SPNs are separated into two distinct populations, either the direct excitatory pathway (dSPN) or the indirect inhibitory pathway (iSPN). The dSPN pathway promotes movement by inhibiting the SNr and the GPi, two main output nuclei of the basal ganglia. In contrast, the iSPN pathway terminates ongoing movement by stimulating the GPe and the STN of the basal ganglia to activate the GPi (Sano et al. 2013).

Although separated into two pathways, both classes of SPNs are GABAergic and synchronize to implement a desired movement following dopaminergic input from the SNpc (Moss and Bolam 2008). This is accomplished via distinct dopaminergic receptor expression.
profiles between dSPNs and iSPNs. DA released into the striatum binds to D1-like receptors (including D5) that are predominantly expressed by dSPNs, or to D2-like receptors that are primarily expressed by iSPNs (Albin et al. 1989; Oorschot 1996; Sano et al. 2013; Tozzi et al. 2011). Continuous binding of D1-like receptors facilitates the induction of Hebbian long-term potentiation (LTP), while suppressing Hebbian long-term depression (LTD) of the dSPNs. On the other hand, binding of D2-like receptors suppresses LTP induction, and promotes LTD of the iSPNs. These Hebbian principles act on cortical glutamatergic inputs, which heavily regulate dSPN and iSPN activity (Keysers and Perrett 2004; Schultz et al. 1997; Song et al. 2000; Zhai et al. 2018). Overall, learned motor behavior stems from this complex complementary balance between the dSPN and iSPN pathways (Figure 1.2).

The remaining 5% of neurons within the striatum are interneurons that project locally to regulate both classes of SPNs and are either the small GABAergic subtypes or the large aspiny cholinergic interneurons (CINs; Figure 1.1) (Ibáñez-Sandoval et al. 2015; Kimura et al. 1980; Tepper et al. 2010). The diverse GABAergic interneurons include fast-spiking GABAergic interneurons (FSN), calretinin-expressing interneurons, and persistent/low threshold spiking interneurons which differ in either their binding protein, neuropeptides or enzyme expression, such as parvalbumin, somatostatin, neuropeptide Y, nitric oxide synthase, or tyrosine hydroxylase (TH) (Ibáñez-Sandoval et al. 2015; Tepper et al. 2010). The larger CINs produce the primary internal source of acetylcholine (ACh) and maintain the highest density of ACh and cholinergic markers in the brain (Kawaguchi et al. 1995; Wilson et al. 1990; Zhou et al. 2002). CINs act as an inhibitory mechanism of hyperactivity and are thought to contribute to the cognitive and hypokinetic motor symptoms of PD (Barbeau 1962; Bordia et al. 2016; McGeer et al. 1961; Tanimura et al. 2018). Although few in numbers, these CINs exert strong control over
SPNs and are critical for the translation of thought into action by integrating glutamatergic, GABAergic, and dopaminergic inputs (Chuhma et al. 2014; Surmeier and Graybiel 2012).
Figure 1.1: Simplified striatal circuitry

SNpc dopaminergic input and cortical glutamatergic input converge onto the striatum to modulate the control of excitatory SPN (D1) and inhibitory SPN (D2), along with interneurons, to elicit a motor output. Glut: glutamate; CIN: cholinergic interneuron; FSN: fast spiking interneuron; SOM: somastatin interneurons; SPN: medium spiny neurons; DAN: dopamine neurons; SNpc: substantia nigra pars compacta.
In the physiological condition, direct (D1 SPN) and indirect (D2 SPN) pathways coordinate learned motor behavior. In Parkinson’s Disease, the balance is shifted toward the indirect pathway and results in motor impairment. During L-Dopa therapy, there is another shift towards the hypoactivity of the direct pathway leading to dyskinesia. SPN: medium spiny neuron; DA: dopamine; ACh: acetylcholine; D1R: dopamine 1 receptor; D2R: dopamine 2 receptor; M1R: muscarinic acetylcholine 1 receptor; M4R: muscarinic acetylcholine 4 receptor.
1.2 Striatal cholinergic interneurons

Properties of striatal CINs

CINs make up for their small numbers with an extensive arborization throughout the striatum that overlaps with dopaminergic and glutamatergic terminals and have dense terminal fields on SPNs (Phelps et al. 1985). Under basal conditions, CINs have a continuous firing pattern of rhythmic bursts and single spike firing rate of 5 – 10 Hz (Aosaki et al. 1995; Raz et al. 1996; Wilson et al. 1990). One main regulatory neurotransmitter of CIN activity comes from DA. CINs have both D1/5R and D2R that act to increase or attenuate ACh release, respectively (Acquas and Di Chiara 1999; Stoof et al. 1982; Straub et al. 2014). Pharmacological studies show that selective D1/D5R agonists increase ACh release, while D1/5R antagonist and D2R agonist reduce ACh release (Acquas et al. 2001; Damsma et al. 1990). The bidirectional action DA holds on CINs allows for the fine-tuning of locomotor activity. Specifically, activation of D2R on CINs produces a “pause” in firing that lasts 60-80ms and further potentiates the DA signal on other striatal neurons (Kharkwal et al. 2016). Quickly following this “pause”, there is a “rebound” burst of 200-300ms in firing of CINs to restore striatal balance. The role of burst-firing activity of CINs is (1) to enhance iSPN suppression of movement and (2) reset cortico-striatal circuit for selecting appropriate behavior. It was recently discovered that glutamate from DANs contribute to this “rebound” of burst firing, as well as other factors (Chuhma et al. 2018). This evidence demonstrates a mechanism by which DA acting on CINs regulates striatal activity, as well as, the possible importance of other signaling factors working to counteract DA inhibition on CINs.
**ACh receptors in the striatum**

The continuous activity of endogenous ACh from CINs maintains motor control within the striatum through tonic activation of muscarinic ACh receptors (mAChR) and nicotinic ACh receptors (nAChR) to regulate dopaminergic, GABAergic, and glutamatergic signaling, collectively (Quik and Wonnacott 2011). The striatum is densely populated by both mAChR and nAChR with multiple subtypes. mAChR are G-coupled protein receptors (GCPR) that are slow acting and have longer modulatory control compared to the ionotropic nAChR, which have a more rapid time scale. Of the two mAChR classes found in the striatum, Gq-coupled mAChR are M1R-like and consist of M1, M3 and M5 mAChR, whereas, Gi-coupled mAChR are M2R-like and consist of M2 and M4 mAChR. M1 and M4 mAChR are expressed by SPNs, M3 and M5 mAChR are on nigrostriatal dopaminergic afferent projections, and M2 and M4 mAChR are on CINs producing an auto-regulatory role. Specifically, M4 mAChR are found primarily in the DLS on CINs and dSPNs. CINs increase excitability of iMSNs through M1 mAChR and decrease excitability of dMSNs through M4 mAChR.

The expression of nAChR are predominately on afferent projection terminals in the striatum (Quik and Wonnacott 2011). Twelve distinct neuronal nAChR subunits exist that are classified as either alpha subunits (α2 to α10) or beta subunits (β2 to β4 (Dani 2015; Zoli et al. 2015)). The alpha subunit has a recognition site for ACh, whereas the beta subunits just promote the binding of ACh to the alpha subunits. For this reason, most nAChR are heteromeric with both alpha and beta subunits together. In DA neurons, binding of ACh to presynaptic nAChR has been shown to facilitate DA release (Drenan et al. 2010; Gotti et al. 2010; Livingstone and Wonnacott 2009). Glutamate, GABA, and serotonergic neuron terminals all express nAChR subunits to promote their neurotransmitter release as well (Grilli et al. 2009; Kaiser and
Wonnacott 2000). The diverse roles of nAChR subunits on different neurotransmitter terminals in the striatum allow for the precise regulation of motor control from CINs. Interestingly, nAChR drugs have been found to be neuroprotective against dopaminergic degeneration in the SNpc, as reflected in a decreased risk of PD development among smokers (Noyce et al. 2012; Quik et al. 2007; Searles Nielsen et al. 2012; Tanner et al. 2002; Wirdefeldt et al. 2011). The abundance of ACh receptors expressed presynaptically and postsynaptically throughout the striatum points to the complex and powerful influence CINs exert on motor function.

1.3 Parkinson’s Disease and L-Dopa induced dyskinesia

*Parkinson’s Disease (PD)*

Motor dysfunctions arise due to the progressive loss of DA neurons in the SNpc and is the cardinal feature of PD (Ungerstedt 1971). The subsequent decrease in striatal DA concentration causes an imbalance between the dSPN and iSPN pathways (Figure 1.2) and results in motor impairments such as bradykinesia, rigidity and slow movement (Dauer and Przedborski 2003). Parkinsonian symptoms emerge following more than 50% loss of DA neurons (Kordower et al. 2013; Ungerstedt 1971) and renders the striatum hypersensitive to DA. As a result, iSPNs become hyperactive and suppress movement, while, dSPNs become hypoactive leading to non-activation of movement. LTD and LTP are therefore lost in the iSPN and dSPN pathways, respectively (Albin et al. 1989; Mallet et al. 2006). This shift in balance favors the iSPN pathway and results in activation of the SNr, which inhibits the thalamocortical circuit and decreases motor output.

In PD patients, both DA and ACh levels fall, but DA diminishes to a larger extent than ACh levels (Barbeau 1962; McKinley et al. 2019). This describes the well-established seesaw
effect of the disequilibrium between DA and ACh with intrinsic cholinergic activity scaling higher than dopaminergic input, resulting in increased inhibition of movement (Barbeau 1962; Duvoisin 1967; Quik and Wonnacott 2011). Optogenetic studies show activation of CINs augments parkinsonian symptoms in mouse models, whereas, inhibition of CINs dampen them (Kravitz et al. 2010; Threlfell et al. 2010). While anticholinergic drugs are an effective therapy in alleviating PD symptoms, they cause too many side effects and are used less frequently in the clinical setting (Maurice et al. 2015). The off-target side effects include cognitive deficits, confusion, constipation, dry mouth, and urinary issues among others. Interestingly, inhibition of CINs in normal animals does not alter locomotion, exhibits no effect on MSN excitability, and has no change in spontaneous or cortically driven activity, suggesting that the role of CIN in motor function critically depends on the dysfunction of dopaminergic tone.

**L-Dopa induced dyskinesia**

To date, there are no disease modifying therapies available for reversing or halting the progression of PD. Instead, symptomatic treatment by DA substitution from Levo-Dopa (L-Dopa) therapy remains the most efficacious management of PD (Lees 1994). L-Dopa therapy is seen as the best treatment because of its ability to cross the blood brain barrier and provide the substrate for DA, fundamentally giving back the neurotransmitter that is lost in PD. However, as much as L-Dopa is an effective replacement for DA, this therapy exhibits many shortcomings. L-Dopa therapy ameliorates most but not all symptoms of PD and, worse, induces uncontrollable L-Dopa induced dyskinesias (LIDs). LIDs are a complicated and debilitating side effect, prompting hyperkinetic abnormal involuntary movements characterized as chorea/choreoathetoid or rapid, irregular, unpredictable, non-stereotyped movements (Bastide et al. 2015; Eriksson et al. 1988; Hechtner et al. 2014; Marsden and Parkes 1977; Quinn et al. 1982). The appearance of
LID reflects “aberrant reinforcement learning” caused by daily L-Dopa induced DA peaks each mimicking a positive reward prediction error that occur at ectopic synapses and are uncoupled from behavioral context (Surmeier et al. 2014; Zhai et al. 2018; Zhuang et al. 2013). The L-Dopa therapy induced spike of unregulated DA leads to hyperactivity of dSPNs via D1R and increased inhibition of the iSPNs through excessive D2R activation after repeated exposure and increased receptor sensitivity. This causes another shift in the balance of the dSPN and iSPN pathways from dSPNs being hypoactive during PD to hyperactive during LIDs, and vice versa for iSPNs (Figure 1.2). Importantly, LIDs appears in almost all patients following 5-10 years of chronic L-Dopa treatment or in advanced stages of PD (Cenci and Lundblad 2006; Fabbrini et al. 2007). Despite years of research, why and how LIDs form is not understood.

Current LID therapy

Considering the multitude of neurons and neurotransmitters within the striatum, anti-dyskinetic drug targets have been vast and ineffective. For example, many studies have looked into targeting postsynaptic modulatory mechanisms such as the opioid, cannabinoid, glutamatergic, noradrenergic, or serotonergic systems without much success (Espay et al. 2018). The difficulty is that these drugs must have anti-dyskinetic properties, while not curtailing the beneficial effects of L-Dopa. The best clinical antidyskinetic drug to date is amantadine, an NMDA antagonist (Pahwa et al. 2017; Rascol et al. 2015). Although the safety of amantadine has been considered “acceptable”, there are additional side effects such as confusion, hallucinations, ankle edema, lethargy, nausea, and vomiting that make it a non-optimal treatment (Fox et al. 2011). Other advanced therapies have been developed but require invasive intervention, such as deep brain stimulation, infusion of levodopa-carbidopa intestinal gel, and continuous subcutaneous apomorphine infusion (Durif et al. 1994; LaHue et al. 2017; Larson
This list exemplifies many limitations in optimally managing LID because of the complexity and many off-target responses these therapies produce.

1.4 CIN involvement in LID

Targeting CINs and ACh receptors in LID

Recently, the focus of interest in LID formation has shifted towards the role of CINs based on several intriguing findings. However, the mechanism(s) of action remain elusive and contradictory. Deciphering whether increased or decreased cholinergic signaling contributes to LID formation and expression has been a challenge because there is strong evidence that high ACh reduces LID as well as low ACh reduces LID. For example, LID expression is attenuated by (1) administration of chronic nicotine, an agonist of nAChR mediated cholinergic signaling ([Quik et al. 2013; Quik et al. 2013; Quik et al. 2013; Zhang et al. 2013] and clinical trial NCT00957918), (2) muscarinic agonists through M4R activation on dSPNs (Shen et al. 2015) and (3) optogenetic “long-duration” stimulation of CINs (Bordia et al. 2016). Whereas, LID formation is augmented by (1) the ablation of striatal CINs (Won et al. 2014), (2) muscarinic antagonist, dicyclomine (Ding et al. 2011) (3) inhibition of RGS4 expression (Ko et al. 2014), a negative modulator of muscarinic feedback regulation of acetylcholine release (Ding et al. 2006), and (4) optogenetic “short-duration” stimulation of CINs (Bordia et al. 2016). How to reconcile these divergent results is not readily apparent but two possibilities are discussed most often: a) chronic nicotine administration could lead to desensitization of endogenous ACh release which could explain functional outcomes that are also achieved by methods of inhibiting cholinergic signaling or b) ACh signaling through specific nicotinic receptors attenuates, while ACh signaling through specific muscarinic receptors facilitates, LID formation (Bordia et al. 2016;
Quik et al. 2018). Together these findings suggest a critical role of cholinergic activity and tone in the formation and expression of LIDs.

*Molecular targets of CINs in LID*

Elucidating the molecular details and functional consequences of the reciprocal relationship of DA neurons and CIN potentially holds the key to understanding the mechanisms behind LID. The phosphorylation of MAP-kinase pathway protein extracellular signal-regulated kinase (pErk1/2; phospho-Thr202/Tyr204–ERK1/2), is involved in many transcriptional processes and has been implicated as a marker for LID (Pavón et al. 2006; Santini et al. 2009; Santini et al. 2007). Upon early L-Dopa dosing, pErk1/2 in the dorsal striatum is found in SPNs and directly correlates to the degree of LIDs (Figure 1.3). However, with continuous L-Dopa treatment a unique patterning of pErk1/2 develops. Upon long-term L-Dopa dosing, there is a shift in pErk1/2 patterning from SPNs to CINs with increased pErk1/2 expression in CINs correlating to increased LIDs (Ding et al. 2011). The understanding of pErk1/2 expression in CINs is unclear, however, when pErk1/2 is pharmacologically inhibited, there is a subsequent decrease in LID (Ding et al. 2011; Schuster et al. 2008). Another biochemical marker implicated in enhanced LID formation is the immediate early gene c-Fos (Cenci and Konradi 2010; Ding et al. 2007). The activity of c-Fos is increased in CINs following optogenetic “long-duration” stimulation of CINs that is correlated to decreased LIDs (Bordia et al. 2016). These changes in gene regulating signaling proteins can potentially alter the basal firing rate of CINs through changes in glutamatergic input and morphology, and, thus, play a role in the formation of LIDs.
Figure 1.3: pErk1/2 patterning in the striatum following LID (Adapted from (Ding et al. 2011))

During acute dosing of L-Dopa, pErk1/2 is expressed in SPNs and not in CINs. Upon chronic L-Dopa dosing and increased LID formation, expression is increased in CINs and decreased in the SPNs. SPN: medium spiny neuron; CIN: cholinergic interneuron; LID: L-Dopa induced dyskinesia.
1.5 Sonic Hedgehog signaling

**DA neurons release Sonic Hedgehog throughout life**

In order to understand the pathophysiology underlining LID formation, it is important to understand how DA neurons communicate. DA neurons are “multilingual”, meaning they communicate to their targets with glutamate, GABA, and other cell signaling factors besides DA throughout life (Chuhma et al. 2018; Trudeau et al. 2014). It therefore follows that all these factors are also lost during DA neuron degeneration in PD. One such signaling factor produced by DA neurons is the trophic factor Sonic Hedgehog (Shh\textsubscript{DAN}), which is found to be critical for the maintenance and survival of the nigrostriatal circuit (Gonzalez-Reyes et al. 2012). Although Shh has been extensively studied in development and patterning of the notochord and floorplate (Briscoe and Ericson 1999; Perrimon 1995; Smith 1994), its critical role in the adult brain is just emerging.

Shh is a 19-kDa protein of the hedgehog family that acts as an intein and cholesterol transferase (Beachy et al. 1997; Fietz et al. 1994). Shh is involved in proliferation of neural precursor cells (Dahmane and Ruiz-i-Altaba 1999; Ruiz i Altaba et al. 2002), neuronal differentiation (Tanabe et al. 1995; Wang et al. 1995), axon outgrowth (Charron et al. 2003), synapse formation, and enhanced neurotransmitter release (Yao et al. 2016), and neuroprotective properties (Zhang et al. 2014). In PD mouse models, viral over-expression of Shh reduces the loss of DA neurons induced by DA-toxins such as methylphenyltetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) (Dass et al. 2002; Gonzalez-Reyes et al. 2012; Tsuboi and Shults 2002). Partial genetic ablation of Shh in DA neurons of mice produces progressive degeneration, whereas those DA neurons with intact Shh survive (Dass et al. 2002; Gonzalez-Reyes et al. 2012; Tsuboi and Shults 2002). While this data points to a cell autonomous function of Shh in
DA neuron survival, this possibility was ruled out by the ablation of the obligate necessary Shh signal transduction component Smoothened (Smo) from DA neurons which did not phenocopy the ablation of Shh from DA neurons (Gonzalez-Reyes et al. 2012). In mice lacking ShhDAN, these DA neurons and their striatal targets, which, in return, provide neurotrophic support to DA neurons via their secretion of the DA neuron trophic factor glia derived neurotrophic factor (GDNF), progressively die as the mouse ages, showing the importance of reciprocal trophic factor support (Gonzalez-Reyes et al. 2012; Hidalgo-Figueroa et al. 2012).

**Shh receptor expression and function**

Although the striatal neurons express DA, glutamate, ACh, and GABA receptors, only CINs and FSNs express the Shh receptor Patched-1 (Ptc-1) (Gonzalez-Reyes et al. 2012; Moss and Bolam 2008). Shh signaling is unique in that activation of the 12-transmembrane resistance-nodulation-division (RND)-like transporter protein Ptc-1 releases the constitutive repression of the 7-transmembrane G-protein coupled receptor (GCPR) smoothened (Smo; Figure 1.4) (Izzi et al. 2011). This Ptc-1/Smo interaction has been shown to be critical for regulating appropriate Shh pathway activity. Activation of Smo recruits Gi protein complex that reduces levels of cyclic AMP (cAMP) (Beachy et al. 1997) and allows for either transcription independent or canonical transcription dependent processes to occur (Bijlsma et al. 2012).

Shh has been shown to have a neuromodulatory effect on glutamatergic neurotransmission in slice preparations (Bezard et al. 2003) and can be secreted from neurons by high frequency burst firing (Su et al. 2017). Therefore, ShhDAN might also have acute effects on CIN and FSN. Previously, we’ve shown that activation of the Shh pathway through Smo inhibits the transcription of muscarinic acetylcholine autoreceptor M2 in the striatum (Gonzalez-Reyes et al. 2012). Among the neuronal subtypes of the striatum, M2 is mainly expressed by CINs, where
it provides negative feedback regulation of ACh release (Lim et al. 2014). Additionally, RGS4 is enhanced in CINs, which inhibits M2 to promote ACh release. In mice with genetic ablation of Shh from DA neurons, basal ACh release is attenuated, as well as, upregulated M2R’s (Gonzalez-Reyes et al. 2012). Taken together, these data suggest that Shh\textsubscript{DAN} plays a role in the release of ACh from CINs and may provide an unexpected link between DAN degeneration and LID development when it is lost in PD.
Figure 1.4: Shh signaling pathway

During the off state, Shh is unbound to Ptc-1 and Smo remained repressed. During the on state, Shh binds to Ptc-1, releasing the repression of Smo and activates downstream mechanisms. Shh: Sonic hedgehog; Ptc-1: patched receptor-1; Smo: smoothened receptor.
1.6 Intent of Thesis

The development of LID is not a natural symptom of PD, but instead, is a major complication of current PD therapy. The current gold-standard PD management only attempts to normalize levels of DA by treatment with the DA precursor L-Dopa and does not augment the other signaling modalities of DA neurons. Hence, L-Dopa therapy must cause an imbalance of DA and non-DA signaling modalities in medicated PD patients. Interestingly, knockdown of TH from DA neurons showed resistance to LID formation (Ulusoy et al. 2010). This study indicates that when other factors secreted by DA neurons remain intact L-Dopa therapy does not pose the risk of LID formation. This suggests that the presynaptic loss of all the factors associated with DA neurons, such as Shh, are critical in the development of LID.

Here, I provide several lines of evidence supporting the hypothesis that the loss of Shh in DA neuron degeneration contributes to the formation of LID during DA substitution therapy and, conversely, that augmenting Shh signaling specifically on CINs can alleviate LID. I tested the physiological relevance of Shh$_{DA}$ regarding LID, through pharmacological, genetic -presynaptic and -postsynaptic manipulation in diverse mouse and non-human primate models. First, I tested the Shh agonist, SAG, and Shh antagonist, cyclopamine, in two classic PD mouse models (1) the DA neuron toxin 6-hydroxydopamine (6-OHDA) unilateral lesion within the DLS (Boix et al. 2015; Lundblad et al. 2005), and (2) a genetic model of Aphakia mice (Pitx3$_{AK/AK}$) with selective loss of DA neurons in the SNpc through deletion of the Pitx-3 gene (Ding et al. 2007). Then I used genetic presynaptic manipulation where mice have conditional ablation of Shh from mature DA neurons (Shh$_{DA}$-/-) (Gonzalez-Reyes et al. 2012). The genetic postsynaptic manipulation approach consisted of (1) ablating Smo from CINs (Smo$_{CIN}$-/-) and (2) constitutively activating
Smo on CINs (SmoM2^+/CIN). Furthermore, in collaboration with Dr. Erwan Bezard, we corroborated the basic tenet of my hypothesis in MPTP treated macaques.

The findings described in this thesis reveal that reduced Shh_{DAN} signaling strength relative to DA signaling onto CIN of the DLS facilitates LID, while augmenting Shh signaling attenuates the formation of LID, as well as, the expression of established LID. My data suggests that Shh_{DAN} is a critical modulator of CIN activity and uncovers an un-anticipated presynaptic mechanism that contributes to LID. Further, Shh pathway agonists inhibit LID to the same degree as Amantadine without curtailing the anti-akinetic benefit of L-Dopa suggesting that the Shh signaling pathway constitutes a valid target for the development for LID inhibiting pharmacology.
Part I: Reduced Sonic Hedgehog by DA neuron degeneration contributes to LID formation that can be alleviated by replacing both DA and Sonic Hedgehog signaling factors
2. Chapter 2: Smo agonists attenuate LID in neurotoxic and genetic models of PD

2.1 Chronic Smo inhibition augments, while Smo activation attenuates LIDs

DA neurons utilize several signaling molecules, in addition to DA, to communicate with their targets, which all must diminish with DA neuron degeneration. Using a genetic tracer allele for Shh confirmed that Shh was reduced in parallel to DA neuron loss in 6-OHDA DA-toxin unilateral lesion model (Figure 2.1 A - C) and genetic aphakia mice lacking the pitx3 gene (AK−/−; Figure 2.1 D, E). Hence, the lack of Shh signaling in L-Dopa therapy might be a critical determinant of LID formation and/or expression in the well-established models of LID.

In the 6-OHDA model of LID, the unilateral, striatal injections of the toxin cause greater than 70% degeneration of nigral DA neurons ipsilaterally resulting in an ipsilateral turning bias (Figure 2.2 A, B). Acute and chronic L-Dopa dosing shifts the turning bias from ipsilateral to contralateral, indicative of the beneficial anti-akinetic effect of L-Dopa, and co-injection with the Smo agonist, SAG, did not affect the L-Dopa induced shift in rotational bias (Figure 2.2 B). Thus, SAG administration does not curtail the effectiveness of L-Dopa. Chronic exposure to L-Dopa in 6-OHDA injected mice results in the appearance of classically defined asymmetric abnormal involuntary movements (AIMs; Figure 2.3 A). Daily injections of L-Dopa (14 days, 5 mg/kg L-Dopa) with the Smo antagonist cyclopamine (5 mg/kg) results in an almost doubling of AIM scores on day 4 and 14 compared to L-Dopa alone (Figure 2.4 A, B). Conversely, co-injection of L-Dopa with SAG (0.8, 2.5, or 7 mg/kg) results in dose dependent, and over time gradual attenuation of AIMs from no effect at the lowest dose on day 4 to a more than 3 fold reduction in AIMs with the highest dose of SAG on day 14 compared to L-Dopa alone (Figure 2.4 B).
In AK<sup>-/-</sup> mice, the absence of the transcription factor Pitx3 results in the bilateral, partial loss of dopaminergic innervation of the dorsal striatum (Figure 2.1 D) and PD-like motor deficits that are responsive to L-Dopa therapy. Daily injection of AK<sup>-/-</sup> mice with 25 mg/kg L-Dopa results in LID. Observed previously, AK<sup>-/-</sup> mice need more days and a higher dose of L-Dopa to establish LIDs than 6-OHDA mice, this may be due to compensational mechanisms in the genetic model (Ding et al. 2007). As in the 6-OHDA model, daily co-injections of L-Dopa (25 mg/kg) with cyclopamine (5 mg/kg) results in a doubling of AIM scores by day 26 compared to L-Dopa only injected controls (Figure 2.4 A, C). In contrast, daily co-injections of L-Dopa (25 mg/kg) with SAG (20 mg/kg) results in an almost 75% reduction of AIM score compared to L-Dopa only injected controls (Figure 2.4 C).

Modulation of Smo activity in either model could potentiate or curtail the anti-akineti
effect of L-Dopa and thereby indirectly effect the display of AIMs. However, daily injections of cyclopamine or SAG with L-Dopa compared to daily injections of L-Dopa alone did not affect spontaneous locomotor activity in response to L-Dopa in 6-OHDA treated animals or AK<sup>-/-</sup> mice suggesting that the observed modulation of LID is not a reflection of an underlying regulation of motor activity level (Figure 2.4 D).

In the 6-OHDA mice, ceasing SAG treatment on day 15 while continuing daily L-Dopa injections resulted in a reappearance of AIMs on day 16, indicating the attenuation of SAG is rapidly reversible (Figure 2.5 A). Escalating the L-Dopa dose from 5 mg/kg to 20 mg/kg caused a concentration dependent increase in the severity of AIMs revealing that the reinstated AIMs remain sensitive to the concentration of L-Dopa dose. Reinstatement of SAG and L-Dopa co-treatment (days 39, 43, 47, 51) as a within subject SAG escalation caused a dose-dependent
attenuation of AIMs. Together, these findings show a bidirectional effect of stimulation and inhibition of Smo on LID formation in well-established models of LID.
Figure 2.1: Shh levels are reduced with DA neuron degeneration in classic models of PD

(A) Schematic showing DLS injection sites (2 ul x 2) of the DA neurotoxin 6-OHDA. (B) 10 x tile scan image of the midbrain following 6-OHDA injections showing SNpc dopaminergic neuron loss on the lesion side compared to the non-lesioned side. (C) Zoomed in 20 x images of SNpc for the non-lesion (top) and lesion side (bottom). TH, tyrosine hydroylase for DA neurons (red), Shh-lacZ (green), and DAPI (purple) were stained. Scale bar = 50 µm. (D) 10 x tile scan images showing one hemisphere of the midbrain for AK<sup>+/−</sup>; Shh<sup>L/L</sup> control mice and AK<sup>−/−</sup>; Shh<sup>L/L</sup> mutant mice with loss of DA neurons from the SNpc. (E) Zoomed in 20 x images of SNpc for the AK<sup>+/−</sup>; Shh<sup>L/L</sup> (top) and AK<sup>−/−</sup>; Shh<sup>L/L</sup> (bottom) mice. TH (red), Shh-lacZ (green), and DAPI (purple) were stained. Scale bar = 50 µm.
Figure 2.2: Dosing with L-Dopa and SAG has similar anti-parkinsonian properties in the 6-OHDA toxin model

(A) Immunohistochemistry showing unilateral loss of TH (red) indicative of a DA neuron lesion 6-OHDA model. (B) Quantification of rotational bias calculated as contra- to ipsi- lateral turn ratio. Mice that turn equally to both sides is represented by the dotted line at 0.5. Classically defined, 6-OHDA mice without L-Dopa (baseline: BL) turn ipsilateral to the lesion and when on L-Dopa mice turn contralateral to the lesion, indicative of the beneficial anti-akinetic effect of L-Dopa. Long-term dosing with both L-Dopa (5 mg/kg) and SAG (7 mg/kg) did not alter contralateral turning compared to controls (n = 8 - 9). RM 2-way ANOVA time effect: F (2,50) = 20.19 p<0.0001. Post hoc Bonferroni’s test: ** P < 0.01; *** P < 0.001 for BL vs. day 1 or day 13. n.s. indicates P > 0.05. Graphs plotted as mean +/- SEM.
Abnormal Involuntary Movement (AIMs)

<table>
<thead>
<tr>
<th>Rating Scale:</th>
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<tbody>
<tr>
<td>0. None</td>
</tr>
<tr>
<td>1. Rare</td>
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<td>2. Occasional</td>
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<tr>
<td>3. Frequent</td>
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<td>4. Continuous</td>
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Figure 2.3: Scoring criteria of abnormal involuntary movements (adapted from (Sebastianutto et al. 2016))

Represented images showing the four types of AIMS: 1) locomotion movement: contralateral rotations, 2) axial movement: dystonic posturing, and severe twisting of the head or neck, 3) limb movement: rapid, jerky movements of the front or hind limbs, and 4) orofacial movement: abnormal chewing, licking, grooming, or sticking out of the tongue. The four types of AIMS were scored on a severity scale of 0 - 4, with 0 exhibiting no abnormality and 4 showing uninterruptable continuous dyskinetic movement.
Figure 2.4: Chronic Smo inhibition facilitates while chronic Smo stimulation attenuates LID in a neurotoxic and a genetic model of PD

(A) Experimental design of chronic drug (cyclopamine: cyclo, or SAG) and L-Dopa daily regimen for 6-OHDA (top) or AK<sup>−/−</sup> (bottom) PD mouse models. (B) AIM score of 6-OHDA mice with either vehicle (n = 6), cyclo (5 mg/kg; n = 6), or SAG (7 mg/kg; n = 9). RM 2-way ANOVA treatment effect: F (4,28) = 12.24, p<0.0001. Post hoc Bonferroni’s test: * P<0.05, ** P<0.01 treatment vs. control. (C) AIM time of AK<sup>−/−</sup> with either vehicle (n = 8), cyclo (5 mg/kg; n = 7), or SAG (20 mg/kg; n = 8). 1-way ANOVA F (2,20) = 20.46 p<0.0001. Post hoc Bonferroni’s test: * P<0.05, ** P<0.01 treatment vs. control. (D) Fold change of distance moved following chronic co-treatment of L-Dopa and either cyclo or SAG in 6-OHDA (day 14; n = 8) and AK<sup>−/−</sup> (day 26; n = 12) mice over L-Dopa and vehicle treated controls. n.s. indicates P > 0.05. All bar graphs plotted as mean +/- SEM.
Figure 2.5: Smo agonist attenuation of LIDs is reversible and scales with L-Dopa dose

(A) Longitudinal AIM scoring during L-Dopa (5 mg/kg) administration with SAG (7 mg/kg) for 7 and 12 days. Followed by SAG washout during days 16 - 27 while maintaining 5 mg/kg L-Dopa. Subsequently, the L-Dopa dose was increased to 20 mg/kg during days 33 - 51, followed by a within subject escalation of SAG from days 39 - 51 from 1, 5, 10, and 15 mg/kg with 3 days of washout with only L-Dopa in between (d 7, 12 n = 9; d 16 - 51 n = 5). Paired 2-tailed student’s t test: * P < 0.05 for treatment vs. vehicle. n.s. indicates P > 0.05. Bar graph plotted as mean +/- SEM.
2.2 Acute activation of Smo decreases AIMs

Clinically relevant, animals with established AIMs were challenged with a single dose of the agonist of Smo to check for reduction in the severity of LID. LID was induced by daily injections of L-Dopa for 14 or 20 days in 6-OHDA lesioned or AK~−~/mice, respectively, and then given a single dose of either Smo agonist or antagonist co-injected with the final dose of L-Dopa (Figure 2.6 A). In the 6-OHDA lesioned animals, there was about a 50% attenuation of AIMs when animals were injected with either the Smo agonists, Purmorphamine (15 mg/kg, Figure 2.6 B) or SAG (10 mg/kg, Figure 2.6 C) along with L-Dopa. The AK~−~/mice were injected with either 5 mg/kg cyclopamine, 20 mg/kg SAG, the clinical effective antidyskinetic drug Amantadine (AM, 60 mg/kg) (Pahwa et al. 2015), or a combination of SAG (20 mg/kg) and amantadine (60 mg/kg) with 25 mg/kg L-Dopa (Figure 2.6 D). There was no change in AIM score following acute cyclopamine injection, but about a 50% reduction with either SAG or Amantadine and a 82% reduction with the combination of SAG and Amantadine (Figure 2.6 D). Acute injection of cyclopamine or SAG had no effect on spontaneous locomotor activity compared to controls (Figure 2.6 E). However, Amantadine by itself or in combination with SAG depresses locomotor activity three-fold compared to controls (Figure 2.4 E). These results indicate that a single dose of SAG significantly attenuates the display of established LID to a degree similar of that achieved by Amantadine without altering overall movement.
Figure 2.6: Acute Smo stimulation attenuates established LIDs in a neurotoxic and a genetic model of PD

(A) Experimental design of acute drug challenge (purmorphamine: PUR, cyclo, SAG, and/or amantadine: AM) regimen following chronic daily L-Dopa. (B) AIM score of 6-OHDA mice challenged with vehicle (n = 6) or PUR (15 mg/kg; n = 7). Unpaired 2-tailed student’s t test * P<0.05 for treatment vs. control. (C) AIM score of 6-OHDA mice challenged with vehicle (n = 5) or SAG (10 mg/kg; n = 7). Unpaired 2-tailed student’s t test * P<0.05 for treatment vs. control. (D) AIM time of AK-/mice challenged with either vehicle (n = 13), cyclo (5 mg/kg; n = 13), SAG (20 mg/kg; n = 13), AM (60 mg/kg; n = 8) or SAG combined with AM (n = 8). 1-way ANOVA F(4,50) = 9.79 p<0.0001, Post hoc Bonferroni’s test: * P<0.05, ** P<0.01, *** P<0.001 for treatment vs. control. (E) Fold change of distance moved on day 20 following chronic L-Dopa and acute treatment in AK-/mice following 5 mg/kg cyclo (n = 9), 20 mg/kg SAG (n = 8), 60 mg/kg Amantadine (AM; n = 9), or combined AM and SAG (n = 8). Unpaired 2-tailed student’s t test: * P < 0.05 for treatment vs. vehicle. n.s. indicates P > 0.05. All bar graphs plotted as mean +/- SEM.
2.3 pErk1/2 activation in CINs is attenuated by Smo activation

As mentioned earlier, activation of the MAP-kinase pathway as recognized by the phosphorylation of Erk1/2 (pErk1/2) has emerged as a biochemical marker of LID (Ding et al. 2011; Pavón et al. 2006; Santini et al. 2009). L-Dopa injection to DA-hypersensitive animals, but not to intact animals, increases the prevalence of pErk1/2 positive neurons in the striatum and the pharmacological inhibition of the MAP kinase pathway attenuates LID in mice models (Ding et al. 2011). Consistent with the behavioral data, there is a two-fold increase of pErk1/2 prevalence in CINs of the DLS but not the DMS after co-injections of cyclopamine and L-Dopa compared to controls in 6-OHDA lesioned animals (Figure 2.7 A - C). Conversely, there is a two-fold decrease of the prevalence of pErk1/2 expressing CINs after SAG and L-Dopa co-injection compared to controls in 6-OHDA lesioned animals (Figure 2.7 A - C). In AK−/− mice, there is a 1.5-fold increase in the numbers of pErk1/2 expressing CINs following cyclopamine and L-Dopa co-injections and, conversely, an almost two-fold decrease following SAG and L-Dopa co-injections in the DLS but not the DMS (Figure 2.7 A – C). Similarly, there is a two-fold decrease in the prevalence of pErk1/2 positive CINs following either acute Purmorphamine or SAG in 6-OHDA treated animals and following SAG in AK−/− mice in the DLS but not in the DMS (Figure 2.7 D). There was no change in the prevalence of pErk1/2 positive CIN in response to acute cyclopamine injection into AK−/− mice compared to controls in the DLS or DMS, similar to their lack of behavioral results (Figure 2.7 D). Thus, consistent with the established increase in the numbers of pErk1/2 positive CIN in the DLS in well-studied models of LID we find that the Smo mediated attenuation of LID is associated with a correlated reduction in the prevalence of pErk1/2 in CIN selectively in the DLS.
Figure 2.7: Smo inhibition enhances while Smo stimulation reduces pErk1/2 expression in CINs following chronic L-Dopa dosing

(A) Schematic showing different compartments of the dorsolateral (DL) and dorsomedial (DM) striatum where images were quantified. (B) Represented images of pErk1/2 (red) colocalization (arrowhead/yellow) with ChAT (green) from animals in the chronic drug experiments in Figure 2.4. Scale bar = 50 µm. (C) Quantification of the prevalence of pErk1/2 in CINs expressed as fold change from animals in the chronic drug experiments in Figure 2.4. Values are expressed as fold change over vehicle treated animals (~36 CINs per condition). Unpaired 2-tailed student’s t test * P<0.05, ** P<0.01 treatment vs. vehicle. (D) Same as above from the acute challenged drug experiments in Figure 2.6. (~36 CINs per condition). Unpaired 2-tailed student’s t test ** P<0.01, *** P<0.001, for treatment vs. vehicle. n.s. indicates P > 0.05. All bar graphs plotted as mean +/- SEM.
2.4 SAG Administration reduced dyskinetic behavior in the MPTP Macaque model of PD

Most pharmacological treatments in clinical development for PD are pre-clinically tested on methylphenyltetrahydropyridine (MPTP) treated macaque monkeys because they phenocopy human symptoms (Bezard et al. 1997). Therefore, in a collaboration with Dr. Erwan Bezard, we tested whether acute treatment with SAG could attenuate the display of established LID in non-human primates (Appendix 1 A). Four Macaques were rendered parkinsonian with repeated injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP; i.v.) and then subjected to a chronic L-Dopa treatment regimen, which induced dyskinesia, a process that overall takes 7 months. These dyskinesias can be blunted by the clinically effective anti-dyskinetic drug amantadine (Ko et al. 2014). Vehicle or SAG (3, 9, and 27 mg/kg) was administered with L-Dopa injection in a within-subject drug escalation design (Appendix 1 A). The acute injections of SAG lead to a dose dependent attenuation of established LID showing significance at the highest dose of SAG at 27 mg/kg during the first 2 hours of drug exposure (Appendix 1 B, C). As observed in the murine models of LID, SAG injections did not diminish the anti-akinetic benefits of L-Dopa treatment in the Macaques (Appendix 1 D).
2.5 The attenuation of LID by Smo stimulation scales with the concentration of L-Dopa

The opposite effect of inhibition and stimulation of Shh/Smo signaling on LID under otherwise constant dosing of L-Dopa suggests that the severity of LID may be correlated to the relative difference between Shh/Smo and DA signaling. Therefore, the dose-response relationship between L-Dopa and SAG was examined. LIDs were induced in three separate groups of AK−/− mice, receiving either 5, 10 or 30 mg/kg of L-Dopa daily leading to distinct, concentration dependent, levels of LID severity. In a probe trial, the degree of LID attenuation was tested using four different concentrations of SAG 0.8; 2.5; 7.5; and 15 mg/kg in each L-Dopa group as a within subject escalation (Figure 2.8 A, B; 3 days of SAG washout in between each incremental SAG increase). In each L-Dopa group, the attenuation of LID by SAG was dose-dependent (Figure 2.8 B). These results allow the calculation of the estimated concentration of SAG that results in half-maximal (EC50) inhibition of LID in each of the three L-Dopa groups. The analysis revealed a correlation between the dose of SAG needed to counteract LIDs that formed in response to escalating concentrations of L-Dopa compared to controls receiving only L-Dopa (R² = 0.965, Figure 2.8 C). These results indicate that the severity of LID is determined by the degree of imbalance between Shh/Smo and DA signaling, suggesting that Shh/Smo signaling impedes DA-inhibition of CINs.
Figure 2.8: Degree of attenuation of LID by Smo agonist scales with L-Dopa dosing

(A) Timeline of dose-response experiment showing three cohorts of AK−/− mice receiving either chronic 5 mg/kg L-Dopa, 10 mg/kg L-Dopa, or 30 mg/kg L-Dopa and within subject escalating doses of SAG (0.8, 2.5, 7.5, and 15 mg/kg; n = 8 for each drug condition). There were 3 days of SAG washout with just L-Dopa dosing between each challenge dose. (B) Dose-response graph. The effect of SAG is expressed as a ratio compared to controls with 1.0 having complete elimination of AIMs, 0 showing the same degree of AIMs as controls and 0.5 as the half-maximal effective concentration (EC50- dotted line) that reduces LID 50%. Linear regression lines were plotted to best fit the data points. Stars represent significance to corresponding controls receiving L-Dopa and vehicle only. Unpaired 2-tailed Student’s t test * P<0.05, ** P<0.01. n.s. indicates P > 0.05. Graph plotted as mean +/- SEM. (C) EC50 response graph showing a positive linear regression (R² = 0.965) with each escalating L-Dopa dose.
2.6 Smo activation potentially acts upstream of M4R activation on dSPN

Considering the implication of aberrant hyperactivity of dSPNs in LIDs, the next obvious question was: Is Smo dependent modulation of LID mediated by CIN regulation of dMSN activity? The finding that boosting cholinergic signaling via the muscarinic M4 receptor, which is expressed by dSPNs, strongly attenuating AIMs in the 6-OHDA model of LID (Shen et al. 2016) offered an experimental opening to test if Shh signaling acts upstream or downstream of dSPN activity. If this thinking is correct, Smo modulatory effect on CIN activity would be upstream of M4R effect on dSPNs, and then cyclopamine (enhances LID) simultaneously administered with M4PAM (attenuates LIDs) can give a readout of LID to determine the pathway sequence of events. Consistent with the previous study, M4 positive allosteric modulator (M4PAM), VU0467154, attenuated L-Dopa induced AIMs in AK−/− mice supporting the idea that LID in AK−/− mice are caused in part by reduced cholinergic signaling onto SPNs (Figure 2.9 A, B). As predicted, the cyclopamine-induced increase in AIMs can be attenuated by M4PAM to the same levels achieved by M4PAM in only L-Dopa treated AK−/− mice (Figure 2.9 B), consistent with Smo mediated modulation of LID occurring presynaptic to M4 signaling on dSPNs. Although CINs have M4R themselves to induce auto-inhibition, cyclopamine would also decrease CIN activity (Gonzalez-Reyes et al. 2012) and both would be presynaptic to the dSPN effects. The attenuation observed by M4R activation of dSPNs was because M4R promotes LTD restoring the LID-induced hyperactivity of dSPNs (Shen et al. 2016). Together, this finding point to Smo mediated modulation of LID in AK−/− mice being upstream of the action of M4R on dSPNs and gives new insight into the mechanisms of how ShhDAN signaling works to counteract LIDs.
Figure 2.9: Shh signaling reduces LIDs by acting upstream of M4R activation on dSPNs

(A) Experimental design displaying that AK⁻/⁻ mice were dosed for several days with L-Dopa (25 mg/kg) or L-Dopa (25 mg/kg) and cyclo (5 mg/kg) until LIDs were established, and then challenged with a single dose of M4PAM, an allosteric M4R agonist (cylo: cyclopamine; M4PAM: VU0467154). (B) AIM time of AK⁻/⁻ mice following daily L-Dopa with either vehicle or cyclo and challenged with M4PAM (n = 7). Paired 2-tailed Student’s t test P<0.05, * P<0.05, ** P<0.01 for Veh vs. M4PAM. Unpaired 2-tailed Student’s t test P<0.05, * P<0.05 for Veh vs. Cyclo. Bar graph plotted as mean +/- SEM.
3. Chapter 3: Presynaptic ablation of Shh<sub>DAN</sub> leads to the development of LIDs that is rescued with Smo activation

3.1 Shh<sub>DAN</sub>-/- mice develop LID in a dose dependent manner

To examine whether Shh signaling specifically originating from DA neurons rather than from other sources in the body is the player involved in LIDs, AIMS were assessed in mice with selective ablation of Shh from DA neurons (Shh<sub>DAN</sub>-/-; Figure 3.1 A; all mouse strains used in this study are referenced in the methods section). As seen in the 6-OHDA and AK<sup>/-</sup> mice, LID behavior was developed in a dose-dependent manner upon chronic treatment with L-Dopa in a dose dependent manner (Bastide et al. 2015; Ding et al. 2007; Lundblad et al. 2004). Daily dosing with 10 mg/kg L-Dopa for 11 days gradually induced AIMS in Shh<sub>DAN</sub>-/- mice starting on day 1 (Figure 3.1 B, C). AIMS were observed only in Shh<sub>DAN</sub>-/- mice and not in control animals. Increasing L-Dopa to 25mg/kg resulted in doubling of AIMS on day 12 in Shh<sub>DAN</sub>-/- mice and induced an almost seven-fold increase from controls suggesting that Shh<sub>DAN</sub>-/- mice develop dose-dependent LID (Figure 3.1 B, C).
Figure 3.1: Genetic ablation of ShhDAN promotes the development of LIDs that are rescued with Smo activation

(A) Shh was ablated from DA neurons using a combination of a Shh conditional allele and Dat-Cre mouse line. (B) Experimental design for chronic L-Dopa regimen in ShhDAN+/− and ShhDAN−/− mice (Baseline: BL, day: d). (C) LID induction by daily injection of 10 mg/kg L-Dopa for 11 days (RM 2-way ANOVA day x genotype: F(2,36) = 4.53, p=0.018. Post hoc Bonferroni’s test: * P<0.05 for BL vs. d11) followed by a 25 mg/kg L-Dopa challenge on Day 12 (RM 2-way ANOVA treatment effect: F(1,18) = 16.23, p=0.001. Post hoc Bonferroni’s test: *** P<0.001 for d11 vs. d12; genotype effect: F(1,18) = 18.36, p=0.0004, **** P<0.0001 for control vs. mutant on d12) in 3 - 4 month ShhDAN−/− and ShhDAN+/− mice (n = 10). (D) Experimental design for pharmacological complementation to restore Shh pathway activity. (E) Pharmacological complementation with 10 or 20 mg/kg SAG (n = 5) or vehicle (n = 10) in combination with L-Dopa after chronic 25 mg/kg L-Dopa for 20 days. 2-way ANOVA genotype effect: F(1,35) = 21.36. Post hoc Bonferroni’s test: p=0.0002, **** P<0.0001 for control vs. mutant on d 20; genotype x treatment effect: F(2,35)=9.15, p=0.001. Post hoc Bonferroni’s test: *** P<0.001, **** P<0.0001 for mutant with and without SAG on d 20 and d 21. n.s. indicates P > 0.05. All bar graphs plotted as mean +/- SEM.
3.2 Smo activation reduces LID in Shh\textsubscript{DAN-/-} mice

To determine whether the acute absence of Shh signaling plays a role in LID expression in Shh\textsubscript{DAN-/-} mice, treatment with SAG was administered to see if restoring Shh signaling levels would counteract LIDs. Using a different cohort of animals, L-Dopa was injected at 25 mg/kg daily for 20 days inducing pronounced AIMs in Shh\textsubscript{DAN-/-} mice but not in control animals as expected (Figure 3.1 D, E). In a probe trial, co-injection of L-Dopa with either 10 or 20 mg/kg SAG did not show any effects in controls but reduced AIMs to control levels in Shh\textsubscript{DAN-/-} mice (Figure 3.1 D, E). These results indicate that the long-term reduction of Shh\textsubscript{DAN} renders the brain susceptible to the formation of LID-like motor activity that can be attenuated by the acute postsynaptic activation of Shh signaling.

3.3 pErk1/2 activation in CINs is attenuated with SAG

Next, Shh\textsubscript{DAN-/-} mice were investigated to see if they would express biochemical markers of LID in response to repeated L-Dopa injection and established LIDs. Consistent with the behavioral data, the pattern of pErk1/2 expression in the striatum shows cell type specific dynamics. There was a 50% increase in the prevalence of CINs with detectable pErk1/2 30 min after L-Dopa (25 mg/kg) injection only in the DLS of Shh\textsubscript{DAN-/-} mice compared to control animals just receiving L-Dopa (25 mg/kg; Figure 3.2 A - C). A single co-injection of 20 mg/kg SAG with L-Dopa 30 min prior to brain harvest on day 21 normalized the prevalence of pErk1/2+ CINs in the DLS to control levels (Figure 3.2 A - C). There was no difference in the prevalence of pErk1/2+ CINs of the DMS in L-Dopa injected Shh\textsubscript{DAN-/-} mice compared to controls, nor did SAG co-injection with L-Dopa decrease the prevalence of pErk1/2+ CIN in the
DMS (Figure 3.2 B, C). No changes were detected in the prevalence of pErk1/2+ FSN, the only other neuronal subtype in the striatum that expresses the Shh receptor Ptc-1 (Gonzalez-Reyes et al. 2012), in L-Dopa treated ShhDA/N-/- mice and controls (Figure 3.2 D). Collectively these results indicate that ShhDA/N-/- mice display LID-like behavior upon chronic L-Dopa administration that results in expression of the classical biochemical LID markers pErk1/2 in the DLS and implicates ShhDA/N as a regulator of the MAP Kinase pathway in CINs.
Figure 3.2: ShhDAN/- mice have increased pErk1/2 expression in CINs following chronic L-Dopa dosing in the DLS

(A) Represented images of mice from the complementary experiment in Figure 3.1, pErk1/2 (red) colocalization (arrows) in CIN (ChAT, green) following 25 mg/kg L-Dopa with and without 20 mg/kg SAG in DLS (Scale bar = 50 µm). (B) Same as above for the DMS. (C) Percent of pErk1/2+ CINs from images in A - B (n = 8 - 12, 35-38 CINs each). RM 3-way ANOVA genotype effect: F_{(1,105)} = 7.09, p=0.009. Post hoc Bonferroni’s test: **** P<0.0001 for control vs. mutant; treatment effect: F_{(1,105)} = 9.79, p=0.002. Post hoc Bonferroni’s test: *** P<0.001 for mutant with and without SAG; and area x genotype effect: F_{(1,98)} = 11.21, p=0.001. Post hoc Bonferroni’s test: ** P<0.01 for mutant DLS vs. mutant DMS. (D) Percentage of pErk1/2 (red) among Parv+ FSNs (parvalbumin, green) following chronic L-Dopa in the DLS and DMS areas (n = 8, ~36 parv+ cells each). n.s. indicates P > 0.05. All bar graphs plotted as mean +/- SEM.
3.4 Shh signaling across the striatum seems to be patterned and not homogenous

Consistent with the marked spatial selectivity of the regulation of MAP kinase pathway by ShhDAN, there was enhanced Shh signaling found in the DLS. Using a gene-expression tracer allele for Gli3, which is a mediator of Shh signaling and is itself up regulated by Shh signaling, revealed a pronounced high lateral to low medial gradient across the DLS (Figure 3.3 A). Conditional labeling of neuropil of Shh expressing neurons by the Shh-Cre dependent activation of myristylated GFP showed a complex pattern with the highest density of Shh carrying neurites in the DLS of the anterior and posterior striatum compared to the DMS (Figure 3.3 B, C). These differences between the DLS and DMS are consistent with habitual learned behavior, the prevalence of DA neuron degeneration in PD, CIN loss in aged ShhDAN-/- mice, and pErk1/2 expression in CINs (Figure 3.3 D). Together, these results give evidence of the spatial selective patterning of Shh signaling in the striatum and further implicates the link between ShhDAN signaling in the DLS and PD-like pathologies.
Figure 3.3: ShhDAN signaling in the striatum has a patterned bias towards the DLS

(A) Using a gene-expression tracer allele for the Shh signaling transcription factor, Gli3, revealed a gradient across the striatum with high lateral and low medial expression. (B) Images showing the expression pattern of myristylated GFP in the anterior and posterior striatum of Shh carrying neuropil. Conditional neuropil labeling of neurons that express Shh driven by Shh-Cre expression of myristylated GFP. Images shown as a heat map in a 16-member pseudocolor palette to depict the intensity of GFP fluorescence. (C) Quantification of the relative intensity of myristylated GFP between the DLS and DMS in both the anterior and posterior part of the striatum. Paired 2-tailed student’s t test * P<0.05, ** P<0.001 for DLS vs DMS (n = 8). (D) Scheme showing the prevalence of behaviors and pathologies restricted to the DLS and not observed in the DMS.
3.5 The prevalence of c-Fos in LIDs is attenuated with Smo agonist

Immediate early genes like c-Fos are strongly induced in response to L-Dopa injection in rodent and primate models of PD (Pavón et al. 2006). Likewise, the optogenetic inactivation of neuronal assemblies in the DLS that previously activated c-Fos expression in response to L-Dopa injection, attenuates LID (Girasole et al. 2018). Consistent, there is a six-fold increase in the prevalence of c-Fos+ cells in the DLS in response to chronic L-Dopa injection in ShhDA N−/− mice compared to controls (Figure 3.4 A, B). Co-injection of 20 mg/kg SAG with L-Dopa 30 min prior to brain harvest normalized the activation of c-Fos to control levels (Figure 3.4 A, B). Interestingly, these c-Fos+ neurons are not CIN+ or FSN+ and must be SPNs, suggesting an indirect role of ShhDA N on overall activity of the SPN pathways.
Figure 3.4: Smo activation reduces overall c-Fos expression following chronic L-Dopa dosing in mice lacking ShhDAN

(A) Represented images of c-Fos+ cells (red) in the DLS following chronic L-Dopa dosing in control and ShhDAN/- mice from Figure 3.1 (Scale bar = 50 µm). (B) Quantification showing the number of total c-Fos+ cells following 25 mg/kg L-Dopa or 25 mg/kg L-Dopa and 20 mg/kg SAG. 2-way ANOVA genotype effect: $F_{(1,80)} = 38.10$, $p<0.0001$. Post hoc Bonferroni’s test: **** $P<0.0001$ for control vs. mutant; treatment x genotype effect: $F_{(1,80)} = 44.90$, $p<0.0001$. Post hoc Bonferroni’s test: **** $P<0.0001$ for mutant with and without SAG (n = 8, ~445 cells per 700 µm x 700 µm area of the DLS). n.s. indicates $P > 0.05$. All bar graphs plotted as mean +/- SEM.
4. Chapter 4: Post-synaptic stimulation of Smo receptor on CINs reduces LID

4.1 Smo ablation on CINs leads to the development of LIDs

To examine whether ShhDAN signaling occurs through CINs, LID-like behaviors were assessed in mice with selective ablation of Smo directly from CINs (Smocin-/-; Figure 4.1 A). Mice were chronically dosed with 25 mg/kg L-Dopa and quantified for LID development (Figure 4.1 B). Continuous L-Dopa (25 mg/kg) dosing for 20 days revealed induction of AIMs in Smocin-/- mice (Figure 4.1 C). Interestingly, control animals with one normal allele of Smo on CINs (Smocin+/-) showed slightly increased AIMs compared to their baseline levels but was significantly lower than Smocin-/- mice (Figure 4.1 C). This suggests an allele dependent relationship and suggests dose-dependent signaling through normal CIN. Examining overall locomotor activity of Smocin-/- mice reveals a reduction in baseline levels compared to controls (Figure 4.1 D). This reduction in movement suggests the importance of Shh/Smo signaling in movement and phenocopies to reduced locomotor activity seen in Shhdan-/- mice (Gonzalez-Reyes et al. 2012). These differences were eliminated following 20 days of L-Dopa dosing, suggesting that chronic L-Dopa restored locomotor deficits to control levels.

Consistent with the behavioral data and earlier results, the pattern of pErk1/2 expression showed increased pErk1/2 expression in CINs following 20 days of chronic L-Dopa (25 mg/kg) in Smocin-/- mice compared to controls (Figure 4.1 E, F). This increase in the prevalence of pErk1/2 expression in CINs is restricted to the DLS and not observed in the DMS, consistent with earlier results from cyclopamine experiments (Figure 2.7 B, C) and in Shhdan-/- mice (Figure 3.2 A - C). Additionally, the activation of c-Fos expression was increased in Smocin-/- mice compared to controls following 20 days of L-Dopa dosing, correlating to their development.
of LIDs (Figure 4.1 G). These findings point to Shh_{DAN} signaling acting through Smo directly on CINs.
Figure 4.1: Genetic ablation of Smo selectively from CINs contributes to the development of LIDs

(A) Construct and scheme of postsynaptic Smo ablation from CINs using a combination of the Smo conditional allele and ChAT-IRESCre mouse line. (B) Experimental design for chronic daily L-Dopa (25 mg/kg) regimen in SmoCIN+/− and SmoCIN−/− mice (Baseline: BL, day: d). (C) Quantification of LID induction by daily injection of 25 mg/kg L-Dopa for 20 days (RM 2-way ANOVA day x genotype effect: F(1,11) = 4.95, p=0.048. Post hoc Bonferroni’s test: ** P<0.01 for control vs. mutant on d20; and day effect: F(1,11) = 52.61, p<0.0001. Post hoc Bonferroni’s test: *
P<0.05 for control BL vs. d20 and **** P<0.0001 mutant BL vs. d20) in 10 - 11 month 
SmoCIN+/- and SmoCIN-/- mice (n = 6 – 7). (D) Overall distance moved shows baseline reduction 
in activity for SmoCIN-/- mice. RM 2-way ANOVA genotype effect: F (1,11)=5.19 p=0.044. Post 
hoc Bonferroni’s test: * P<0.05 for control vs. mutant BL. L-Dopa dosing for 20 days showed no 
change in activity between SmoCIN+/- and SmoCIN-/- mice. n.s. indicates P > 0.05. (E) 
Representative images of pErk1/2 (red) colocalization (yellow, white arrows) in CIN (ChAT, 
green) following 25 mg/kg L-Dopa in the DLS and DMS (Scale bar = 50 µm). (F) Quantification 
of percent of pErk1/2+ CINs (n = 6 - 7, 35-38 CINs each). RM 2-way ANOVA area x genotype 
effect: F (1,135) = 7.95, p=0.006. Post hoc Bonferroni’s test: ** P<0.01 for control vs. mutant DLS. 
n.s. indicates P > 0.05. (G) Images (left; Scale bar = 50 µm) and quantification (right) of the 
overall number of c-Fos+ cells (red) in the DLS following 25 mg/kg L-Dopa (n = 6 - 7, ~445 
cells per 700 µm x 700 µm area). Unpaired student’s t-test ** P<0.01 for control vs. mutant. All 
bar graphs plotted as mean +/- SEM.
4.2 Constitutively active Smoothened receptor on CINs ameliorates LIDs

The next complementary experiment investigated the gain of function of Smo using a constitutively active form of Smo called SmoM2 on CINs, independent of Shh source or other Smo+ cells, such as FSN(Figure 4.2 A; SmoM2+/− AK−/−_chat-cre mice). SmoM2 is fused with an eYFP protein and is expressed only in those cells that are recombined (Figure 4.2 B, showing expression of SmoM2 in CINs). Upon chronic dosing with 25 mg/kg L-Dopa, LIDs emerge in control AK−/−_chat-cre mice by day 12 but not in SmoM2+/− AK−/−_chat-cre mice (Figure 4.2 C, D). Hence, these mice show an opposite phenotype compared to ShhDAN−/− and SmoCIN−/− mice. Additionally, SmoM2+/− mice without the presence of a Cre promoter were assessed to determine if the SmoM2 allele disrupted AIM induction. There were no differences observed in AIM formation between both controls (SmoM2+/− mice or AK−/−_chat-cre mice) for all 20 days of dosing (data not shown). Locomotor activity was unchanged in SmoM2+/− AK−/−_chat-cre mice compared to controls during baseline and all days of L-Dopa dosing revealing that the observed resistance of LID formation is not a reflection of an underlying reduction of motor activity (Figure 4.2 E).

The expression of pErk1/2 in CINs was evident in control mice by day 20 but was reduced in SmoM2+/− AK−/−_chat-cre mice, mimicking their AIM behavior (Figure 4.2 F, G). Interestingly, pErk1/2 was reduced in both the DLS and DMS. This suggests that regulation of Erk1/2 by Smo signaling can occur in all CINs. Thus, the observation of a selective increase of pErk1/2 in the DLS in ShhDAN−/− animals is consistent with the idea that Shh signaling predominantly occurs on CIN of the DLS in the undisturbed brain.
Figure 4.2: Constitutively active form of SmoM2 in CINs provides resistance to LID development

(A) Construct and scheme of mice with postsynaptic constitutively active version of SmoM2 on CINs using a combination of a SmoM2-YFP conditional allele and ChAT-IRES-Cre mouse line in the AK\(^{-/-}\) mice. (B) Images of the DLS showing recombination of constitutively active version SmoM2 specifically in CINs using eYFP (green) co-labeled with ChAT (red, white arrows) that is not seen in AK\(^{-/-}\)ChAT-Cre control mice. (C) Experimental design for chronic daily L-Dopa (25 mg/kg) regimen for 20 days (day: d). (D) LID quantification by daily injection of 25 mg/kg L-Dopa for 20 days in 2 - 4 month control AK\(^{-/-}\)ChAT-Cre and mutant SmoM2\(^{+/+}\)AK\(^{-/-}\)ChAT-Cre mice (n = 6). RM 2-way ANOVA time x genotype effect: F\(_{(5,50)}\) = 7.91, p<0.0001. Post hoc Bonferroni’s test: * P<0.05, **** P<0.0001 for control vs. mutant. (E) Overall distance moved was unchanged between control and experimental mice during both baseline and over 20 days of daily L-Dopa dosing. (F) Representative images of pErk1/2 (red) colocalization (yellow, white arrows) in CIN (ChAT, green) following 25 mg/kg L-Dopa in the DLS and DMS (Scale bar = 50
µm). (G) Percent of pErk1/2+ CINs (n = 8 - 12, 35 - 38 CINs each). RM 2-way ANOVA genotype effect: F (1,68) = 27.42, p<0.0001. Post hoc Bonferroni's test: *** P<0.001, **** P<0.0001 for control vs. mutant. n.s. indicates P > 0.05. All bar graphs plotted as mean +/- SEM.
5. Chapter 5: Potential mechanisms: Lack of Smo activation reduces CIN activity by dampened cortical glutamatergic input

5.1 Lack of ShhDAN attenuates while Smo agonist rescues basal p-rpS6^{240/244} levels of CINs

Reduced CIN activity might be a contributing factor in LID formation and expression considering the evidence that increased nicotine use, boosting signaling by the muscarinic ACh receptor M4, and optogenetic activation of CINs attenuate LID in mouse models (Perez et al. 2018). Therefore, the role of ShhDAN on CIN activity was explored to understand its function in LIDs. In order to allow comparative measurements of cholinergic activity across populations of CINs, p-rpS6^{240/244} immunoreactivity was used. p-rpS6^{240/244} is part of the m-Torc pathway and is a widely used neuronal activity marker for CIN, which has been shown to follow the spiking pattern of CINs under basal and stimulated conditions (Bertran-Gonzalez et al. 2012; Matamales et al. 2016; Matamales et al. 2016). Analysis of CIN activity through p-rpS6^{240/244} activity has been used in studies investigating reinforcement learning, aging, and catalepsy induced behaviors (Bertran-Gonzalez et al. 2012; Kharkwal et al. 2016; Matamales et al. 2016). To account for across subject variability, NeuN immunoreactive marker was used as an internal control to normalize housekeeping levels between subjects. There was a 37% reduction in p-rpS6^{240/244} reactivity in CINs of ShhDAN/- mice compared to controls in the DLS (Figure 5.1 A - raw images showing immunostaining; heat map images in Figure 5.2 A and quantification in Figure 5.2 B, presynaptic side). Consistent with this, cholinergic tone, measured by in vivo dialysis of ACh release, was reduced 8-fold in ShhDAN/- mice (Gonzalez-Reyes et al. 2012). Remarkably, acute injection of one dose of SAG (20 mg/kg) into ShhDAN/- mice normalizes p-rpS6^{240/244} levels (Figure 5.2 A, B, presynaptic side). Injection of L-Dopa (25 mg/kg) shows similar degree of activity in CIN of ShhDAN/- mice but acute injection of SAG (20 mg/kg)
counteracted CIN activity in the presence of L-Dopa and normalizes levels in $Shh_{DA^+/-}$ mice to control basal levels (Figure 5.2 A, B, presynaptic side). These results suggest that reduced Shh signaling emanating from DA neurons might contribute to the formation and expression of LID by dampening cholinergic activity in the DLS.
Figure 5.1: Activity Marker p-rpS6$^{240/244}$ in CINs

(A) Large 20x raw images showing p-rpS6$^{240/244}$ co-localized in CINs for basal levels of $Shh_{DAN}^{+/−}$ mice (top), $Shh_{DAN}^{-/−}$ mice (bottom; Scale bar = 50 um). Smaller panels show zoomed in image of square (Scale bar = 10 um) with markers for p-rpS6$^{240/244}$ (red), ChAT (green), NeuN (blue) and a heat map of p-rpS6 showing a 16 member pseudocolor palette to depict the intensity of p-rpS6$^{240/244}$ fluorescence. Left scheme shows the DL area within the striatum where the images were taken from.
Figure 5.2: Lack of ShhDAN signaling reduces activity marker p-rpS6\textsuperscript{240/244} in CINs that is restored with Smo activation directly

(A) Representative high-magnification confocal images of p-rpS6\textsuperscript{240/244} in CINs of DL striatum as a heat map (Scale bar = 10 um). The corresponding smaller image shows ChAT (red) and DAPI (blue) double stain. A region of interest (ROI) is shown around the ChAT staining in the white dotted line. Each CIN has its own ROI and is quantified based on the mean gray value intensity (m.g.v.) per cell of p-rpS6\textsuperscript{240/244} staining normalized to NeuN staining, to account for between subject differences, in individual somata. A 16-member pseudocolor palette depicts the intensity of p-rpS6\textsuperscript{240/244} fluorescence. Left side represents images of mice with genetic pre-synaptic manipulations and saline, SAG, or L-Dopa dosing, while the right side represents images of mice with genetic post-synaptic manipulations and saline or SAG dosing. (B) Quantification of p-rpS6\textsuperscript{240/244} intensity of CINs. Each dot represents one neuron, and red lines indicate the mean ± SEM. In order on the pre-synaptic side, there’s Shh\textsubscript{DAN}+/- mice (white), Shh\textsubscript{DAN}-- mice (red), Shh\textsubscript{DAN}-- mice with 20 mg/kg SAG (blue), Shh\textsubscript{DAN}+/- mice with 25 mg/kg L-Dopa (grey) and Shh\textsubscript{DAN}+/- mice with 25 mg/kg L-Dopa and 20 mg/kg SAG (cyan). In order on the post-synaptic side, there’s Smo\textsubscript{CIN}+/+ mice (white), Smo\textsubscript{CIN}-- mice (orange), Smo\textsubscript{CIN}-- mice with 20 mg/kg SAG (blue), and Smo\textsubscript{M2}+/--\textsubscript{ChAT-cre} mice (cyan). Kruskal-Wallis
nonparametric 1-way ANOVA followed by Dunn’s post-test shows **** P<0.0001 for controls vs. mutants, or mutants with and without treatment, n.s. indicates P > 0.05. (n = 3-4, 60-100 cells per n). (C) Scheme showing possible mechanism of Shh signaling counteracting DA inhibition of CINs that lead to balanced control of SPNs and restored motor output.
5.2 Genetic ablation of Smo on CINs attenuates p-rpS6\textsuperscript{240/244} that is not rescued with Smo agonist

Examining the postsynaptic manipulation of Smo on CIN activity revealed reduced activity in Smo\textsubscript{CIN-/-} mice consistent with results from the ligand ablation experiments (Figure 5.2 A, B). Importantly, acute injection of SAG (20 mg/kg) did not restore p-rpS6\textsuperscript{240/244} levels as seen in Shh\textsubscript{DAN-/-} mice (Figure 5.2 A, B, postsynaptic side). This finding established that SAG acts through Smo on CINs rather than through indirect mechanisms to regulate CIN activity. Conversely, Smo\textsubscript{M2+/-} expression in CINs showed similar levels of CIN activity compared to controls suggesting that baseline levels of Shh signaling results in maximal p-rpS6\textsuperscript{240/244} levels in CINs (Figure 5.2 A, B, postsynaptic side).

Additional evidence for the acute regulation of CIN activity by Shh signaling comes from viral injection of AAV9-eGFP-cre into the DLS of Smo\textsuperscript{L/L} mice (Figure 5.3 A). To eliminate compensational effects of genetic ablation of Smo on CINs from birth, viral injections allowed the study of induced ablation of Smo in the adult. This also allowed for a within-subject control, comparing the contralateral side to the ipsilateral side, as well as, within the ipsilateral side GFP+ recombined CINs vs. GFP- CINs. A drawback to viral injections is that recombination is not cell specific for CINs and results could stem from indirect mechanisms. However, consistent with the genetic ablation findings, p-rpS6\textsuperscript{240/244} levels were reduced in ipsilateral GFP+ CINs compared to both controls (Figure 5.3 B, C). The ipsilateral GFP- CINs were slightly reduced compared to the contralateral side suggesting feedback and crosstalk mechanisms, including lateral inhibition, between CINs within the ipsilateral side (Figure 5.3 C).

Together, both genetic and viral ablation of Smo on CINs results point to the conclusion that Shh\textsubscript{DAN} acting to increase or restore CIN activity by the activation of Smo specifically
located on CINs and that mechanisms might act to counteract L-Dopa inhibition of CINs that leads to balanced regulation of both SPN pathways on motor output.
Figure 5.3: Acute viral ablation of Smo reduces neuronal activity marker p-rpS6$^{240/244}$ in CINs validating genetic findings

(A) Schematic of AAV9-Cre GFP viral injection into the DLS. Dotted box represents area imaged and quantified. (B) Representative high-magnification confocal images of p-rpS6$^{240/244}$ in CINs of DLS as a heat map (Scale bar = 10 um). The corresponding smaller image shows ChAT (red) and Dapi (blue) double stain. Underneath shows the same image with ChAT (red) and viral induced GFP (cyan) indicating recombination of the Smo$^{L/L}$ allele. Each CIN has its own ROI and is quantified based on the mean gray value intensity (m.g.v.) per cell of p-rpS6$^{240/244}$ staining normalized to NeuN staining, to account for between subject differences, in individual somata. A 16-member pseudocolor palette depicts the intensity of p-rpS6$^{240/244}$ fluorescence. (C) Quantification of p-rpS6$^{240/244}$ intensity of CINs. Each dot represents one neuron, and red lines indicate the mean ± SEM. The contralateral (con) CINs (white), ipsilateral (ipsi) cre+ CINs (red), and ipsilateral cre- CINs (grey) are compared ($n = 3$, 60 - 80 cells per n). Kruskal-Wallis nonparametric 1-way ANOVA followed by Dunn’s post-test shows **** $P<0.0001$ for con vs. ipsi, or * $P<0.05$ for ipsi GFP+ vs. ipsi GFP-, n.s. indicates $P > 0.05$. 
5.3 ShhDAN regulates number of cortical glutamatergic synapses on CINs

These changes in basal firing rate of CINs, through reduced ShhDAN levels, can reflect alterations in glutamatergic input and morphology, and, thus, contribute to the formation of “aberrant learning” observed in LIDs. This “aberrant learning” posits that L-Dopa dosing mimics “phasic-like” DA release and disrupts normal spike-time dependent plasticity leading to unwanted strengthening or stripping of random synapses that result in involuntary movements or dyskinesia (Beeler et al. 2010; Zhai et al. 2018). Consistent with this, there is increased activity of dSPN, which is associated with an increase in the numbers, and strength of vGluT1 synapses associated with the development of LIDs. Since CINs of the DLS are thought to be important regulators of SPNs but also targets of learning related synaptic plasticity (Aoki et al. 2015; Bradfield et al. 2013; Matamales et al. 2016; Zhang et al. 2013; Zhang et al. 2018), ShhDAN was tested as a critical component for the glutamatergic synaptic rearrangement of CINs.

CINs in the DLS receive glutamatergic input from the cortex (marked selectively by expression of the vesicular glutamate transporter 1, vGluT1) and the thalamus (marked selectively by vGluT2; Figure 5.4 A). The number and size of vGluT1 and vGluT2 boutons on soma proximal dendrites that are in juxtaposition with a postsynaptic density, recognized by PSD95 to mark electrically active synapses (Goyal and Chaudhury 2013; Romorini et al. 2004), was quantified (Figure 5.4 B, C). ShhDAN−/− mice display synapse type, structure, and striatal domain specific deficits compared to controls. The numbers of vGluT1, but not vGluT2, terminals on CIN in the DLS were reduced (Figure 5.4 C), and only the smallest class of vGluT1 boutons were affected (Figure 5.4 D). The number of glutamatergic terminals has been shown to reflect learning (Cheng et al. 2011) and reduced numbers of these synapses might
indicate disturbed learning caused by altered glutamatergic drive of striatal CIN in Shh\textsubscript{DAN-/-} mice.

Together, these results suggest a scenario in which the lack of Shh signaling from DA neurons in disease or during L-Dopa therapy results in disturbed glutamatergic drive of CINs, which in turn result in reduced CIN activity. These findings begin to give potential insight into the underlying cause of aberrant CIN activity in LIDs by Shh\textsubscript{DAN} acting as a dynamic modulator of glutamatergic synapses.
Figure 5.4: Lack of ShhDAN reduces number of cortical synapses on CINs in the DLS

(A) Cortical (vGluT1: blue) and thalamic (vGluT2: magenta) input into the striatum can be distinguished by their respective glutamate transporter isoforms. Synapses were analyzed in the DLS and DMS (boxes). (B) Confocal images (Z-stack 20 µm thick at 63x; scale bar = 20 µm) of vGluT1 (blue) labeled terminals on CIN soma and primary dendrites (ChAT, green) adjacent to PSD95 (red, white arrows) for Shh

DAN+/+ and ShhDAN−/− mice. Bottom images show enlargement of white dotted box in overview above (scale bar = 5 µm). (C) Quantification of number of vGlut1 and vGlut2 synapses on CINs with juxtaposition of postsynaptic density marked by PSD95, revealing a deficit of cortical synapses in Shh

DAN−/− mice compared to Shh

DAN+/+ mice (n = 4, 6 CINs per each area, 120 – 165 synapses per 90 µm x 90 µm area). Unpaired 2-tailed student’s t test ** P<0.01, for mutant vs. control. n.s. indicates P > 0.05. (D) Size histograms of vGlut1 boutons adjacent to PSD95 reactivity in CIN of the DLS and DMS striatum of Shh

DAN+/+ and Shh

DAN−/− mice (n = 4, 6 CINs per each, 120 – 165 synapses per 90 µm x 90 µm area). Unpaired 2-tailed student’s t test * P<0.05. vGluT2 analysis not shown. All graphs plotted as mean +/- SEM (Bar graphs: red lines; Line graphs: shaded area).
6. Chapter 6: Long-term optical stimulation of DAN result in LID-like motor activity that is rescued with Smo activation

6.1 Prolonged stimulation of DAN increase rotation number that is blocked by Smo activation

High frequency stimulation of hippocampal neurons elicits Shh release (Su et al. 2017). Guided by these studies we expressed Dat-Cre dependent channelrhodopsin (ChR2) selectively in mesencephalic DA neurons of the ventral lateral midbrain and implanted an optical fiber terminating above the SNpc (Figure 6.1 A). Selective ChR2-eYFP expression in TH positive DA neurons was confirmed by postmortem analysis (Figure 6.1 B). TH fiber density in the striatum was not altered by ChR2 expression and/or 20 days of daily 1-hour optical stimulation sessions (Figure 6.1 C). Interestingly, unilateral high frequency stimulation (20mW/473nm; 5s of 60hz 10ms pulses) evoked contralateral rotations that were time-locked with stimulation (Figure 6.1 D). Intensity of rotational activity was increased in a linear fashion during one-hour long stimulation sessions when comparing the first 10 minutes to the last 10 minutes.

A gradual escalation of the intensity of the locomotion response is commonly observed upon injection of dopaminergic psychostimulants, DA agonists and L-Dopa and is thought to be caused by behavioral “priming” or progressive sensitization towards repeated dopaminergic signaling. Since we propose that Shh\textsubscript{DAN} signaling counteracts DA inhibition on CINs, we tested whether dynamic changes in Shh\textsubscript{DAN} signaling, with Shh levels exhausted by the end of the hour, could contribute to the gradual increase in rotational response to unilateral photostimulation of DA neurons. Consistent with greater Shh signaling at the beginning and diminished Shh signaling at the end of photostimulation, we find that blocking Shh signaling by injection of the Smo inhibitor cyclopamine (5 mg/kg) elicited a maximal rotational response immediately at the beginning of stimulation rather than only after one hour as seen in controls (Figure 6.2 A).
Injection of cyclopamine did not alter locomotion by itself in absence of stimulation (Figure 6.2 B) suggesting that the activation of Shh_{DAN}/Smo becomes relevant only upon concomitant DA signaling. This data points to the possibility that cyclopamine further decreases CIN activity and increases rotation to a similar degree during the first 10 mins as the last 10 mins in controls (Figure 6.2 C). Conversely, activating Shh signaling by injection of the Smo agonist SAG (20 mg/kg) blocked the gradual increase in rotational behavior seen in controls (Figure 6.2 D). SAG did not alter locomotor behavior during baseline before stimulation similar to cyclopamine (Figure 6.2 E). This data points to SAG enhancement of CIN activity throughout the one-hour stimulation resulting in increased control from CINs on DA-induced rotational output (Figure 6.2 F).

Together, these observations suggest that the gradual increase in locomotion is caused by progressively diminished Smo activation during the one-hour stimulation sessions. A parsimonious explanation for gradually reduced Smo activity in this paradigm is the possibility that releasable Shh_{DAN} stores exhaust in the course of forced DA neuron stimulation which in turn would result in the progressive relief of repression of locomotion by progressively reduced CIN activity. Thus, forced DA neuron stimulation might produce over time a situation of reduced Shh_{DAN} signaling relative to DA signaling onto CIN, which, in the the 6-OHDA lesioned animals, the AK\(^{-}\) mice, Shh_{DAN}\(-/-\) mice, and, Smo_{CIN}\(-/-\) mice facilitate LID formation and expression.
Figure 6.1: Optical stimulation of DA neurons increases rotation in a time dependent manner

(A) Construct and experimental design showing injection of Cre dependent AAV5 expressing ChR2-eYFP fusion protein in Dat-Cre mice into the SNpc. Photostimulation of the SNpc DA neurons lasted one-hour using a 473 nM laser for 5 seconds every 30 seconds. (B) Co-localization of ChR2 (green- viral induced eYFP) with TH (red) in the SNpc. (C) Represented images (left) and quantification (right) showing that both unilateral expression of ChR2 and prolonged stimulation does not alter TH fiber density in the ipsilateral striatum compared to the contralateral hemisphere (n = 8; n.s. indicates P > 0.05). (D) Quantification of contralateral rotations during periods of DA neuron stimulation (blue ticks) and non-stimulation in the first and last 10 minutes of one-hour period (n = 8). RM 2-way ANOVA stimulation: F (1,7) = 67.33 p<0.0001. Post hoc Bonferroni’s test: * P<0.05, ** P<0.01 for stimulation vs. non-stimulation; time: F (1,7) = 22.56 p=0.002. Post hoc Bonferroni’s test: *** P<0.001 for 0 - 10 vs. 50 - 60 minute time points. All bar graphs plotted as mean +/- SEM.
Figure 6.2: Reduced Shh\textsubscript{DAN} augments rotational behavior in optical stimulation of DA neurons

(A) Number of contralateral rotations during stimulation in 10-minute bins over the course of one-hour of stimulation in animals injected with vehicle or 5 mg/kg cyclo (n = 5). RM 2-way ANOVA treatment effect: $F_{(1,8)} = 7.40$ p=0.026. Post hoc Bonferroni’s test: ** P<0.01 for vehicle vs. cyclo; time effect: $F_{(4,32)} = 5.69$ p=0.001. Post hoc Bonferroni’s test: *** P<0.001 for vehicle 10-20 vs. 50-60. n.s. indicates P > 0.05. (B) Baseline locomotion shows no change between mice treated with vehicle or 5 mg/kg cyclo in open field arena during the 10 minutes before optogenetic stimulation (n = 7). (C) Potential scheme suggesting cyclo action on Shh\textsubscript{DAN} signaling by inhibiting CINs and promoting the dSPN pathway to increase rotation. (D) Contralateral rotations over one-hour following vehicle or 20 mg/kg SAG (n = 7). RM 2-way ANOVA time effect: $F_{(4,44)} = 6.96$ p=0.0002. Post hoc Bonferroni’s test: ** P<0.01 for vehicle 10 - 20 vs. 50 - 60. n.s. indicates P > 0.05. (E) Baseline locomotion shows no change between mice treated with vehicle or 20 mg/kg SAG in open field arena during the 10 minutes before optogenetic stimulation (n = 7). (F) Possible schematic suggesting SAG actions on Shh\textsubscript{DAN} signaling to increase CIN activity and restore balance of the SPN pathways on motor output. All bar graphs plotted as mean +/- SEM.
6.2 Long-term DA neuron stimulation produce AIM-like behavior that is reduced by Smo agonist

Next, we tested whether long duration optogenetic stimulation would lead to LID-like dyskinesia. To this end, LID-like movements were identified and quantified during 10-minute time bins following the one-hour forced stimulation sessions (**Figure 6.3 A**). As described earlier for scoring LID in the unilateral 6-OHDA model, stereotypical and contra-lateral rotations, axial, limb, and orofacial movements were measured. Remarkably, the first hour of pulsatile, forced stimulation induced LID-like rotational, axial and contra-lateral AIMS time-locked with stimulation in the last 10 minutes, but not in the first 10 minutes (**Figure 6.3 B**). Because of the qualitative and quantitative similarity to the motor activity observed in the unilateral 6-OHDA paradigm, this behavior was termed optogenetic-induced dyskinesia (OID). Daily repeated one hour stimulation sessions resulted in an escalation of the OID response: on day 9 mice exhibited (1) orofacial (tongue protrusions) movements in addition to abnormal axial and limb movements observed on day 1 and (2) AIM-like behavior was noted during the first 10 minutes of the stimulation; this behavior further increased gradually during the remainder of the session (**Figure 6.3 B**). OID on day 9 was of greater intensity compared to the OID seen during the last 10 minutes of the stimulation session on day 1, indicating that repeated stimulation leads to progressive and long lasting sensitization to forced firing.

If OID would emerge due to gradual exhaustion of Shh\textsubscript{DAN} then postsynaptic activation of Smo signaling during the one-hour stimulation should be attenuated OID. Saline injection 15 min prior to forced stimulation on day 10 did not alter the display and profile of OID compared to day 9 (**Figure 6.3 C**). On day 11, animals were injected with SAG (20 mg/kg) 15 min before stimulation. As observed before, AIM-like behavior time-locked with forced stimulation (**Figure
Remarkably, SAG treatment led to a significant attenuation of AIMs during the first and last 10 minutes compared to day 10 (Figure 6.3 C). The greatest reduction of AIM-like activity is among abnormal axial- (twisting of the body, red sections of the bars in Figure 6.3 B, C, D), abnormal limb- (green sections of bars in Figure 6.3 B) and orofacial- movements (yellow sections of bars in Figure 6.3 B). Rotational behavior (blue sections of bars in Figure 6.3 B) was not significantly reduced by SAG. OID was also scored on a timescale, quantifying the total duration of time an animal spent displaying AIM-like behaviors and shows a highly similar stimulation dependent display of AIMs (data not shown) compared to the more specific AIM scale scoring (Figure 6.3 B, C). These results show that forced firing of DA neurons results in progressive AIM-like activity within minutes of onset of pulsatile stimulation that escalate in magnitude by repeated daily session that can be attenuated partially by concomitant Smo activation.
Figure 6.3: Long-term optical stimulation of DA neurons results in LID-like behavior

(A) Experimental paradigm for Opto-induced dyskinesia (OID). (B) OIDs mimicked AIM-like behavior and were scored as such during the first 10 and last 10 mins for day 1 and 9 (n = 8). AIM scores were quantified during off stimulation and on stimulation (blue ticks) and broken down into rotation (blue), axial (red), limb (green), and orofacial (yellow) under the same criteria as 6-OHDA AIM scoring. RM 3-way ANOVA stimulation effect: F (1,14) = 56.11 p<0.0001. Post hoc Bonferroni’s test: **** P<0.0001 for stimulation vs. non-stimulation; day x stimulation effect: F (1,14) = 15.6 p=0.002. Post hoc Bonferroni’s test: ** P<0.01 for stimulation during d1 vs. d9 at 50-60, *** P<0.001 for stimulation during d1 vs. d9 at 0-10. (C) AIM scores following day 10: saline (n = 6) and day 11: SAG (20 mg/kg; n = 6). RM 3-way ANOVA stimulation effect: F
(1,12) = 124.2 p<0.0001. Post hoc Bonferroni’s test: **** P<0.0001 for d10 stimulation vs. non-stimulation, * P<0.05, *** P<0.001 for d11 stimulation vs. non-stimulation; day x stimulation effect: F (1,12) = 13.56 p=0.003. Post hoc Bonferroni’s test: ** P<0.01 for stimulation d10 vs. d11.

(D) Video still image of mouse posture during stimulation showing axial AIM on day 10 and non-axial on day 11 following SAG treatment.  (E) Immuno-strain of c-Fos (red) expression in the stimulated vs. non-stimulated DLS in saline and 20 mg/kg SAG treated animals (Scale bar= 50 um).  (F) Quantification of c-Fos+ cells (red).  RM 2-way ANOVA treatment effect: F (1,27) = 41.41 p<0.0001.  Post hoc Bonferroni’s test: **** P<0.0001 for saline treated group stimulation d10 vs. SAG treated group stimulation d11; stimulation effect: F (1,27) = 114.3 p<0.0001.  Post hoc Bonferroni’s test: *** P<0.001, **** P<0.0001 for stimulation vs. non-stimulation for d10 and d11 (saline: n = 2, SAG: n = 6 ~445 cells per 700 um x 700 um area).  (G) Images and (H) quantification of CIN soma mean p-rpS6\textsuperscript{240/244} gray intensity normalized to NeuN in the DLS following one-hour stimulation on day 10 (saline n = 2, 80-100 cells per each) or day 11 (SAG n = 6, 40-60 cells per each).  Each dot represents one neuron and mean/SEM indicated by red line.  Paired 2-tailed Student’s t test ** P<0.01 for stim- vs. unstim- side, ****P<0.0001 for saline vs. SAG.  n.s. indicates P > 0.05.  All bar graphs plotted as mean +/- SEM.
6.3 Prolonged stimulation of DAN increases c-Fos activation and reduces p-rpS6 in CINs

As shown before, c-Fos expression is induced by L-Dopa and optogenetic inhibition of neuronal assemblies in the hypo-dopaminergic striatum that expressed c-Fos in response to a previous dose of L-Dopa blocks LID (Girasole et al. 2018). Consistent, we find increased numbers of c-Fos expressing cells in the stimulation ipsilateral striatum compared to the contralateral striatum in the vehicle group (Figure 6.3 E, F). Importantly, we find that SAG decreased the total number of c-Fos activated cells in the stimulated striatum when given 15 minutes prior to onset of pulsatile stimulation (Figure 6.3 E, F).

The attenuation of LID-like behavior in response to Smo activation is correlated with an increase in p-rpS6<sup>240/244</sup> expression in CIN of the DLS in the Shh<sub>DAN</sub>/Smo paradigm (Figure 4.2 A, B). Consistent with the inhibition of CINs in response to optogenetic stimulation of DA neurons, there is decreased p-rpS6<sup>240/244</sup> reactivity in CIN of the stimulation ipsilateral DLS compared to the contralateral unstimulated control side (Figure 6.3 G, H). Stimulation in the presence of SAG restores p-rpS6<sup>240/244</sup> levels in CINs of the stimulated DLS compared to saline treated controls (Figure 6.3 G, H).

Together, these experiments indicate that prolonged firing of DA neurons results in reduced Shh<sub>DAN</sub>/Smo signaling, dampening CIN activity, and causing LID-like motor activity within minutes, which then further intensifies in response to daily stimulation sessions.
7. Chapter 7: Part I Discussion

The findings described here point to diminished ShhDAN being one critical factor in LID. Reduced ShhDAN levels and/or diminished Shh signaling relative to DA levels facilitate while increased Shh signaling attenuates LID formation and expression suggesting a novel therapeutic approach for the treatment and avoidance of LID in PD patients. These findings support the idea that Shh/Smo signaling are key mediator of CIN function in the DLS. There are three important points that these studies strongly suggest: (1) Shh, along with DA, must be considered to be a physical component of CIN modulation that emanates from DA neurons, (2) the relative balance of DA and ShhDAN signaling is a critical determinant of CIN activity, and (3) Smo pharmacology can normalize PD related dysfunction of CIN upstream of ACh signaling.

7.1 Deficits in ShhDAN/Smo signaling onto DLS CIN is critical in causing LIDs

A fundamental unresolved issue in understanding the mechanisms leading to the appearance and expression of LID is whether and how this phenomenon is related to the underlying pathology of PD, namely DA neuron degeneration (Borgkvist et al. 2018). We tested the possibility that reduced ShhDAN/Smo signaling could be a significant cause of LID formation and expression as: (1) progressive DA neuron degeneration must lead to reduced ShhDAN in addition to the loss of DA in the brain, and (2) DA substitution therapy without augmenting Shh/Smo signaling must result in a distortion of the relative strength of Shh and DA signaling. Consistent with these expectations we found that reducing Shh/Smo signaling in any of four ways – (1) inhibiting Smo by the antagonist Cyclopamine in either the AK−/− or the 6-OHDA model of LID, (2) ablating Shh from DA neurons, (3) ablating Smo from CINs, and (4) exhausting DA neuron of stored Shh via optogenetic stimulation of DA neurons- facilitated or
increased LID or LID-like OID. Conversely, boosting Smo signaling with two different and selective agonists, SAG or Purmorphamine, attenuated established LID or LID-like OID in (1) 6-OHDA mice, (2) AKc mice, (3) L-Dopa treated parkinsonian Macaques, (4) Shh_{DAN/-} mice, (5) Smocin/- mice, and (6) mice with exhausted Shh stores in DA neurons. Consistent and remarkable, the conditional genetic expression of the constitutively active form of SmoM2 in CINs in the AKc animal background, which is prone to produce LIDs, creates LID-resistant mice. The acute or long-term systemic dosing with Smo agonists did not curtail the symptomatic benefit of L-Dopa treatment. While the attenuation of LID by Smo agonists in the mouse paradigms was almost complete, the anti-dyskinetic effect in the primate model was modest. However, the temporal profile of drug action suggests that matching the pharmacokinetics of Smo agonist with that of L-Dopa could result in enhanced attenuation of LID in primates as well.

There is overwhelming evidence that implicates altered physiology and neuronal activity of CINs in DLS in LID in PD models. For example, (1) repeated dosing with L-Dopa, and correlation with progressively increased LID expression, results in increased pErk1/2 prevalence in CIN of the DL striatum (Ding et al. 2011), (2) boosting ACh signaling mediated by the muscarinic ACh receptor, M4 (Shen et al. 2015) and, (3) long duration, optogenetic stimulation of CIN ameliorates LID (Bordia et al. 2016). Consistent with these reports and mirroring the behavior in the 6-OHDA, AKc, Shh_{DAN/-}, and Smocin/- paradigms we found that ablation of Shh from DA neurons or the ablation of Smo in CINs resulted in dampened neuronal activity of CIN, and an increase in the prevalence of CIN with detectable pErk1/2 in the DLS but not the DMS. Conversely, stimulation of Smo resulted in increased CIN activity, which was not observed when the Smo receptor is ablated from CINs, and a decrease in the numbers of
CIN with pErk1/2 expression selectively in the DLS. It is noteworthy that mice with OID do not display increased pErk1/2 despite their CINs showing reduction in CIN neuronal activity. While pErk1/2 is a well-established marker for DA hypersensitivity in the striatum (Gerfen et al. 2002) our observations suggest that DA hypersensitivity is not sufficient for LID development per se. Consistent, there was no evidence that mice with OID develop DA hypersensitivity in contrast to 6-OHDA, AK\(^{-/-}\), Shh\(\text{DAN}^{-/-}\), and Smo\(\text{CIN}^{-/-}\) mice. This observation might be explained by a physiological stress response in CINs correlating to MAP-kinase activation due to L-Dopa therapy that is alleviated with Shh signaling (Dai et al. 2012; Darling and Cook 2014; Zhang and Liu 2002).

Among all the DA neuron projection targets and constituent neuronal subtypes of the striatum, only CIN and FS neurons express detectable levels of the Shh receptor Ptc-1 (Gonzalez-Reyes et al. 2012) suggesting that modulation of neuronal activity and MAP Kinase activity in CIN by Shh signaling is direct. In 6-OHDA, AK\(^{-/-}\), Shh\(\text{DAN}^{-/-}\), and Smo\(\text{CIN}^{-/-}\) mice, we find that those CINs that express pErk1/2 in mice displaying LIDs reside in the striatal domain of greatest density of Shh carrying neuropil. These observations reveal a remarkable overlap of the cytohistological and neurochemical pathology in PD and LID with patterned Shh signaling in the striatum that selectively effects CINs.

How could Shh\(\text{DAN}/\text{Smo}\) effects on CIN be connected to LID relevant plasticity of striatal output neurons? A critical feature of LID pathophysiology is the aberrant DA dependent LTP of glutamatergic synapses on dSPN (Zhai et al. 2018). It was found that ACh signaling via M4 mAChR can counteract DA dependent LTP and instead promote LTD of glutamatergic synapses in dSPN (Shen et al. 2015). Consistent, boosting M4 mAChR signaling with positive allosteric modulators (PAM) alleviates LID (Shen et al. 2015). We find that the facilitation of
LID in response to the Smo inhibitor, cyclopaamine, was normalized by co-injection of the M4PAM (VU0467154). Thus, the LID promoting reduction of CIN activity by diminished ShhDAN signaling can lead to reduced M4 mAChR signaling. This reduced M4 activity leads to diminished LTD of glutamatergic synapses on dSPN. Hence, our experiments provide a novel and unexpected mechanism by which DA neuron degeneration can underlie aberrant glutamatergic synapse plasticity on dSPNs through diminished ShhDAN signaling on CINs.

7.2 ShhDAN counter DA-mediated inhibition of CIN residing in the DLS by multiple mechanisms

A central and persistent question in understanding basal ganglia function in the control and PD brain is how dopaminergic signaling controls CIN function (Barbeau 1962; Zhang and Cragg 2017). DA blunts ACh release from CIN through (1) Goi coupled D2 receptors and GABA signaling promoting D5 receptors (Yan et al. 1997; Yan and Surmeier 1997) and by (2) eliciting excitatory withdrawal by modulating GABAergic and glutamatergic input onto CIN by presynaptic mechanisms (Zhang et al. 2018). In turn, ACh release, acting via nicotinic receptors present in DA nerve terminals, facilitates DA release (Threlfell et al. 2012). While the rapid desensitization of nicotinic receptors (Giniatullin et al. 2005) and the induction of LTP of glutamatergic synapses onto CIN by DR5 signaling (Oswald et al. 2015) might reduce the DA mediated inhibition on CIN, together these mechanisms seem to provide inescapable negative control of CIN by DA. In contrast, how CIN activity recovers from DA induced “pauses” of firing is not well understood. Interestingly, previous studies implicate DA and glutamate in the recovery, or “rebound”, of CIN activity after a DA induced pause, by optogenetic stimulation of dopaminergic afferents in vivo (Chuhma et al. 2018; Straub et al. 2014). It is tempting to speculate that another unidentified signaling factor might be ShhDAN, since the present findings
suggest that Shh_{DAN} is released by burst firing of DA neurons and that Shh_{DAN} increases cholinergic activity marker p-rpS^{6240/244} and normalizes L-Dopa induced dampening of CIN activity (Figure 7.1 A, B). How then could Shh_{DAN} curtail DA driven inhibition of CIN?

The present studies offer several mechanistic insights: First, in all of these paradigms the acute effect of Smo-agonist or -antagonist treatment is only seen upon activation of DA signaling. This suggests that Smo signaling must impinge on a component of D2/D5 receptor signaling after pathway activation by DA. Shh signaling activates the GPCR Smo by inhibiting the transmembrane transporter Ptc-1 whose unidentified cargo blocks Smo from coupling to the G-proteins G\alpha_i or Rho, and/or from activating the Gli class of transcription factors (Riobo et al. 2006) Second, Shh_{DAN}/Smo signaling is likely to act in a stoichiometric manner on the D2/D5 receptor pathways since the half maximal effective dose of the Smo agonist that counteract LID, is dose-dependent with the L-Dopa dose used to induce LID. Interestingly, both, D2R and Smo couple to G\alpha_i. Coupling to the same signaling effector could provides a mechanism by which delayed activation of Smo by Shh_{DAN} relative to D2R activation by DA could terminate DA signaling by recruiting limited pools of G\alpha_i away from D2Rs by activated Smo. Third, the ablation of Shh from DA neurons causes a profound loss of small glutamatergic synapses that originate from the cortex on CIN and provide excitatory input to CINs. Since DA can also inhibit CIN through presynaptic processes that block glutamatergic drive (Zhang et al. 2018), these findings leave open the possibility that Shh_{DAN} could counteract the DA-induced inhibition of CIN by stabilizing glutamatergic synapses on CIN long term.

On first sight, these data and the finding that boosting muscarinic (Shen et al. 2016) or nicotinic signaling can ameliorate LIDs (Bordia et al. 2016) are at direct odds with the well-established increase in cholinergic tone compared to dopaminergic tone in PD (Barbeau 1962;
McGeer et al. 1961; McKinley et al. 2019). Why does the elevated cholinergic tone of the PD brain not protect from LID? Recent findings demonstrate that DAN degeneration leads to an absolute reduction of both, DA and ACh (McKinley et al. 2019) a result also consistent with the earlier finding that Shh\textsubscript{DAN-/-} mice reveal CIN degeneration and a profound reduction in cholinergic tone (Gonzalez-Reyes et al. 2012). Hence, there appear three possibilities why the relative increase in ACh tone in the PD brain does not protect from LID: (1) Some modes of cholinergic signaling may facilitate LID. In support, brief pulses of muscarinic mediated cholinergic signaling increases LID (Bordia et al. 2016) and the ablation of CIN reduces LID (Won et al. 2014). (2) L-Dopa dosing shifts the balance of ACh and DA towards higher DA than ACh. (3) ACh levels only protect from LID if they are at or above normal levels. My studies support the latter possibility and, importantly, offer an un-anticipated, additional but critical mechanism by which DA and ACh homeostasis is maintained in the striatum: First, acute Shh\textsubscript{DAN}/Smo signaling counteracts DA signaling mediated inhibition of CIN but does not alter CIN activity independent of DA. Thus, Shh\textsubscript{DAN-/-} mice, exhibit reduced cholinergic tone (Gonzalez-Reyes et al. 2012). Second, if the mechanism of action of Shh\textsubscript{DAN}/Smo signaling in CIN is dependent on activated DA effectors as discussed above, then the reduced levels of DA signaling in the PD brain will make the concomitant diminished Shh\textsubscript{DAN} irrelevant. Thus, the net effect of DA neuron degeneration is a relief of repression from DA leading to a rise in ACh. Third, diminished levels of Shh\textsubscript{DAN}/Smo signaling become relevant upon L-Dopa dependent activation of D2/D5R signaling that will provide a substrate through which Shh\textsubscript{DAN}/Smo signaling can interfere with DA signaling dependent inhibition of CIN. In essence my data is compatible with the idea that Shh signaling only effect CINs that are concomitantly exposed to DA, which could explain both the relative increase in ACh tone in the PD brain in the absence
of ShhDAN, as well as, the inhibition of LID via stimulating CIN activity by counteracting L-Dopa dependent inhibition of CIN.

Overall, my results suggest the following model by which diminished ShhDAN/Smo signaling facilitates LIDs. In the normal brain, there is coordinated balance between DA and Shh signaling on CIN activity. This leads to coordination between DA and ACh levels in the striatum that is essential for undisturbed behavior (Figure 7.2 A). PD creates a progressive dampening of DA neuron input. This slow reduction may allow cells to adapt accordingly, however, long-term loss of DA neurons induce corresponding changes to striatal neurons in this circuit. In PD, ACh levels fall in parallel to DA levels but not to the same extent, which causes an imbalance between the ratios of each with ACh above DA (McKinley et al 2019). This imbalance results in motor impairments. Then L-Dopa therapy floods the system with uncoupled DA without the administration of Shh signaling to counteract DA-induced inhibition on CINs (Figure 7.2 B). This enhances striatal DA concentrations and shifts the balance in the opposite direction resulting in high DA and low ACh. This relative difference between striatal DA and ACh concentrations determine the degree of LID. There may still be an increase in ACh levels upon L-Dopa dosing compared to the PD-state, but it is not enough to counterbalance DA levels (McKinley et al 2019). My present work shows that co-administration of Smo agonist and L-Dopa restores the balance of DA to Shh signaling on CINs and may do this by counteracting D2R mediated inhibition of CINs (Figure 7.2 C). This increases striatal ACh concentrations to counterbalance high DA levels and leads to a reduction in LIDs. In summary, ShhDAN mediates DA neuron control of CIN activity in healthy and in parkinsonian states. Further, the pharmacological augmentation of ShhDAN/Smo signaling in PD
patients could ameliorate formation of LID in response to DA substitution therapy by modulating CIN activity.
Figure 7.1: Bidirectional effect of Smo on DA-induced inhibition of CIN activity

(A) Potential scheme showing that DA/ L-Dopa activation causes “pause” or decrease in ACh release of CINs. The lack of Smo activation to counteract this inhibition leads to increased LIDs. (B) Upon Smo activation, DA-induced inhibition is repressed, which leads to increases ACh release. This increase in CIN activity decreases LIDs.
(A) In the normal brain, Shh_{DAN} mediated Smo signaling gates DA mediated D2R signaling with a bidirectional effect on CIN activity. The balance of DA and Shh signaling on CINs produces controlled striatal output of ACh and DA concentrations for normal motor output. (B) In the absence of Shh_{DAN}, L-Dopa enhances D2R mediated inhibition of CINs. L-Dopa therapy alone creates an imbalance of DA and Shh signaling with high DA and low Shh. This results in striatal output imbalance of ACh and DA concentrations with more DA than ACh that leads to increased LIDs. (C) Co-administration of Smo agonist and L-Dopa counteracts D2R mediated inhibition of CINs, restoring the balance of DA and Shh signaling on CINs. This increases striatal ACh concentrations to counterbalance high DA levels and leads to a reduction in LIDs.
Part II: Cortical source of Sonic Hedgehog from glutamatergic input to the DLS does not reveal the same phenotype as Shh_{DAN} in LIDs
8. Chapter 8: Motor cortex source of Shh does not potentiate LID formation

The cortex is critical for action selection and decision making in motor function. Glutamatergic input from the cortex molds synaptic strength and is involved in plasticity and learning. The teaching signaling from the cortico-striatal loop acts on CINs and is involved in cognitive flexibility through interactions with DA (Bamford and Bamford 2019; Wang et al. 2013). Many cortical nuclei target the striatum to crystalize a planned behavior that demands constant updating. There are six layers within the cortex that comprise a number of cell types and are organized in distinctive columns (Boraud et al. 2018). The output of each column allows for behavioral tuning within motor and cognitive areas. Accordingly, cortical influence over behavior is another aspect of striatal motor learning that may be dysregulated in PD and LID states.

8.1 Sonic Hedgehog from cortical neurons communicate with the DLS

The results thus far implicate Shh\textsubscript{DAN} as an important regulator of neuroplasticity in the DLS during normal and disease states (Su et al. 2017; Yao et al. 2016; Zhang et al. 2014), but Shh\textsubscript{DAN} is only one of several potential sources of striatal Shh. Cortical pyramidal tract (PT) neurons either express Ptc-1, the Shh signaling receptor, or Shh (Shh\textsubscript{PT}) itself (Figure 8.1 A). Specifically, there is a population of Motor cortex (M1/2) PT neurons that project Shh exclusively to the DLS (Figure 8.1 B – C). Therefore, it posits that Shh\textsubscript{PT} could represent an additional source of Shh signaling that contributes to trophic support and neuromodulation of CINs in the DLS in a similar manner as Shh\textsubscript{DAN}. It is further hypothesized that the loss of Shh originating from multiple sources potentiates the severity of LID formation.
Within the cortex, there seems to be cells that either receive and/or express Shh signaling (Figure 8.1 A). Examining cortical Ptc-1 mRNA levels, shows expression of positive cells in all layers of cortex, and Shh-nLacZ expression reveals positive Shh expressing neurons within layer V. In a more sophisticated analysis, the use of the viral mapping tool AAV9-CAG-FLEX-tdTomato into Shh-Cre+ mice was used (Figure 8.1 B, C). Recombination of tdTomato occurs only in those neurons expressing Shh and can be used to visually map connections between different brain regions. Here, separate mice were injected at two different sites, one in the DLS and the other into the M1/2 cortex. The DLS injection revealed distinct connections to the M1/2 cortex, however, the viral spread overlapped with the DMS and cannot be differentiated (Figure 8.1 B). Therefore, the M1/2 cortex injection site was used to determine specific projections to the striatum (Figure 8.1 C). This injection revealed M1/2 cortical projections specifically to the DLS and not to the DMS. Together, these mapping experiments uncover direct connections between Shh expressing neurons in the cortical-striatal pathway, exclusively to the DLS, the area involved in PD and learning pathologies (Figure 3.3 D).
Figure 8.1: Glutamatergic neurons from the Motor cortex 1/2 express Shh to the DLS

(A) Scheme of the anatomy of the motor cortex (M1/2: motor cortex 1 and motor cortex 2) in proximity to the striatum. Images representing the M1/2 cortex showing the Shh receptor Ptc-1 mRNA expression in all layers (left) and Shh-nLacZ expression in layer V (right) of the glutamatergic pyramidal tract (ShhPT) neurons. (B) Viral injection of AAV9-CAG-FLEX-tdTomato into Shh-Cre+ mice uncover recombination of tdTomato in only those neurons expressing Shh. Injection into the DLS showed direct neuronal connections to the M1/2 cortical PT neurons. Right: zoomed in image of the M1/2 from the white box. (C) Injection of AAV9-CAG-FLEX-tdTomato into the M1/2 cortex of Shh-Cre+ mice revealed Shh carrying projections only to the DLS and not the DMS. Right top: zoomed in image (top white box) of the tdTomato+ expressing neurons (red) from the M1/2 cortex. Right bottom: zoomed in image (bottom white box) of the targeted DLS (red) from these tdTomato+ expressing neurons from the M1/2 cortex.
8.2 ShhPT is upregulated in AK<sup>-/-</sup> PD mouse model

Shown previously, expression levels of Shh by DA neurons were variable in the undisturbed brain and were in part regulated by negative feedback from CIN since Shh<sub>DAN</sub> expression can be induced 10-fold by physiological cell stress in CINs or axonal injury (Gonzalez-Reyes et al. 2012). These observations point to the possibility that Shh expression among multiple sources within the CIN centered connectome could be coordinated by feedback signals from CIN in such a way that lowered expression by any one particular source is compensated by other sources. We therefore hypothesized that Shh expression across the CIN centered connectome is homeostatically regulated and that CINs act as sensors for ambient levels of Shh expression.

Therefore, compensatory mechanisms were explored by looking at expression levels of Shh in the corresponding intact area of AK<sup>-/-</sup> mice. Since AK<sup>-/-</sup> mice have loss of the nigrostriatal pathway and thus, have reduced Shh<sub>DAN</sub> input, Shh levels were examined in the intact cortical area using rt-PCR. All measures indicate comparable levels in AK<sup>-/-</sup> mice compared to AK<sup>+/+</sup> mice for midbrain SNpc, cortex, and Amygdala (Figure 8.2 A). Shh expression levels were slightly upregulated in SNpc and cortex compared to AK<sup>+/+</sup> controls. The increase in Shh expression in the SNpc, despite the loss of the nigrostriatal projection, can be from compensation of the intact VTA DA neuron and/or the lack of negative feedback loop results in the increase of surviving Shh+ neurons in the SNpc (Figure 8.2 B). This increase of Shh expression in the SNpc is consistent with previous findings in 6-OHDA lesioned mice, AF64a (toxin for CINs) injected mice, and Shh<sub>DAN</sub>-/- mice (Gonzalez-Reyes et al. 2012). These results point to the key players involved in the feedback loop between DA neurons and CINs, because ablation of either DA
neurons, CINs, or ShhDAN causes dysregulation of this loop. The results here suggests that ShhPT also plays a role and there is crosstalk between the two circuits.

Shh levels in the amygdala was measured as a control because this area is Shh+ but is outside of the nigrostriatal and corticostriatal circuit. Consistent, there was no change in Shh expression levels in the amygdala from AK−/− mice compared to controls (Figure 8.2 A). Quantification of TH expression levels showed extensive reduction in the SNpc because this is the area of DA loss in the AK−/− mice. Together, these findings point to a potential compensatory mechanism and indicates some cross talk between the two converging sources of striatal Shh. When one source is lost, the other source may increases expression to compensate. Therefore, if these sources communicate to each other, then the loss of both sources should enhance the display and expression of LIDs.
Figure 8.2: ShhPT is upregulated following loss of SNpc DA neuron connections

(A) Fold change of gene expression for either Shh (left- SNpc, Cortex, or Amygdala) or TH (right- SNpc) of AK−/− mice compared to AK+/− controls (n = 10). Shh levels from the amygdala and TH levels from the SNpc was used as within subject controls. Unpaired 2-tailed non-parametric Mann-Whitney test ** P<0.01, **** P<0.0001, for AK−/− vs. AK+/− mice. n.s. indicates P > 0.05. Bar graph plotted as mean +/- SEM. (B) Scheme depicting the loss of ShhDAN source leads to the upregulation of ShhPT expression, indicating crosstalk between the two sources. Shh increase in the SNpc could be due to VTA DA neuron compensation and/or loss of the negative feedback loop from CINs.
8.3 Viral ablation of Sonic Hedgehog from motor cortical PT neurons does not alter LIDs

To test for the role of Shh<sub>PT</sub> in LID development, Shh was ablated from the Motor cortex (M1/2) using the AAV5-eGFP-Cre virus of Ak<sup>-/-</sup>; Shh<sup>L/L</sup> mice (Figure 8.3 A-B). The Motor cortex was targeted because of the direct projections to the DLS (Figure 8.1 C), its involvement in the expression of behaviors, and this area is the major interface between the CNS and the musculoskeletal system (Alexander and Crutcher 1990). Animals were injected twice bilaterally to infect both M1 and M2 cortices. GFP labeling showed viral infection in the M1/2. Control mice received AAV5-eGFP virus to account for viral infection. Mice were rested for 4 weeks to allow for appropriate recombination and then dosed daily with 25 mg/kg L-Dopa (Figure 8.3 C). There was no change in AIMS between Cre+ or Cre- virally injected animals for day 1, 4, and 11 (Figure 8.3 D). These results point to Shh from M1/2 not being involved in the development of LIDs. However, some limitations for this experiment include the differences between viral spread and infection between animals. The varying loss of Shh<sub>PT</sub> in the M1/2 could account for the lack of difference in LID development. Alternatively, these results point to the M1/2 not being involved in LID but may point to involvement of other cortical areas besides the motor cortex, such as the Orbito-frontal cortex (OFC), the Prefrontal cortex (PFC), or the anterior Cingular cortex (ACC). Additional experiments are needed, either injection of AAV5-eGFP-Cre into these other cortical areas or the use of Shh<sup>L/L</sup> Emx-Cre (Shh<sub>PT</sub>-/-) mice, which have loss of Shh from all cortical PT neurons. These mice will not have the issue of viral spread and would be an ideal option to look at all Shh<sub>PT</sub> expressing neurons to the DLS.
Figure 8.3: Shh ablation from the motor cortex has no effect on LIDs

(A) Scheme of AAV5-eGFP-Cre injection into motor cortex 1 and motor cortex 2 (M1/2). (B) Representative images showing viral infection (green) into the M1/2 cortex. Left: 10x tile scan. Right: zoomed in image of white box. (C) Timeline of chronic 25 mg/kg L-Dopa dosing 4 weeks after viral injection of either Cre+ or Cre- virus for 11 consecutive days of AK+/−; ShhL/L mice. (D) AIM analysis showing no difference between Cre+ and control injected mice at day 1, 4, or 11 following daily dosing with L-Dopa. n.s. indicates P > 0.05. Bar graph plotted as mean +/- SEM.
8.4 Shh\textsubscript{PT} ablation does not attenuate CIN survival rate

Previous findings showed a 50% loss of total number of CINs in the DLS when Shh is ablated from DA neurons (Gonzalez-Reyes et al. 2012). Therefore, both Shh\textsubscript{DAN} and Shh\textsubscript{PT} sources may contribute 50% of Shh input each to the striatum and/or there may be two neuron populations that are sensitive to the different sources of Shh. To explore if Shh\textsubscript{PT} communicates to CINs in the same fashion as Shh\textsubscript{DAN} does, CINs of Shh\textsubscript{PT}-/- mice were assessed to see if Shh\textsubscript{PT} impinges on their survival rate. However, there was no difference in CIN numbers (ChAT+ cells) of Shh\textsubscript{PT}-/- mice compared to controls at 2 months of age (Figure 8.4 A, B). Older mice (15 months) did not reveal any differences as well. These findings suggest that Shh\textsubscript{PT} is not necessary for the survival of CINs as seen with Shh\textsubscript{DAN}. The lack of increase in LIDs of M1/2 Shh ablated mice and the lack of change in CIN survival rate indicates that Shh\textsubscript{PT} is not critical for the development of LID, and instead, lends greater impact to the importance of the Shh\textsubscript{DAN} source in LIDs.
Figure 8.4: ShhPT has no effect on CIN survival rate

(A) Images of CINs (ChAT: black) in the DLS following ablation of Shh from PT neurons (Shh\textsubscript{PT-/-} mice) and controls (Shh\textsubscript{PT+/-} mice) at 15 months of age. Dotted box represents DLS area where CINs were counted. (B) Quantification of CIN number in the DLS of either 2 month or 15 month old Shh\textsubscript{PT-/-} mice compared to controls (n= 2 – 4; Six striatal areas were counted for each that spanned the anterior-posterior striatum).
8.5 ShhPT ablation increases CIN activity and may target FSN

To determine if ShhPT alters CIN activity, p-rpS6^{240/244} levels were assessed in ShhPT/- mice. Unexpectedly, there was an increase in p-rpS6^{240/244} levels in CINs of ShhPT/- mice compared to controls (Figure 8.5 A - B). This result is the opposite of findings seen in ShhDAN/- mice in CINs. Therefore, it posits that ShhPT does not act directly on CINs and explains why there is no reduction in CIN survival rate when ShhPT is ablated. ShhPT can potentially be released to specific neurons within the DLS that act to inhibit CINs resulting in their increase in activity when ShhPT is ablated. Thus, perhaps ShhPT input acts on the other Ptc-1+ neurons in the DLS, FSNs, which have been known to regulate SPN activity and may indirectly, through lateral inhibition, alter CINs (Klug et al. 2018; Koós and Tepper 2002; O’Hare et al. 2017; Owen et al. 2018). The crosstalk between CINs and FSN could explain this increase in CIN p-rpS6^{240/244} levels if ShhPT/- mice have reduced FSN activity (Figure 8.5 C). Additional experiments are needed to examine the survival rate and activity levels of FSN in ShhPT/- mice. This finding suggests the possibility that ShhDAN target CINs while ShhPT target FSN, and are important for their survival and maintenance, respectively.
Figure 8.5: Lack of ShhPT increases CIN activity and may potentially regulate FSNs

(A) Heat map images and (B) quantification of CIN soma mean p-rpS6$^{240/244}$ gray intensity normalized to NeuN in the ShhPT/- mice and controls (saline n = 2 - 4, 80-100 cells per each). Each dot represents one neuron and mean/SEM is represented as red line. Unpaired 2-tailed Student’s t test ****P<0.0001 for mutant vs. control. (C) Scheme of potential distribution of Shh source: ShhDA/N projecting Shh to CINs and ShhPT projecting Shh to FSN, the other Ptc-1+ neurons, in the DLS (left). Then when ShhPT is ablated FSN inhibition of CIN is disrupted (right).
9. Chapter 9: Part II Discussion

Considering the evidence that Shh\textsubscript{DAN} is critical to avoid LIDs, other sources of Shh might be necessary as well. However, when looking at Shh\textsubscript{PT} or more specifically Shh\textsubscript{PT} from the Motor cortical areas M1/2, the evidence does not suggest a role from this source in CIN survival or in the development of LIDs. These findings point even more to the importance of Shh\textsubscript{DAN} and that not all sources of Shh play a role in CIN maintenance and LID formation.

Although Shh\textsubscript{PT} directly communicates with DLS and Shh\textsubscript{PT} increases Shh expression levels when the nigrostriatal Shh\textsubscript{DAN} source is lost, there does not seem to be a direct role of the Motor cortex in LID development. One possible explanation may be due to the possibility that LIDs are habitual-aberrantly learned behaviors, and habitual behaviors are thought to reflect a switch from cortical controlled behavior to automated subcortical control (Boraud et al. 2018; Wilkinson et al. 2009). Thus, Shh\textsubscript{PT} from the cortex may not participate in the automated process of LIDs. However, the possibility of the involvement of other areas of the cortex in LID formation cannot be ruled out, since all areas express Shh and Shh\textsubscript{PT} might regulate FSNs. Indeed, there is emerging evidence showing the involvement of FSN in LIDs (Alberico et al. 2017; Girasole et al. 2018; Gittis et al. 2011). Therefore, investigating LID development in Shh\textsubscript{PT}/- mice with 6-OHDA lesion would be the next logical step. Shh\textsubscript{PT}/- mice have ablation of Shh in all cortical areas. The indirectly and opposite regulation of CIN activity from Shh\textsubscript{PT}, showing an increase in activity when Shh\textsubscript{PT} is ablated, demonstrates the complexity of this system and perhaps may dysregulate both CINs and FSN when both Shh sources are ablated.

Furthermore, Shh\textsubscript{PT} does not play a role in the survival of CINs and does not dampen CIN activity, suggesting that Shh\textsubscript{PT} does not modulate CINs directly, as seen with Shh\textsubscript{DAN}. The above evidence shows the importance of Shh\textsubscript{DAN} instead of Shh\textsubscript{PT} because: (1) acute
cyclopamine in PD mouse models would exacerbate LIDs, (2) there would be increased LIDs in $Smo_{CIN}\text{-/-}$ mice (receptor ablation on CINs removing all potential sources) compared to $Shh_{DAN}\text{-/-}$ mice (ligand ablation from one source), and (3) there would be a loss of 50% CINs in $Shh_{PT}\text{-/-}$ mice. Moreover, the pathology of PD points to the importance of $Shh_{DAN}$, because these DA neurons are the neurons that degenerate, and LIDs develop because of the lack of Shh signaling during L-Dopa therapy. Thus, the role of $Shh_{PT}$ may potentially be involved in LIDs but not to the same extent as $Shh_{DAN}$ because $Shh_{DAN}$ is the presynaptic mechanism that contributes to the development of LIDs.
10. Chapter 10: Conclusion

In conclusion, the assembly of evidence provided here show that Shh$_{DAN}$ signaling regulates CIN activity to normalize action selection and abnormal movement of the DLS. Following L-Dopa therapy, stimulation of Shh signaling restores hyperactivity of dSPNs and allows CINs to reset the balance of both SPN pathways in motor output. Shh$_{DAN}$ regulates CIN by providing trophic support, counteracting DA-inhibition, and sculpting cortical glutamatergic synapses within the DLS that are involved in normal and aberrant motor learning. The exact role of Shh$_{PT}$ in the DLS needs to be explored further, but evidence begins to show that Shh$_{PT}$ does not regulate CINs directly and possibly regulates FSN instead. Collectively, these results provide novel insight into the development of LIDs by the neurodegeneration of DA neurons reducing Shh$_{DAN}$ levels, which are critical for the regulation of CINs. Whereas, Shh$_{PT}$ from the Motor cortex does not appear to influence LIDs, thus giving more weight to the importance of the Shh$_{DAN}$ as the source in LID development. These findings have direct translational implications.

The development of LID is not a natural occurrence of the disease but only a side effect that emerges during the treatment with L-Dopa. These results show the benefit of considering other signaling factors produced by DA neurons in addition to DA. Shh signaling therapy with L-Dopa can provide relief to patients suffering from LIDs. The finding that the SAG dose scales with the dose of L-Dopa can provide reliable guidance to physicians in accurately reducing LIDs in patients by precisely restoring the balance of DA and Shh signaling within the DLS.
11. Chapter 11: Future directions

The studies described here open a number of possibilities for future directions. The obvious next step would be to see if SAG therapy is effective in the clinical setting. However, considering the potential oncogenic properties of SAG, downstream targets of Smo signaling in CINs may be a better option. Deep sequencing of CIN profiles in the absence of Shh or presence of SAG may give insight into which mechanisms to explore further. These analyses may give rise to potential pharmacological target(s) that may achieve similar results as stimulation of Shh/Smo signaling.

I show robust evidence for the role of Shh$_{DAN}$ in LID development in the diseased state, therefore, the role of Shh$_{DAN}$ must be important in the normal non-diseased learning and should be explored further. Investigating the role of Shh$_{DAN}$ and Smo$_{CIN}$ in reinforcement learning tasks such as T-maze could give insight into the basic role of Shh signaling in the adult brain. Preliminary evidence shows that $Shh_{DAN/-}$ mice cannot override the habitually learned behavior in the T-maze following devaluation and points to Shh$_{DAN}$ as a critical component in flexible learning capabilities. This together with the reduction of vGluT 1 terminals on CINs in $Shh_{DAN/-}$ mice, points to Shh, along with DA, acting as a physical component of the “teaching signal” that emanates from DA neurons. Investigating the exact role of Shh in plasticity in these learning tasks will open many doors. If $Smo_{CIN/-}$ mice have similar unbreakable habitual patterns then Shh$_{DAN}$’s potential role as a “teaching signal” acting directly through CINs is consistent with my results described here.

Additionally, the role of D2R on CINs has not been explored in the context of LID. It will be interesting if D2R ablation on CINs leads to a reduction in LID because of the lack of
“pause” in CIN activity. To explore if Smo and D2R interact, SAG should have no effect on mice lacking the D2R on CINs.

As described earlier, to exclude the role of Shh from cortical sources, assessing LID in Shh<sup>PT-/</sup>- mice would address this issue. In order to induce AIMs, a 6-OHDA lesion would have to be administered and animals dosed with chronic L-Dopa. These animals would show increased LID development compared to controls because of the dysregulation of both CINs and FSNs. This approach would address the question as to the role of Shh<sub>PT</sub> in LID establishment.
12. Methods

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### Virus Strains

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### Chemicals, Peptides, and Recombinant Proteins

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### Experimental Models: Organisms/Strains

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### Software and Algorithms

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EXPERIMENTAL MODELS AND SUBJECT DETAILS

Animals

6-OHDA experiments

Wild-type C57/Bl6 mice weighing 22-28 g (2 months old) were purchased from Jackson Laboratory for the 6-OHDA lesion experiments. L-Dopa treatment began at 6-10 weeks of age. All mice were maintained on a 12 h light/dark cycle, with ad libitum food and water. Animal use and procedures were in accordance with the National Institutes of Health guidelines and approved by CCNY’s Institutional Animal Care and Use Committees (IACUC).

Genetically modified mice experiments

Homozygous ak/ak mice (Abbreviated as AK+/−) were generously provided by Dr. Un Jung Kang (Columbia University; (Ding et al. 2007; Hwang et al. 2003)). The generation of the Shh-nLZ+/−Dat-IRESCre (Abbreviated as ShhDan+/−) mouse strain was described previously (Gonzalez-Reyes et al. 2012). Homozygous Pitx3 All genetic conditional mice used in experiments were purchased from Jackson’s Laboratory (reference numbers listed above) and further bred in house. Animals were dosed with L-Dopa at 22-32 g weight and roughly 3-10 months old (specified in main text). SmoCIN−/− mice were made using conditional SmoL/L mice bred with ChAT-IRESCre (Rossi et al. 2011). SmoM2+/−ChAT-cre AK+/− mice were made using conditional SmoM2-YFP+/− mice (Jeong et al. 2004; Xie et al. 1998) bred with ChAT-IRESCre and AK+/− mice. Recombination was checked using eYFP. For the neuropil carrying Shh experiment, Shh-cre eGFP mice (Harfe et al. 2004) were bred with Rosa26mTmG (Muzumdar et al. 2007). Gli3-nLacZ mice were used for expression experiments (Bai et al. 2004). Emx1-IRESCre mice were bred with ShhPT−/− mice to ablate Shh from the cortex (ShhPT−/− mice). All mice
were maintained on a 12 h light/dark cycle, with ad libitum food and water. Animal use and procedures were in accordance with the National Institutes of Health guidelines and approved by CCNY’s Institutional Animal Care and Use Committees (IACUC).

Non-human primate experiments

The non-human primate experiments were performed in accordance with the European Union directive of September 22, 2010 (2010/63/EU) on the protection of animals used for scientific purposes in an AAALAC-accredited facility following acceptance of study design by the Institute of Lab Animal Science (Chinese Academy of Science, Beijing, China). The four Macaca fascicularis male monkeys (Xiexin, Beijing, PR of China) were housed in individual cages allowing visual contacts and interactions with other monkeys in adjacent cages. Food and water were available ad libitum. Animal care was supervised daily by veterinarians skilled in the healthcare and maintenance of NHPs.

METHOD DETAILS

Drugs

In mice, all pharmacological agents were administered intraperitoneal (i.p.). Animals were treated daily in a volume of 10 mL/kg of body weight with a combination of L-Dopa (5-25 mg/kg) and the peripheral L-amino acid decarboxylase antagonist, benserazide (12.5-20 mg/kg) diluted in 0.9% sterile saline (referred to as just “L-Dopa” and specified doses described in main text). Sonic Hedgehog agonist (SAG; 0.8-20 mg/kg) and antagonist (Cyclopamine; 2.5-5 mg/kg) were dissolved in DMSO and brought into solution with 45% HPCD in 0.9% sterile saline for 6-OHDA and AK−/− AIM studies. SAG-HCl was diluted in 0.9% sterile saline and used for the
Shh<sub>DAN</sub><sup>−/−</sup> and Smoc<sub>CIN</sub><sup>−/−</sup> mice studies. Both cyclopamine and SAG were administered 15 minutes before L-Dopa injections. Amantadine was dissolved in 0.9% sterile saline and given 100 minutes prior to L-Dopa administration. M4PAM was dissolved in 0.9% sterile saline and given at the same time as L-Dopa. Purmorphamine was dissolved in a cocktail of Polyethylene glycol (PEG) and ethanol in PBS and given 15 minutes before L-Dopa dosing. Control mice were treated with a mixture of carrier (DMSO, HPCD etc.) in similar proportions to the treatment drugs.

**Mouse unilateral 6-OHDA model**

According to the well-established method, we applied the unilateral 6-OHDA injection into the dorsolateral striatum (Francardo et al. 2011). Mice were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg) administered i.p. and the surgical field was prepared with betadine. Bupivacaine (Marcaine), a local anesthetic, was subcutaneously (s.c.) injected near the incision site. Bregma was visualized with 3% hydrogen peroxide There were two unilateral injections (2 x 2ul each) of 6-OHDA into the left striatum using the following coordinates based on the mouse brain atlas of Paxinos and Franklin (2001): Anteroposterior (AP) +1.0 mm; Lateral (ML), + 2.1 mm; Dorsoventral (DV), -2.9 mm; and AP +0.3 mm; L +2.4 mm; DV -2.9 mm. 6-OHDA-HCl (3.0 mg/ml) was dissolved in a solution containing 0.2 g/L ascorbic acid and 9 g/L NaCl and injected via a Hamilton syringe with a 33-gauge needle attached to a micro-syringe pump at a slow rate of 0.4 ul/min and left in place for 3 minutes and then retracted slowly. Following surgery, animals were injected with 5% sucrose (10 ml/kg, s.c.) and saline (10 ml/kg, i.p.) and recovered on a heating pad. To avoid dehydration and significant weight loss, hydrogel pouches and hi-fat chow were given to the mice ad lib. Behavioral testing and drug
treatment began three weeks following surgery. Lesion assessments were quantified by ipsilateral paw-use bias in the cylinder test and verified histologically, post-mortem, by quantification of tyrosine hydroxylase (TH) fiber density at the end of experiments. Only animals with 70% or more of TH depletion were included in the analyses.

**Asymmetric forelimb-cylinder test**

The cylinder test was used to assess the anti-akinetic effects of L-Dopa for the 6-OHDA lesioned mice by measuring forelimb paw placement. Mice were placed in a glass cylinder (10cm wide x 14cm high) 30 minutes post L-Dopa injection to be analyzed for 4 minutes. Two mirrors were placed in the back to see mice from all angles. The limb use asymmetry score was quantified as a ratio of contralateral to the lesion paw use over the total number of wall contacts with both paws (Lundblad et al. 2002).

**Open field test**

In the open field test (OFT), whole body movements were measured using Noldus Ethovision XT video tracking system. The animals head, body, and tail positions were tracked in a transparent Plexiglas 4-chamber box apparatus (50 x 50 cm for each chamber). Freely locomotive movements were measures as distanced moved through the chamber following baseline (no drug) and 45 minutes following drug administration. Contralateral to the lesion turning behavior was monitored as a percentage over ipsilateral turning. All OFT lasted 5 minutes to assess both the anti-akinetic effects of L-Dopa in concert with treatment drugs and to determine the potential sedative effects.
Abnormal involuntary movements

Abnormal involuntary movement (AIMs) scoring was rated on the established behavioral rating scales (Cenci and Lundblad 2007; Lundblad et al. 2002). Briefly, there are four types of AIMs: 1) abnormal rotational locomotion, 2) axial AIMs, showing dystonic posturing, and severe twisting of the head or neck, 3) limb movements, with rapid, jerky movements of the front or hind limbs, and 4) orofacial movements of abnormal chewing, licking, grooming, or sticking out of the tongue. The four types of AIMs were scored on a severity scale of 0-4, with 0 exhibiting no abnormality and 4 showing uninterruptable dyskinetic movement. 1-2 blinded experimenters rated AIMs. AIM scores were assessed by a blind observer to the treatment group, 35 minutes post treatment injection and 20 min post L-Dopa injection. Animals were single caged during AIM assessments. Scores for each animal were recorded every 20 minutes, for 1 min each, over 2 hours. The total sum of AIM score throughout 2 hours was calculated as the “Total AIM” score.

AK−/− forelimb and 3-paw dyskinesia

Animals were recorded in a clear plastic cylinder (16 cm in diameter and 25 cm in height) 30 minutes following L-Dopa injection. Mirrors were placed behind the cylinder to allow for ventral views of the behavior. Each trial was five minutes with a 30-second habituation period followed by behavioral scoring. The duration of abnormal paw movement was quantified for each trial similar to previously described (Ding et al. 2007) and included sliding and shaking of the forelimb paws on the cylinder, and “three-paw” dyskinesia (two forelimbs and one hindlimb). “Four-paw” dyskinesia was observed in extreme dyskinetic cases.
Primate MPTP model

Model preparation

The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication protocol, chronic L-Dopa treatment and the clinical assessments was conducted in four male macaques (Macaca fascicularis, Xierxin, Beijing, PR of China), as previously published. The macaques were first rendered parkinsonian with MPTP-hydrochloride (0.2mg/kg, i.v., Sigma) dissolved in saline. Then daily (at 9 am) assessment of parkinsonism in home cages for 30 min by two blinded observers was done using a validated rating scale assessing tremor, general level of activity, body posture (flexion of spine), vocalization, freezing and frequency of arm movements and rigidity (for each upper limb). Once parkinsonism was stable, levodopa (Madopar®, Roche, Levodopa/carbidopa, ratio 4:1) was administered twice daily for 4-5 months at an individually-tailored dose designed to produce a full reversal of the parkinsonian condition (p.o. by gavage). Over this period, animals developed severe and reproducible dyskinesia, presenting choreic–athetoid (characterized by constant writhing and jerking motions), dystonic and sometimes ballistic movements (large-amplitude flinging, flailing movements), as seen in long-term L-Dopa-treated PD patients. O, 45% HPCD in 0.9% saline and administered i.v.. Within subject escalation was performed with washout period of three days.

Immediately after drug administration, we transferred the monkeys to an observation cage (dimensions - 1.1m x 1.5m x 1.1m) as per guidelines. The total duration of observation was 240 min post-gas exposure. We performed a battery of behavioural observations as previously described. Experts blinded to the treatment observed 10-min video recordings taken every 30 min throughout the duration of the experiment and scored the severity of the parkinsonian condition using the parkinsonian disability score. The parkinsonian disability score is a
combination of four different scores: (i) the range of movement score, (ii) bradykinesia score, (iii) posture score, and (iv) tremor score. These four scores are combined using formula: (4 - range of movement) + bradykinesia + postural abnormality + tremor. We rated the severity of dyskinesia using the Dyskinesia Disability Scale: 0, dyskinesia absent; 1, mild, fleeting, and rare dyskinetic postures and movements; 2, moderate, more prominent abnormal movements, but not interfering significantly with normal behaviour; 3, marked, frequent and, at times, continuous dyskinesia intruding on the normal repertoire of activity; or, 4, severe, virtually continuous dyskinetic activity replacing normal behaviour and disabling to the animal.

Statistical analysis

Statistical analyses were carried out using Graphpad Prism (version 8). Time course of parkinsonian disability and dyskinesia scores was analysed in 30 min time bins over the 4 hours observation period. The median of the total scores of disability, dyskinesia, chorea and dystonia at 0–2 hours following treatment was analysed. The parkinsonian and dyskinesia scores were statistically compared between different conditions using a Friedman’s test followed by Dunn’s multiple comparison test.

Optogenetic manipulations

Implant construction and implantation

Implants and patch cables were constructed and polished as previously described (Sparta et al. 2012). Mice were anesthetized with isoflourene and the surgical field was prepared with betadine. Bupivacaine was injected in the scalp prior to incision. Bregma was visualized with 3% hydrogen peroxide and a craitonomy was made over the injection site. The AAV5-EF1a-DIO-
hChR2(H134R)-eYFP-WPRE virus (UNC Vector Core, titer) was injected at AP -3.2 mm, ML +1.5 mm, DV -4.3 mm by pressure injection with a pulled glass pipette and allowed to dwell for 10 minutes. A ferrule implant was placed at AP -3.2 mm, ML +1.5 mm, DV -4.2 mm, secured with metabond, and protected with a dust cap (Thor Labs). Following surgery, mice were given 0.05 mg/kg buprenex and recovered on a heating pad. At least one month was allowed for viral expression.

**Optogenetic stimulation paradigms**

One trial of stimulation consisted of a ten-minute habituation period followed by an hour of intermittent stimulation. Simulation consisted of five seconds of stimulation at the beginning a seven second Ethovision module at 60Hz with 10ms pulses with a laser power of 20mW from the tip of the implant. After 30s passed the next stimulation commenced. Stimulation was controlled via a TTL pulse from Ethovision’s software to a Doric four channel pulse generator controlling a 710nm Laserglow DPPS laser. All sessions were recorded and analyzed with Ethovision. Pharmacological agents were given 30 minutes prior to the beginning of the trial.

**Optogenetic-induced dyskinesia**

For optogenetic-induced dyskinesia (OID) experiments, stimulation occurred in a 5L glass cylinder and consisted of one hour of intermittent stimulation as previously described. Stimulation occurred daily for the duration of the experiments. A second camera (Casio EX-FH100) recorded behavior from the side of the cylinder during the first and last ten minutes of the trial. OIDs were assessed on rotation, limb, orofacial, and axial movements using the exact scoring scale with the 6-OHDA lesion paradigm (Cenci and Lundblad 2007). OIDs were scored
during both stimulation and non-stimulation periods and contributed to the total AIM score independently. Pharmacological agents were given 15 minutes prior to the beginning of the trial.

**Viral injections**

**AAV9-eGFP-Cre injection into the DLS**

Using the same surgical protocol and coordinates for 6-OHDA lesion injection mice (2x 2ul injections at AP +1.0 mm; ML +2.1 mm; DV -2.9 mm; and AP +0.3 mm; ML +2.4 mm; DV -2.9 mm), AAV9-CMV.HI-eGFP-Cre-WPRE-SV40 was injected unilaterally into Smo<sup>L/L</sup> mice for acute recombination in the adult brain. GFP was used to differentiate between cells affected with the virus and those not. The contralateral side was used as a within-subject control.

**AAV5-eGFP-Cre injection into the M1/M2 cortex**

Two injections (1 ul each on both hemispheres) were made into the motor cortex of AK<sup>-/-</sup>; Shh<sup>L/L</sup> mice. M1: AP +0.8 mm; ML +/-0.8 mm; DV -1.0 mm; M2: AP +1.8 mm; ML +/-1.5 mm; DV -1.0 mm. AAV5-eGFP-Cre was used to ablate Shh from areas infected (GFP+ cells). AAV5-eGFP was used as a control virus. Animals were rested for 4 weeks for recombination and then tested for LIDs.

**AAV9-flex-tdtomato injection into the M1/M2 cortex**

Two injections (0.5 ul each, unilaterally) were made into the motor cortex of Shh-Cre mice. M1: AP +1.6 mm; ML +1.9 mm; DV -1.5 mm; M2: AP +1.6 mm; ML +0.8 mm; DV -1.5 mm. AAV9-flex-tdtomato was used to map Shh expressing neuron connections. Two injections (0.5 ul each, unilaterally) were made into the DLS. AP +1.0 mm; ML +2.2 mm; DV -2.9 mm;
Sample preparation and Immunohistochemistry

Animals were anesthetized with pentobarbital (10 mg/ml) 30 minutes after last injection and transcardially perfused with 50 ml of 4% paraformaldehyde (PFA) in 0.2M Phosphate buffer PH 7.2. The brains were extracted and fixed overnight in 4% PFA and 48 hours in 30% sterile sucrose. Brains were mounted using OTC mounting agent and sliced with a cryostat at 40 μM. Slices were incubated with antibodies using the protocol described previously (Alcacer et al. 2012). Sections were mounted using vectashield (Vector Laboratories Inc.).

Confocal microscopy was performed using a Zeiss LMS 810 laser-scanning confocal microscope using the same diameters for all the sections from one experiment. Counting was performed manually in 375 x 375 μm confocal images taken in the dorsolateral and dorsomedial striatum in blind conditions to the mouse treatment and/or genotype. TH immunofluorescence intensity was quantified in the striatum with MacBiophotonics Image J, and the data represented mean gray levels above background value in 375 x 375 μm confocal images. Co-stained and single stained cells were manually counted in FIJI/ImageJ software (NIH) from the primary and secondary antibodies used in the list above. Prevalence of pErk1/2 positive CINs was quantified in a percentage of pErk1/2+ CIN out of total number of CIN in one plane (375 x 375 μm) in three striatal slices: anterior (AP: 1.34 mm), middle (AP: 0.74 mm), and posterior (AP: 0.14 mm) per animal. The criteria and settings were identical between experimental and control tissue for each experiment. myrGFP stained neuropil analysis was done by transforming full striatal sections into heat maps in Image J then creating a region of interest (ROI) around the...
dorsolateral and dorsomedial striatum (represented in extended Fig. 7). Heat map images reveal the intensity level of myrGFP comparing anterior and posterior striatal sections.

**p-rpS6^{240/244} Analysis**

The mean grey value of p-rpS6^{240/244} intensity was measured in FIJI/ImageJ software (NIH) using a freehand ROI area around the CIN somata similar to the protocol described by (Bertran-Gonzalez et al. 2012). NeuN intensity of the same cell was used as a control for fluorescence viability between subjects and numbers were plotted as a ratio of p-rpS6^{240/244} intensity over NeuN intensity of CINs. A 16 pseudocolor palette highlights the intensity of p-rpS6^{240/244} fluorescence. Heat map images are shown to emphasize the intensity levels and taken within the dorsolateral striatum. About 200-300 CINs were quantified per each condition.

**Glutamatergic Analysis**

Glutamatergic terminals were quantified selectively on CIN only by quantifying vGluT1 or vGluT2 puncta that abutted within 0.5um a postsynaptic density recognized by postsynaptic density (PSD95) antibody co-stained with the cholinergic antibody ChAT. Z-stacks were taken at about 20um thick. Terminals were analyzed on 6 CINs in both the dorsolateral and dorsomedial of ShhDA/- mice and controls at high resolution (63x oil). vGluT+ terminals were counted in ImageJ using ROI and mask analysis of particles between the sizes of 0.1 to 5 microns. Only those vGluT+ puncta fitting the criteria of being directly around the soma and primary dendrites of CINs and juxta positioned to PSD95 immunoreactivity co-stained with ChAT were counted. vGluT1 and vGluT2 were analyzed on adjacent coronal sections.
Quantification of gene expression

Total RNA from cortex, amygdala, and lateral vMB containing the entire SN (may include some 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) and calculated by the ΔΔCt method.

Statistics

Multiple comparisons were analyzed using 2-way, 3-way, or repeated measures (RM) ANOVA with GraphPad Prism 8.0 software, followed by post hoc Bonferroni’s test for specific comparisons. For 2-group comparisons, 2-tailed paired or unpaired Student’s t test was used. Nonparametric statistics were used to verify all main effects using Statistical analysis for MPTP primate study was carried out using a parametric one-way repeated measure analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test or Mann-Whitney test when appropriate.
Appendix 1: SAG Administration reduces dyskinetic behavior in the MPTP Macaque model of PD (Collaboration with Dr. Erwan Bezard)

(A) Drug regimen of methylphenyltetrahydropyridine (MPTP) injected Macaques following establishment of LID and challenged with vehicle or SAG (3, 9, and 27 mg/kg) administered intravenously (i.v.) concomitantly to oral L-Dopa in a within-subject drug escalation design. (B) Quantification of dyskinesia score binned for the first 2 hours of vehicle or SAG (3, 9, and 27 mg/kg) administration. Friedman’s nonparametric RM 1-way ANOVA followed by Dunn’s post-test $p=0.037 \ *P< 0.05$ plotted as mean +/- SEM (n = 4). (C) Time course of behavioral scores of dyskinesia over 4 hours for vehicle or SAG (3, 9, and 27 mg/kg). Error bars excluded for clarity. (n = 4). (D) Time course of disability score of primates during L-Dopa treatment with either vehicle or SAG (3, 9, and 27 mg/kg) over 4 hours. Parkinson’s Disease (PD) scoring consists of a range of movements including bradykinesia, postural abnormality, and tremor giving a maximum global parkinsonian disability score of 10.
References


