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Abstract

Early SHH-Dependent Telencephalic Patterning Disruptions in Tourette Syndrome

Melanie Victoria Brady

2021

Ventral telencephalic development gives rise to the basal ganglia, a subpallial brain region responsible for motor function and coordination. This brain region is implicated in many movement disorders, including Tourette Syndrome (TS). TS is a heterogenous neurodevelopmental disorder and its etiopathophysiology is unknown. To date, TS has been investigated in animal models and postnatal human subjects, but early development of this disorder has not been studied. Previous work in adult TS post mortem basal ganglia tissue has shown a reduction in striatal interneurons, which serve to largely regulate striatal output. However, possible mechanisms for this neuronal loss and whether or not these findings originate in early development are poorly understood.

This study examines TS etiology by modeling basal ganglia development in tridimensional human induced pluripotent stem cell-derived neural organoids. Basal ganglia organoids were generated from and compared across healthy unaffected control individuals and adult unremitting TS patients. We found early telencephalic patterning disruptions in TS-derived basal ganglia organoids, showing a preference for dorsal-posterior specification instead of the expected ventral-anterior commitment seen in healthy control-derived organoids. The aberrant fate shift in the TS-derived basal

ganglia organoids was seen at both RNA and protein levels, confirmed across three separate assays, with consistency across three distinct time points. Transcriptome analyses in the organoids further identified categories of neuronal deficits that show overlap with a manually curated list of differentially expressed genes uncovered by transcriptome analyses at the post mortem level, reiterating the relevance of the bioassay utilized in this study.

This work also investigated a potential mechanism for the early developmental phenotypes observed in the TS organoids. We found significant alterations in sonic hedgehog (SHH) signaling components at both RNA and protein levels that are essential for distinguishing dorsal-ventral patterning in the human brain. Additionally, transcriptome analyses reveal a potential role for cilia, the cellular protrusions that facilitate SHH signal transduction. We found disruptions in genes that are required for cilia formation and function in the TS basal ganglia organoids that were absent from the healthy controls.

This study leads an early developmental examination of TS in humans and offers a bioassay applicable to modeling basal ganglia-related disorders. These results reveal new biomarkers of interest in TS etiology and describe a new implication for SHH signaling. These results indicate that TS patients may exhibit altered telencephalic development, which yields deficits in neurons that ultimately populate the basal ganglia and regulate optimal circuitry function.

Early SHH-Dependent Telencephalic Patterning Disruptions in Tourette Syndrome

A Dissertation

Presented to the Faculty of the Graduate School

Of

Yale University

In Candidacy for the Degree of

Doctor of Philosophy

By

Melanie Victoria Brady

Dissertation Director: Flora M. Vaccarino, MD

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Publications by the Author and Corresponding Thesis Chapters

Fasching, L.#, **Brady, M.V.#**, Bloch, M.H., Lombroso, P. and Vaccarino, F.M.: Tourette Syndrome. In: Rosenberg's Molecular and Genetic Basis of Neurological and Psychiatric Disease (2020) 6th Edition, Vol 2, edited by Roger N. Rosenberg and Juan M. Pascual. (Chapter 1)

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Introduction

Specific Aims

During embryonic development, the ventral telencephalon gives rise to the three ganglionic eminences – medial, lateral and caudal; these regions generate various classes of interneurons that eventually migrate to the cortex and olfactory bulb, and even the amygdala and hippocampus, essentially populating the forebrain [1-3]. The transcriptional signature of the ventral telencephalon is first initiated and controlled by the sonic hedgehog (SHH)-secreting notochord, which in turn specifies the medial ganglionic eminence and other ventral regions. This ventral specification is pioneered by the direct activation of the transcription factor NKX2.1, fostering a transcriptional program that is distinct from that of the dorsal telencephalon [4]. These dorsal-ventral axes detail a region-specific topography of the forebrain that houses specific cellular lineages. As an early determinant of ventral telencephalic fate, NKX2.1, in direct response to SHH signaling, antagonizes dorsal gene expression in this ventral region[5]. The ventral telencephalon ultimately develops into the basal ganglia, and this region's functional circuitry is critical for movement and motor coordination.

Basal ganglia circuitry dysfunction is implicated in several movement disorders, a common one being Tourette Syndrome (TS), which is characterized by motor and vocal tics [6]. However, current approaches in the field to advance our understanding of TS often have several limitations. First, classified as a neurodevelopmental disorder, TS is postulated to manifest early in development; however, most human-based strategies

investigate adult individuals, or examine adult post mortem brain tissue. For instance, neuroimaging evidence suggests a decrease in striatal volume in TS patients [7], and our lab has previously shown a significant decrease in several classes of inhibitory GABAergic interneurons in post mortem basal ganglia tissue of adult TS patients [8-10]. While these approaches yield critical information to aid in our understanding of this complex and heterogeneous disorder, the optimal developmental period fails to be captured, and therefore these studies leave several questions to be addressed when ascertaining disorder etiology. Second, alternative model systems for studying TS include rodents and non-human primates, each with their own limitations. In these organisms, the animals are genetically modified to artificially present with neurobiological and behavioral symptoms aimed to mimic the human phenotypes. In these synthetic approaches, however, disrupting the natural development of these organisms to force a disorder that these subjects would otherwise not experience can yield unintended results; further, deciphering and decoding the non-human behavioral findings in an attempt to match a human disorder is challenging and often yields unclear results and conclusions, as well. Third, the aforementioned complexities arise from the ethical fact that we do not have access to human brain tissue from early developmental time points. Therefore, in order to model TS, an early developmental human system is necessary.

Tridimensional (3D) human induced pluripotent stem cell (iPSC)-derived neural organoids have emerged as a versatile *in vitro* model with vast therapeutic potential due

to the system's capability to mimic early aspects of human brain development [11-13]. This system is optimal for the investigation of human cellular fates and brain region-specific commitments at the genomic, transcriptomic, molecular and cellular level that cannot be achieved with current rodent models or human patients. However, most organoid strategies give rise to neuronal cells mainly comprised of cortical excitatory lineages, resulting from a dorsal telencephalic commitment [11-13].

The goal of this thesis is to investigate the development of basal ganglia organoids across TS patients and healthy control individuals to identify the underlying neurobiology and mechanisms of disorder active during neurodevelopment in order to contribute to the field's understanding of the disease's etiopathophysiology. First, a new iPSC-derived 3D organoid strategy to recapitulate human basal ganglia development at the embryonic and fetal stages is developed. Next, the novel *in vitro* approach is utilized in a series of experiments to explore differences in neurodevelopment at both RNA and protein levels across TS patients and control subjects. Finally, further investigations at the RNA and protein level guide the identification of a developmental mechanism that explains the *in vitro* phenotype observed. This work establishes a versatile model system to facilitate the study of the wide range of neurodegenerative diseases and neurodevelopmental disorders perturbing the basal ganglia. Further, this research elucidates potential biomarkers of TS to provide possible avenues for therapeutic advancement and improvement of clinical assessment of the disorder.

Aim I: Develop a protocol to generate organoids that resemble the basal ganglia, resulting from a ventral telencephalic commitment.

Most current organoid strategies give rise to dorsal telencephalic cortical fates [11-15]. Additionally, the current few that give rise to ventral telencephalic fates [16, 17] do not generate the broad range of neuronal types that are essential components of the basal ganglia and, more specifically, cholinergic interneuron populations. As evidenced from previous findings, cholinergic expression is of particular interest for investigating cellular and molecular abnormalities linked to TS [8-10], making these existing protocols suboptimal for the goals stated here. We previously developed a cortical organoid model to examine pathogenesis of ASD [13, 18], and I have since employed modifications to enrich for a basal ganglia-like cellular commitment within the organoids. In this aim, and detailed in Chapter 4 of this thesis, I have carefully optimized the type, concentration and timing of a novel cocktail of ventral morphogens, such as fibroblast growth factor (FGFs), in order to culture 3D organoids that favor ventral basal ganglia development over dorsal cortical or caudal hindbrain. Extensive analysis of data obtained via three separate assays – immunocytochemistry and stereological counts, real-time qPCR, and bulk RNA sequencing – reveals the emergence of ventral telencephalic transcriptional patterning within the basal ganglia organoids, with a preference towards medial and lateral ganglionic eminence development. Additionally, these basal ganglia organoids show a variety of ventral progenitors as well as the maturation of ventral telencephalon-originating GABAergic neurons for the striatum and cerebral cortex. Further, two-photon imaging of late-stage organoids show robust and extended maturation up to a year in

culture, as well as the development of putative synaptic spines, suggesting functionality of the system. The analyses provided here suggest the achievement of a model for human embryonic/fetal ventral telencephalon, fostering the neuronal diversity integral to the human cortico-basal ganglia network. This model is applied to investigate TS, in which this delicate cellular milieu is perturbed.

Aim II: Generate basal ganglia organoids from TS patients and unaffected healthy control individuals, and determine the possible developmental underpinnings of morphological, cellular and transcriptional differences found in adulthood.

The iPSC lines were generated from five (n=5) adult individuals with severe, unremitting TS. Basal ganglia organoid cultures were differentiated from both TS probands and unaffected healthy control (CT, n=10) individuals using two separate clonal lines per subject. In this aim, and detailed in Chapter 5 of this thesis, I compare the ability of the CT and TS iPSC lines to differentiate into ventral telencephalic progenitors and post-mitotic GABAergic and cholinergic neurons by using the protocol designed in Aim I. Using immunocytochemistry, real-time qPCR and bulk RNA sequencing, a developmental deficit is revealed whereby TS-derived basal ganglia organoids are mispatterned at both RNA and protein levels and across three time points. Furthermore, the findings reveal alterations in the expression of synaptic molecules that intensify over time in the TS-derived basal ganglia organoids, suggesting potential functional disruptions. The transcriptome results from the TS-derived basal ganglia organoids were compared to the previously published transcriptome data from adult TS post mortem brain tissue to find

considerable overlap in differential gene expression between patients and controls. Together, these data identify a developmental deficit in TS-derived organoids that can help elucidate the human phenotype clinically observed.

Aim III: Identify a developmental mechanism for the observed mispatterning of the ventral telencephalon in TS-derived basal ganglia organoids.

Given the developmental deficits observed under Aim II (as detailed in Chapter 5 of this thesis), I attempted to investigate the earlier molecular occurrences within the organoids that could bring further understanding to the findings. In this aim, and detailed in Chapter 6 of this thesis, I target the SHH pathway to uncover differences across the TS and CT basal ganglia organoids that provide an explanation for the phenotype observed in the TS samples. To achieve this, I focus on the earliest time point at which basal ganglia patterning occurs within the organoids, with a shift towards a ventral telencephalic commitment, to identify potential reasons for the TS phenotype observed across time. The data are obtained at both RNA and protein levels utilizing three separate assays to explore these alterations. The results indicate disruptions in the SHH signaling pathway, with enhanced expression of proteins that are typically exclusive to the dorsal telencephalon in TS organoids as compared to controls.

Background and Significance

TS is a chronic neurodevelopmental disorder manifesting in childhood and affecting roughly 0.6% of children [19]. Repetitive motor and vocal tics, such as eye-blinking or

throat clearing, are the defining symptomatology of the disorder [20]. Approximately one-fourth of the children diagnosed with TS present with tics that continue into adulthood. Across patients, adults largely experience the most severe, refractory and incapacitating cases of tics [6]. The behavioral characteristics of the disorder suggest a complex integration of diverse sensory, motor and cognitive functions yielding TS onset. While there have been substantial empirical efforts by international associations contributing to the examination of more than 1400 TS and 5200 control (CT) blood samples, a limited number of risk genes have been discovered according to genome-wide statistical thresholds [21-24]. Further, no causative or predisposing biomarkers have been identified. Although analyses of rare family-specific copy number variants and genomic variants implicate a few genomic loci in TS, these remain unfitting as potential causes for commonly occurring TS, the majority of which remain unknown [25-29].

Dysregulation of basal ganglia circuitry is a primary hypothesis to describe TS pathophysiology. The basal ganglia reside beneath the cortex, comprised of the striatum, globus pallidus internal and external segments, substantia nigra and subthalamic nucleus. Functional circuitry emerging from these nuclei are critical for proper execution of coordinated motor behavior, and initiation and maintenance of efficiently patterned motor programming [30]. The basal ganglia receive glutamatergic cortical and thalamic projections innervating the striatum. Cortical input ultimately drives GABAergic medium spiny neuron (MSN) activity, regulated by different basal ganglia

interneuron populations. Basal ganglia output to the thalamus is regulated by two major striatum-originating anatomical pathways: the direct and indirect pathways, which elicit opposing functional effects [30]. The direct pathway promotes voluntary motor behavior by relieving inhibition of thalamic neurons and facilitating excitatory feedback to the cortex. In an opposite manner, the indirect pathway thwarts motor behavior via inhibiting thalamic neuronal activity and diminishing excitatory feedback to the cortex. In TS, tic release elicits prominent activity in the basal ganglia, the anatomical and neuronal modification of which are correlated with the disorder [6, 20]. Neuroimaging data suggest decreases in volume of the striatum (comprising the caudate and putamen) in TS patients (See Chapter 1B and Figure 11.1 for a depiction of basal ganglia regions) [31, 32]. This is consistent with our post mortem findings of significant decreases in cholinergic (CH/TAN) [8, 9], parvalbumin (PV) [8, 9, 25, 26], and SST/NOS/NPY [10] GABAergic interneuron immunoreactivity in these basal ganglia regions, suggesting loss and/or dysfunction of interneurons in adult TS patients [25, 26].

We hypothesize that decreases in several classes of TS patients' striatal interneurons disrupt the equilibrium of functional basal ganglia pathways, encouraging tic release. Cortical innervation of the striatum excites the fast-spiking GABAergic PV+ interneurons [33-35], selectively inhibiting MSNs designated for the direct pathway [36, 37]. Additionally, intralaminar thalamic nuclei project to the striatum, activating the tonically active CH/TAN+ interneurons [38], subsequently exciting and regulating PV+ interneurons [39]. Conceivably then, alterations in striatal PV+ and CH/TAN+ number

and activity should disrupt the thalamic output regulated by direct pathway activation, and to that effect, both thalamic and cortical influences on direct pathway activity.

An accurate basal ganglia model is critical for both basic and clinical studies, with implications for TS and several other neurological diseases and neurodevelopmental disorders. Basal ganglia dysfunction and TS have been modeled in various organisms – from rodents to non-human primates – but heterogeneity of the disorder limits the relevance of certain animal systems. For instance, rodent models generated by different disorder's mutations can have the same resultant increased grooming behavior, which has been evaluated as a key phenotype of trichotillomania [40], Autism Spectrum Disorder (ASD) [41] and OCD [42], in addition to TS. The commonality of such repetitive behaviors greatly weakens the claim that a given behavioral abnormality is characteristic of a particular disorder. Additionally, with humans possessing the most complex neural development and circuitry, the anatomical differences across species impact the degree to which disease pathophysiology can be convincingly captured. Thus, the use of a developmental *in vitro* human model system, with the potential to differentiate into whole brain regions, is uniquely suited to address the pathophysiology of disease in the absence of useful models for early human development.

Neural organoids are 3D multilayered aggregates capable of recapitulating human embryonic development. Comparison of the organoids' transcriptome at various developmental stages with post mortem data found our neural organoids correlated best

with the dorsal telencephalon and spanning 8-16 postconceptional weeks of human fetal development [15, 18]. However, representation of the ventral forebrain is lacking from the majority of existing organoid protocols. Generating organoids that model basal ganglia development will facilitate investigation of TS and other neurodevelopmental disorders targeting this brain region.

Summary

This thesis aims to advance our understanding of TS etiology and human basal ganglia development. First, I aim to develop an iPSC strategy to model human basal ganglia development in 3D organoids – a system which will foster a clinically-relevant assessment of how TS arises and unfolds. Next, I am to use this technology to compare basal ganglia development across control and TS individuals by conducting cellular, molecular and transcriptome analyses and uncover new underpinnings of the disorder. Finally, I aim to identify a developmental mechanism for the differences in development that are observed. Results of these studies will be presented in Chapters 4, 5, and 6 of this thesis, respectively, followed by a discussion in Chapter 7 including implications of the studies' findings and future directions.

Chapter One:

Tourette Syndrome & its Cellular and Molecular Pathology

Chapter 1A: Tourette Syndrome

The following review examines the phenomenology of TS, detailing the clinical presentations and the evolution of our empirical and medical understanding of the disorder. Additionally, limitations in current treatment methods are described. This section reveals the complexities of TS that have contributed to the challenges surrounding studying this disorder. Comprehensively, this section outlines the current and historical information necessary to understand the neuropsychiatric disorder that this thesis investigates.

1. Summary

1.1 Disease Characteristics, Hallmark Manifestations and Inheritance

Tourette syndrome (TS) is a childhood-onset psychiatric disorder characterized by chronic motor and phonic tics [43]. Symptoms typically vary in nature and frequency over time and, in the majority of cases, decrease with age [6, 44]. Approximately one-third of the children diagnosed with TS present with tics that continue into adulthood. Across patients, adults often experience the most severe, refractory and incapacitating cases of tics [6]. There is strong evidence of a genetic contribution; however, candidate genes revealed by family studies are not significant risk genes for the majority of the population, and association studies have not identified common variants above threshold significance [22, 25, 26, 45-48].

1.2 Diagnosis and testing

TS is diagnosed when both motor and vocal tics are present for more than 12 months, with onset before age 18, and is estimated to occur in 0.1-1% of children [49]. As there are currently no physiological or genetic tests, diagnosis occurs through clinical evaluation [50].

1.3 Current research

Several brain regions have been implicated in TS, primarily the caudate nucleus and putamen in the basal ganglia of the striatum, but also the cerebral cortex, hippocampus and cerebellum [7-9, 32, 51, 52]. Research includes structural and functional analysis of gross anatomical differences across patients, evaluation of cellular phenotypes and neurotransmitter systems in post-mortem brain studies and discovery of TS-associated gene mutations [47, 53-56]. Neuroimaging studies have revealed a five percent volume reduction of the striatum across TS patients, as well as a correlation between volume reduction of the caudate nucleus and putamen in children and increased tic severity in adulthood [7]. The presence of anti-neuronal antibodies in some patients, high rates of maternal autoimmune disorders, and a sub-category of children in which infection is coincident with tic onset have prompted investigations of neuroimmune interactions [51, 57-66]. Studies in human post-mortem brain tissue echo defects in the aforementioned basal ganglia regions, suggesting cellular abnormalities in the striatum, and in particular, decreased numbers of cholinergic tonically active neurons (CH/TAN) and two

types of GABAergic interneurons, the parvalbumin+ and somatostatin/nitric oxide synthase /neuropeptide Y+ in TS versus matched normal controls [9]. In contrast, the majority of striatal neurons, the medium spiny neurons, were not affected. Transcriptome analysis is consistent with the interneuron loss and also revealed prominent inflammatory changes in TS brain tissue [10].

2. Clinical Features

2.1 Historical overview

TS was first classified as a neurobiological disorder in the mid-1880's by Gilles de la Tourette and Jean-Martin Charcot working at the Salpetriere Hospital in Paris [67, 68]. In the subsequent century clinical classification continued to be refined leading to current diagnostic criteria for childhood onset of chronic motor and vocal tics [68].

2.1.1 Disease identification

General tic disorders affect 4% of children and are characterized by sudden, repetitive motor or vocal tics, which can be transient (2 weeks to 12 months) or chronic (more than 12 months). The first symptoms are often simple motor tics, such as eye blinking and facial movements, which later progress to involve additional muscles of the face, neck, and shoulders. Vocal tics, such as throat clearing, grunting, coughing, or sniffling, typically appear several years after the motor symptoms. A minority of patients has complex vocalizations, such as swearing (coprolalia), echolalia, and the utterance of words or word parts. Patients have described a premonitory sensation that occurs immediately prior to tics, describing tic expression as a way to relieve the premonitory

urge [69]. Tics typically occur in bouts, vary over time with the worst period occurring when the child is 10-12 years, and decrease with age [6, 70]. A minority of patients are chronically disabled with severe tics that continue into adulthood [71].

2.1.2 Gene identification

To date, no gene has emerged as definitively causal. No common gene variants above threshold significance were identified, which could be attributed to the low risk conferred by such common variants, and by the limited power of discovery of rare gene candidates using these methods [22, 46]. Significant association of TS with chromosome 2 was reported, and the strength of this association increased when diagnosis of TS and chronic tic disorder were combined, suggesting that symptoms of both emerge from common underlying genetic vulnerability [72, 73]. Additional regions on chromosome 9 [74], 18 [75], and 22 [76-78] have been implicated. Given the difficulty of localizing genes via traditional linkage approaches, family-based association strategies are being used and have found several genes that associate with the risk of developing TS, including the dopamine receptor, DRD2; the dopamine transporter gene, DAT1; serotonin receptor, HTR2C; and gain of function alleles in the setotonin transporter gene, SLC6A4 [76-78].

Rare mutations were identified that appear to confer high risk of developing TS in particular families. Mutations in Slit and Trk-like 1 (SLITRK1) were identified after characterization of a chromosomal inversion in a child with TS [25]. SLITRK1 was found

to be expressed in brain regions implicated in TS, and *in vitro* assays revealed that wild-type SLITRK1 enhanced dendritic growth compared to protein generated by a mutated allele [25, 47]. Very rare loss of function mutations have also been described in histidine decarboxylase (HDC), which catalyzes the production of histamine; IMMP2L, a mitochondrial pepsidase gene; and CNTNAP2, a neurexin superfamily member [25, 26, 47, 79, 80]. It is presently unclear to what extent such rare mutations segregate with TS, versus co-morbid disorders such as OCD [81], or contribute to TS in the majority of patients [79, 80]. A recent genome wide association study of 2,434 patients identified rare copy number variations (CNVs) in two genomic loci: *NRXN1* and *CNTN6,* where *NRXN1* deletions and *CNTN6* dublications were associated with a significantly higher risk for developing TS [23]. Furthermore, DNA sequence analysis of 674 non-multiplex TS trios and over a thousand control families recently confirmed an increased rate of de novo CNV and sequence variants in TS indviduals, suggesting that such de novo gene disrupting variants could be responsoible for approximately 5% of TS cases. These analyses resulted in the identification two high confident TS risk genes, *CELSR3* and *WWC1*. Interestingly, proteins encoded by *NRXN1, CELSR3,* and *CNTN6* are involved in the control of cell adhesion and/or polarity, suggesting convergent functional pathology [82].

2.1.3 Early treatments

Neuroleptics became the earliest, and remain the most frequently used pharmacological treatment for TS, albeit with varying degrees of efficacy [83, 84]. Due to frequent co-

morbidity of TS with obsessive compulsive disorder (OCD) and attention deficit hyperactivity disorder (ADHD), other early treatments include the use of adrenergic receptor agonists such as clonidine [84]. Standards of treament continue to be refined and are described in Section 7.1.

2.2 Mode of Inheritance and Prevalence

Early segregation analyses indicated that inheritance is transmitted vertically as a single gene of major effect. However, while some data are consistent with an autosomal dominant locus with sex specific penetrance, there is also evidence for intermediate inheritance, and many favor a multifactorial or polygenic model, suggesting that TS is the result of multiple genes acting together. The original description of TS included a familial expression pattern, and the rate of TS in relatives of probands is 10-15%, 10-fold higher than in the general population. Inheritance is strongly genetic: tics occur in a second twin in 77% of monozygotic, but only 23% of dizygotic twins [45]. Yet environmental factors do contribute, as monozygotic twin concordance is not 100%. Retrospective analyses revealed lower birth weights in the more severely affected twin in monzygotic pairs, suggesting that prenatal factors contribute to symptom expression later in life [85].

2.3 Natural History

2.3.1 Age of onset

Based on current classification standards, onset of TS symptoms begin in childhood before age 18. The mean age of onset is between 5 and 7 years, with simple motor tics typically preceding the onset of verbal tics by several years [86].

2.3.2 Disease evolution

TS symptoms typically vary in frequency and intensity, increase during periods of fatigue or stress. Symptoms may also wax and wane spontaneously, independent from triggers or medication [87]. Lifetime severity may be influenced by perinatal exposures that presumably affect early brain development. These observations have led to a stress-diathesis model in which the interaction of genetic vulnerability factors with environmental stressors during critical periods of development affects disease onset and severity [69].

2.3.3 Disease variants

A potential sub-category of TS has emerged but remains controversial. Initially an increased prevalence of OCD was observed in Sydenham's chorea patients with a late manifestation of rheumatic fever associated with a prior streptococcal infection. After an epidemic of streptococcal pharyngitis in Providence, Rhodes Island, tic disorders increased in affected children. Swedo and colleagues then proposed the existence of a subgroup of children with tics and/or OCD now known by its acronym PANDAS or Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal infections [88]. PANDAS is defined by 5 criteria: (1) the presence of a tic disorder and/or

OCD; (2) prepubertal onset of neuropsychiatric symptoms; (3) history of a sudden onset of symptoms and/or an episodic course with abrupt symptom exacerbation interspersed with periods of partial or complete remission; (4) evidence of a temporal association between onset or exacerbation of symptoms and a streptococcal infection; and (5) adventitious movements (e.g., motoric hyperactivity and choreiform movements) during symptom exacerbation [89]. In an epidemiological study, patients were more likely than controls to have had streptococcal infection in the 3 months preceding onset; having multiple GABHS infections in the preceding 12 months was associated with an increased risk for TS; and overall, 12% of TS cases appeared to be GABHS-related [90]. The proposed pathophysiology is based on the molecular mimicry hypothesis, wherein antibodies produced to fight infection cross-react with epitopes in previously unaffected tissues. This model suggests that neuronal function is compromised in a manner analogous to the sequence of events proposed for Sydenham's chorea.

Therapeutic benefit was described in TS patients treated with plasmapheresis [91], and multiple groups found evidence for autoantibodies in the blood serum of a subgroup of patients [92], and additional studies have examined the involvement of cytokines, reporting decreased numbers of regulatory T cells [57, 58]. However, other studies have not supported the PANDAS hypothesis; one report found no significant difference in neuronal binding capacity *in vitro* of IgG isolated from PANDA versus TS or control cases [64], and in another study, PANDA cases had lower than expected exacerbation of symptoms and GABHS infections compared to non-PANDA cases [65]. Further

investigation is needed to elucidate the relationship between GABHS and TS, and to predict when interventions such as antibiotic treatment or IgG replacement might be of benefit.

2.3.4 Comorbidities

Clinically ascertained cases of TS are associated with a variety of other disorders. More than 60% of TS patients reaching clinical attention have problems with inattention, impulsiveness and hyperactivity, and may warrant a separate diagnosis of ADHD [49, 93]. While the high frequency of co-occurring features (aggressiveness, depression, learning disabilities, and anxiety disorders) may reflect an ascertainment bias, surveys in community samples have also observed a higher than expected frequency of ADHD and disruptive behavior among children with TS [49]. As many as 30% of individuals with TS have sufficiently severe symptoms to warrant diagnosis of OCD, and research suggests that the two may be etiologically related [94-98].

3. Disease mechanisms: Pathophysiology and current research

Several brain regions are implicated in TS, including the basal ganglia, cerebral cortex, hippocampus, and cerebellum [7-9, 32, 51, 52]. The basal ganglia have long been presumed to be central for the pathophysiology of TS, based on anatomical and functional studies, as well as the effectiveness of D2 blockers for the treatment of tics. The basal ganglia form a crossroad of converging and diverging routes for neural pathways projecting from the cerebral cortex to the thalamus and back to cortex [99].

These pathways are somatotopically organized in parallel circuits regulating motor, somatosensory, cognitive, and emotional functions. When a movement is initiated, the inhibitory output of the direct pathway medium spiny neurons, which project from the striatum to the internal globus pallidus (GPi) and substantia nigra pars reticulata, is enhanced, releasing the inhibitory GPi brake to the precise thalamic target [99, 100]. These pathways are somatotopically organized in parallel circuits regulating motor, somatosensory, cognitive, and emotional functions. Disruption of one or more of these pathways is currently proposed to underlie TS symptoms.

In the striatum, there are 4 main classes of striatal interneurons, identified by expression of calcium binding proteins and co-transmitters. These include 3 sub-classes of GABAergic interneurons: (1) fast-spiking parvalbumin; (2) calretinin; and (3) somatostatin/nitric oxide synthase/neuropeptide Y; and (4) tonically active cholinergic interneurons (TAN)[101-103]. Glutamatergic, cholinergic, GABAergic, and dopaminergic inputs converge on the spines and dendrites of the medium spiny neurons to regulate their output. Dopamine input and the GABAergic and cholinergic activities within the striatum regulate the excitatory glutamatergic input from the cortex, and ultimately the information processing to the thalamus [100, 104, 105]. Inhibitory parvalbumin interneurons are directly stimulated by the cortex and convey strong feed-forward and widespread inhibition to the striatal medium spiny neurons, focusing their activity on the most relevant inputs. A deficiency in striatal interneurons was reported in cases of severe unremitting adult TS, suggesting that these cells may play a role an important

role in the genesis and/or persistence of tics Reductions of GABAergic and cholinergic interneurons in the basal ganglia in TS subjects have been found, based on immunohistochemical labeling of post-mortem tissue with antibodies to parvalbumin, choline acetyltransferase [8, 9, 106], and nitric oxide synthase (**FIGURE 1**) [10]. The deficiency in striatal interneurons suggests that these cells may play a role an important role in the genesis or persistence of tics [8, 9, 106]. Parvalbumin^{+}-GABAergic interneurons form a widespread inhibitory network responsible for maintaining inhibition within the striatum, particularly in sensorimotor territories [34, 35].

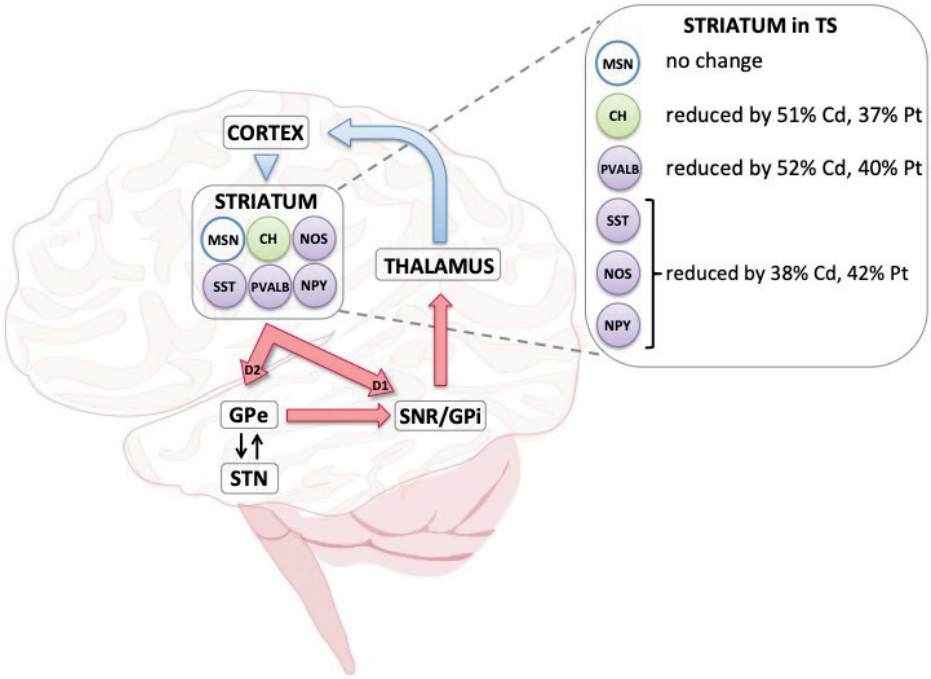

FIGURE 1. Cortico-striatal-thalamic circuitry and altered neuronal composition of the caudate nucleus (Cd) and putamen (Pt) of the basal ganglia in Tourette syndrome [10].

In addition to decreases of parvalbumin^{+} interneurons in the caudate nucleus, a relative "excess" was found in the GPi [8]. While it is unknown whether this could be due to changes in cell migration, cell genesis or cell death, it is plausible that discharge patterns from the caudate, the GPi, or both could disrupted, leading to aberrant inhibition of discrete thalamic neurons and their cortical targets, resulting in tics [8]. Thus, deficient regulation of medium spiny neuron activity leading to disruption of their modulatory influence upon the thalamus and cortex is currently proposed to underlie TS symptoms.

4.1 Human neuroimaging

Neuroimaging of TS patients revealed larger brain volumes in prefrontal regions and parieto-occipital cortical regions, smaller inferior occipital volumes, and smaller caudate volumes [32], and caudate volume in childhood was found to inversely correlate with tic severity in adulthood [7]. Functional studies have found alterations in cortical-basal ganglia circuits, suggesting increased activity in motor, premotor, sensorimotor cortical regions and striatum in TS subjects [107, 108]. Compared to controls, TS subjects displayed heightened activity in the left caudate, which correlated with tic severity during pre-pulse inhibition to startle tasks [109]. In addition, increased metabolic activity in the right caudate was found during tic suppression, which negatively correlated with tic severity [110]. As tic severity increased, the signal changes decreased, suggesting that tics are due to a failure of inhibitory systems [110, 111]. This finding concurs with the

decreased number of inhibitory interneurons found in the TS caudate in the -mortem studies described above [9, 10].

Further support for the influence of sensorimotor cortical regions on TS is evident from additional imaging studies that revealed cortical thinning in the sensory and motor regions in children with TS [112]. And while some regions – including the lateral temporal and inferior parietal cortices and the inferior primary sensory cortex – exhibited comparable cortical thickness across both TS children and control children, longitudinal analysis of these cohorts revealed that control children exhibited cortical thickening with age while the TS children did not [112]. Previous findings via transcranial magnetic stimulation showed decreased intracortical inhibition in motor cortices in TS children, suggesting a potential reduction in GABAergic interneurons in these regions [113]. Postmortem studies are needed to investigate the cellular abnormalities underlying these neuroimaging findings. The correlation between cortical thickness of sensorimotor regions and tic symptoms reiterates a prominent role for these brain regions in the clinical manifestation of TS.

PET and SPECT studies have suggested increased density of striatal dopamine receptors in TS subjects [107, 108]. Involvement of dopamine signaling is also indicated by considerable clinical pharmacological data: (1) the most consistently effective drugs for suppressing tics are dopamine D2 receptor blockers; (2) tics may worsen following exposure to high doses of stimulants known to increase central dopaminergic activity;

(3) withdrawal from neuroleptic treatment is often accompanied by a rebound in tic symptoms presumably due to heightened receptor sensitivity and/or up-regulation of post-synaptic dopamine receptors; (4) elevated levels of a dopamine metabolite, homovanillic acid, have been found in the cerebrospinal fluid of TS subjects [114, 115].

Other neurotransmitter systems have been implicated based on immunohistochemical and transcriptome studies, as described earlier, which include GABA, glutamate and acetylcholine, that are all known to play a role in cortico-striato-thalamo-cortical circuitry (**FIGURE 1**) [104].

4.2 Animal models

In vitro models enable the advantage of studying the genomics and genetics of complex circuitries involved in mechanisms underlying Tourette syndrome, but meet their limitations when we try to understand the downstream effects of genetic alterations especially in regard to tics and behavioral comorbidities. Modeling Tourette syndrome in rodents and primates allows us to mimic a disorder by introducing genomic or drug induced alterations, study the resulting effects and subsequently develop strategies to rescue the phenotype. The gain of those critical insights is of tremendous value to be able to improve current therapies and thus the quality of life for patients.

As an example, current research includes genetic modeling to understand the roles of SLITRK1 and histidine decarboxlyase (Hdc) in mice [25]. Tic-like behavior in form of excessive grooming was observed after the induction of acute stress by cued fear

conditioning in Hdc knockout mice [26, 56]. Administration of low doses of picrotoxin into the dorsal striatum and the sensorimotor cortex induce non-rythmic tic-like movements in mice. This phenotype was rescued by the administration of muscimol, a GABA-A agonist, again suggesting a connection between alteration of the GABAergic system and the development of tic-like movements [116, 117]. Models of striatal interneuron ablation are being evaluated for their potential to evoke stereotypic behaviors. Stereotopy can be induced through stimulant exposure, which affects expression of immediate early genes and generates an imbalance of striatal activity within striosome versus matrix compartments [118, 119]. In rodents and monkeys, low doses of agents that activate the dopamine pathways (i.e., amphetamine, apomorphine, and cocaine) increase locomotion while elevated doses produce stereotypic behaviors. Animal models manipulating the serotonin system by administrating serotonin agonists should be considered with caution due to potential nonspecific effects [117].

Neuroimmune modeling has revealed induction of stereotypic behavior in rodents following striatal infusion of serum from TS patients with high levels of anti-neuronal autoantibodies, but not after infusing serum from controls or TS patients with low levels of anti-neuronal autoantibodies [120, 121]. Subsequent studies were not able to reproduce these results, and additional research is necessary to determine whether autoantibodies play a role in some TS cases [122]. Interestingly, *in vitro* studies have shown that immune modulation is sufficient to alter signaling mechanisms critical for parvalbumin neuron function [123, 124]. Interestingly, *in vitro* studies have shown that

immune modulation is sufficient to alter signaling mechanisms critical for parvalbumin neuron function [123, 124].

4.3 *In vitro* models

Currently, with the rise of stem cell-derived neural organoids, there is great promise for disease modeling in *in vitro* systems to elucidate the etiology of TS. Although there are no studies published to date featuring such TS model systems, the potential is vast for stem cell strategies to uncover new biomarkers and/or neural mechanisms involved in TS. Human induced pluripotent stem cell (iPSC)-derived neural organoids are 3D multilayered cellular aggregates capable of recapitulating human embryonic development. With its childhood on-set as early as 3 years of age, TS is categorized as a neurodevelopmental disorder; however, symptoms may be delayed with respect to the causal mechanisms underlying the disorder, and therefore the actual “age of onset” is unknown. Human neural organoids could provide the first accessible window into human embryonic and fetal development, thereby facilitating study across patient and control groups to untether the complex etiopathophysiology of TS. Recent advancement in this technology mimic both cortical and basal ganglia development in these neural organoids [16, 18, 125], allowing the assessment not only of individual regional development across patients and controls, but also the more complete telencephalic forebrain – including the dorsal cortex and ventral basal ganglia components of the circuitry.

The additional advantage of using stem cell strategies include the important foundational fact that the cell samples are obtained directly from living TS individuals. While large-scale therapies are possible from this approach as well, the patient-specificity of the technology allows for an uncovering of an individual's neurodevelopment that could ultimately benefit the most severe of TS patients to potentially illuminate individualized treatment methods.

Both scientific and clinical fields could benefit from investigation and modeling of TS in neural organoids. Such research could elucidate critical biomarkers in disease pathogenesis as well as therapeutic targets, and could even contribute to new diagnostic opportunities in children.

5. Differential Diagnosis

Many diseases can manifest with tics, such as Huntington's disease and stroke, but do not typically have pediatric onset. Others movement disorders, including Sydenham's chorea, the dystonias, and some rare genetic conditions may require more careful evaluation [126, 127]. Events such conditions as carbon monoxide poisoning are germane to children and may present with transient tics; however, these are distinguishable as TS involves chronic symptoms [127].

6. Testing

There are presently no molecular, neuroimaging, or physiological mechanisms that can reliably aid in TS diagnosis. Instead, diagnosis relies on clinical evaluation, using rating systems such as the The Yale Global Tic Severity Score [50]. The YGTSS rates multiple features of symptoms including the number, frequency, intensity, and complexity of motor and vocal tics, as well as the level to which tics interfere with daily function.

7. Management

7.1 Standard of care

The goal of treatment is to achieve a balance between adequate tic control and minimizing side effects, rather than tic eradication. Several medications (**Table 1**) have been used for the treatment of tics, although few have been adequately tested in placebo-controlled studies [128]. Typical antipsychotics such as haloperidol and pimozide were the first medication demonstrated to be effective for tics. Haloperidol is a potent postsynaptic D2 receptor blocker often effective at low doses but associated with a high frequency of adverse effects. Pimozide is also effective but at high doses, or in combination with drugs that inhibit CYP3A4, QT prolongation is a potential consideration and cardiac monitoring is warranted [128].

Atypical neuroleptics have also been used for tic treatment. Tiapride and sulpiride, unavailable in the United States, have shown positive results in controlling tics; however, both require more study to confirm their efficacy. The D2- and 5HT2- blocking

agents Risperidone and Ziprasidone were found to be effective in placebo controlled studies [129, 130]. Ziprasidone has a unique receptor profile including 5-HT1A-agonist and modest norepinephrine and serotonin reuptake blocking properties, suggesting that it may have additional anxiolytic and antidepressant effects [128]. Aripiprazole, which acts a partial agonist at the D2 receptor also has demonstrated efficacy in treating tics [131, 132]. Atypical antipsychotics are associated with a poor side-effect profile including weight gain, increased risk of metabolic syndrome and diabetes so even though they are the medications with the greatest demonstrated efficacy in reducing tics, they are often not used first-line, especially in children [133].

Adrenergic alpha 2 agonists are frequently used in the treatment of tics or ADHD that is commonly associated with TS. Clonidine was introduced as a treatment for tics in the 1970s and has shown modest benefits for ADHD. Although unlikely to be as effective as D2 blocking antipsychotics in reducing tics, it is commonly used in children with TS due to concerns about long-term exposure to neuroleptics. In the 1990s another alpha-2 agonist, guanfacine, was introduced for the treatment of ADHD in children with TS, and in a randomized clinical trial was superior to placebo for ADHD and tic symptoms [134]. Alpha-2 agonist medications have been demonstrated to be effective in reducing tic symptoms but primarily for children with comorbid ADHD [135].

Several other medications have been tried with varying success in their capacity to suppress tics including topiramate [136], cannabis-derived medications [137, 138],

vesicular monoamine transporter 2 (VMAT2) inhibitors [139-141] and ecopipam (a D1/D5 receptor antagonist) [142]. These emerging treatments are discussed in section 7.3. In addition to pharmacological strategies, there is growing interest in behavioral interventions such as parent training, anger control training and habit reversal training [143, 144]. Clinical trials have found behavioral therapy particularly habit reversal therapy to be effective, with some patients demonstrating continued improvement 6 months post-treatment [145-147]. The benefits of habit reversal therapy for the treatment of tics in terms of effect size seem to be similar to the most effective anti-tic medications [148]. However, dissemination remains a major challenge to the widespread use of behavioral treatments for tics.

TABLE 1. Current pharmacological treatments for Tourette syndrome

Class/Mode of action	Agent	Typical dosage	Note
Typical neuroleptics (D2 blockers)	Haloperidol	0.5-2.0 mg/day	Many side effects including sedation, dysphoria, cognitive dulling, weight gain, parkinsonism, dystonia, dyskinesia, and akathisia
	Pimozide	0.5-4 mg/day	QT prolongation at high doses or in combination with CYP3A4 inhibitors
Atypical neuroleptics (D2 blockers)			
	Aripiprazole	5-20 mg/day	Low frequency of neurological side effect and less weight gain than most antipsychotics.
	Risperidone [149]	1.0-3.0 mg/day in 2 divided doses	Low frequency of neurological side effects; drowsiness and weight gain are common. Also 5HT2 blocker
	Ziprasidone [129]		Low frequency of neurological side effect and less weight gain than most antipsychotics; monitor for QTc prolongation;
	Tiapride	*Unavailable in USA*	Rhabdomyolysis, QT prolongation
	Sulpiride	0.1-0.8 g/day *Not available in the US*	Low frequency of neurological side effects
Adrenergic alpha 2 agonists	Clonidine [150, 151]	0.1-0.3 mg/day in 3-4 divided doses	Minimal side effects; modest benefit for ADHD
	Guanfacine [134]	1.5-3 mg/day in 3 divided doses	Minimal side effects; benefit for ADHD

7.2 Failed therapies and barriers to treatment development

Treatment of TS is challenging, as its natural course is variable. Even without treatment, symptoms reduce or resolve by young adulthood in the majority of patients. While emerging studies continue to support a standard of care for TS, these have also revealed an unusually robust placebo response [150-153]. This presents a potential

barrier to identifying effective therapies, and has been noted in other disorders that involve the basal ganglia [154].

7.3 Therapies under investigation

Preliminary results in severe, refractory TS cases suggest that procedures such as neurofeedback, transcranial magnetic stimulation, and deep brain stimulation may have a place in the treatment of TS [100, 155-158]. Although offering great promise for the treatment of severe TS cases, consensus has not yet been achieved, for instance, on the optimal placement of the deep brain electrodes, and much needs to be learned about how best to apply these technologies [159]. Several medications such as topiramate [136], dronabinol [160], VMAT2 inhibitors [139-141], ecopipam (a D1/D5 antagonist) [142] have demonstrated efficacy in small, randomized-controlled trials or in large open-trials. Larger, multi-site, placebo-controlled studies are currently ongoing to more clearly determine the efficacy of these agents in the treatment of tics.

8. Conclusions

Research over the next decade is likely to build on the recent discoveries in neurobiology, epidemiology, genomics, genetics, and neuroimaging, and lead to better treatments. New psychopharmacological agents based on a better understanding of the neurobiology and will be used in combination with behavioral interventions. Moreover, longitudinal clinical investigations into the phenomenology and natural history may provide additional clues on the biological processes involved. The cellular abnormalities that have been detected in patient's post-mortem brains will serve as a guide for the

development of animal models, which will allow investigators to explore specific hypotheses. In addition, induced pluripotent stem cells can be generated directly from patients, which may allow to reproduce aspects of TS neurobiological abnormalities *in vitro* using the organoid system, facilitating the generation of large scale genetic and drug screen to find patient-tailored treatments [161]. Through these combined efforts, we will eventually come to a more complete understanding of the neurobiological mechanisms and treatment of TS.

Acknowledgements

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Chapter 1B: Cellular and Molecular Pathology of Tourette Syndrome

The following section focuses on the current postulates in the field surrounding TS etiology. This section compiles current data that detail deficits and abnormalities seen across TS individuals, especially unremitting adult patients. Through this information, the heterogeneity of the disorder can be appreciated and the gaps in our understanding are explained. This section establishes a critical empirical foundation for the motivation of the study pursued in this thesis and for ascertaining the goals of the aforementioned thesis aims.

Introduction

The basal ganglia are considered a central station in the regulation of motor programs. Although the function of the basal ganglia is not entirely known, their circuitry has been extensively investigated. The basal ganglia are a set of nuclei situated deep within the cerebral cortical hemispheres. In the basal ganglia, a large number of cortical and subcortical excitatory inputs converge on a relatively small number of neurons whose firing represent complex temporal coincidences of neural activity in a vast number of afferent regions. The main function of the basal ganglia is to exert negative regulation by tonically inhibiting ongoing neural activity in the thalamus. However, arousing stimuli that travel through the ascending reticular activating system, and those that, having been associated with the anticipation of rewards, activate the dopamine system, temporarily suppress the negative regulation of the basal ganglia, resulting in facilitation

of sensory and motor functions. In this way the basal ganglia regulates the initiation and execution of learned actions, such as habits, and in general facilitate the integration of voluntary and involuntary segments of cognitive and motor activity.

Patients with Tourette syndrome (TS) have a decreased volume of the caudate (CD) and putamen (PT) nuclei in the striatum [31, 32], the first station of the basal ganglia receiving inputs from the cerebral cortex, the thalamus and midbrain dopamine neurons (Figure 11.1). Although both cortical and cerebellar anatomical abnormalities have been described in neuroimaging studies of TS individuals [112, 162-164] (reviewed in [68], the decrease in caudate volume has particular relevance since the reduced volume of the caudate nucleus in childhood correlates significantly and inversely with the severity of tic and obsessive compulsive disorder (OCD) symptoms in early adulthood [7]. Furthermore, in monozygotic twins discordant for severity of TS, the right CD was smaller in the most severely affected twin pair [165]. Overall, the neuroimaging studies suggest that the basal ganglia are a central component of the pathophysiology of TS (see also Chapter 12).

Basal ganglia anatomy and circuitry

The hypothesis that an inherited or developmentally acquired dysfunction of the basal ganglia causes TS is in agreement with studies in a variety of animal models suggesting that the basal ganglia are the main “integrators” of higher motor and cognitive functions (see also Chapter 15). One of their key functions is to select among ongoing

sensorimotor activities based on their salience and motivational value, by integrating the activity of the dopamine system, which is activated by the anticipation of rewards, with sensory, motor and attentional systems, which are channeled to the striatum via the thalamus and the cerebral cortex. Indeed, the CD and PT receive direct inputs from the cerebral cortex, the thalamus and midbrain dopamine neurons. The final common output of the basal ganglia is the Internal Segment of the Globus Palludus/Substantia Nigra Reticulata (GPi/SNr), which tonically inhibits the ventral anterior and ventral lateral (VA/VL) and intralaminar (IL) thalamic nuclei, suppressing motor activity (Figure 11.1A). It has been hypothesized that failure to integrate sensorimotor information within the basal ganglia may lead to the development of tics (see Chapter 12 on Electrophysiology).

The medium spiny neurons (MSNs) of the CD and PT are projection neurons of the striatum that form two major pathways, the indirect and direct pathway, which have opposite effects on the GPi/SNr and behavior [30]. The *direct pathway* inhibits the GPi/SNr, resulting in excitation of thalamic neurons and facilitation of motor behavior; conversely, the *indirect pathway* indirectly excites the GPi/SNr by inhibiting the subthalamic nucleus, inhibiting motor behavior (Figure 11.1A).

The striatum, based on its cortical inputs, contains three major functional territories, the associative, sensorimotor, and limbic (see Figure 10.1B). These functional domains are largely segregated throughout the striatum. The associative territory comprises almost

the whole extension of the CD and the precommissural PT. The sensorimotor domain includes the dorsolateral aspect of the CD head, part of the dorsal precommissural PT and the entire postcommissural PT. The main component of the limbic striatum is the nucleus accumbens, the deep layers of the olfactory tubercle, and the ventral part of both the CD and the PT [166]. The cortico-striatal projections terminate in the form of clusters of various sizes, whose distribution closely follows that of the two major striatal chemical compartments – the striosomes and the extrastriosomal matrix. Studies of the non-human primate have shown differential patterns of connectivity for the striosome and matrix compartments in the striatum [167, 168]. Striosomes are interconnected with regions linked to limbic circuitry, and the matrix is interconnected with sensorimotor and associative cortical circuitry.

Interneurons in the basal ganglia and their functional implications for disorders

Recent evidence has shown important modulatory roles of striatal interneurons on the direct and indirect pathways. There are 4 classes of interneurons within the striatum, identified by their expression of calcium binding proteins and co-transmitters: (1) Parvalbumin (PV^+); (2) Calretinin (CR^+); (3) Somatostatin/Nitric Oxide Synthase /Neuropeptide Y ($SST^+/NOS^+/NPY^+$); and (4) Cholinergic or Choline acetyl transferase ($ChAT^+$).

The fast-spiking, PV^+ interneurons form a non-habituating striatal inhibitory network [34, 35], which is the main source of feed-forward inhibition from the cortex to the striatum.

PV+ cells receive strong cortical synaptic input [33] and respond with shorter latency that MSNs to cortical stimulation, preferentially suppressing the activity of MSNs of the *direct pathway* [36, 37] (Figure 11.1 A).

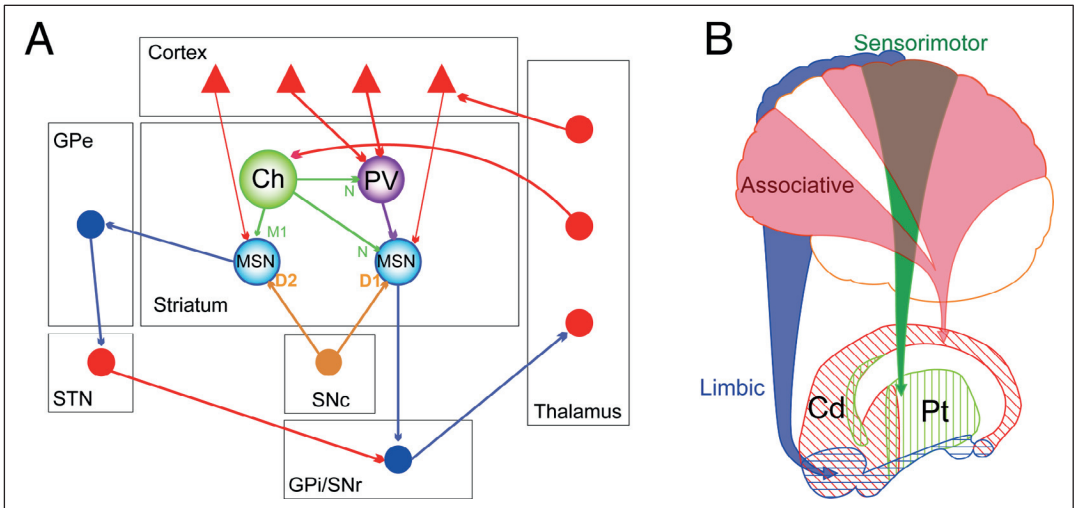

Figure 11.1. The Basal Ganglia.A, Schematic outline of the basal ganglia circuitry.Red, excitatory neurons; blue, inhibitory neurons; green, cholinergic neurons, orange, dopaminergic neurons. Parvalbumin (PV) interneurons mediate the cortical feed-forward inhibition upon medium spiny neurons (MSNs) of the striatopallidal direct pathway, resulting in inhibition of voluntary movements. Cholinergic (Ch) interneurons enhance the responsiveness of medium spiny neurons (MSNs) of the striatonigral indirect pathway, resulting in movement suppression. B, Functional subdivisions of the basal ganglia projected on a lateral view of the basal ganglia andtheir cortical afferent systems. Based upon Parent et al (1990) and Bernacer et al (2007).

The cholinergic interneurons have been long thought to play a role in associative learning [169] and fire tonically with periodic pauses, which temporally coincide with burst firing in dopamine neurons. Cholinergic neurons receive a strong excitatory input from the median-parafascicular complex (IL) nuclei of the thalamus [38] (Figure 11.1 A). Cholinergic interneurons excite SST+/NOS+/NPY+ striatal interneurons, which in turn inhibit MSN [170]. Hence, MSNs can be inhibited directly by PV+ interneurons and indirectly by cholinergic neurons via the SST+/NOS+/NPY+ interneurons.

Most striatal MSNs are silent except for those MSNs that are involved in the initiation of particular movements and related cognitive activity. This global suppression of MSN activity is caused by activation of the cortex and intralaminar nuclei of the thalamus, which, in turn, feed upon the above-described prominent inhibitory systems, the PV^+, $SST^+/NOS^+/NPY^+$, GABAergic, and cholinergic interneurons, respectively. Consequently, any interference with striatal interneuron activity, or with their activation by their cortical and thalamic afferents, is expected to cause aberrant movement initiation, such as dyskinesias and tics.

Unbiased and systematic neuropathological studies of TS revealed a disturbance in number or maturation of regulatory basal ganglia interneurons

Our group reported the first unbiased and systematic neuropathological study of TS in 2005 [8]. It showed a disturbance in number or maturation of PV^+ interneurons using stereology in postmortem basal ganglia tissue from three adult subjects with severe, persistent TS as compared with age- and sex-matched normal controls (NCs). Immunostaining for SP and met-enk, neuropeptides contained within MSN of the direct and indirect pathways, respectively, showed no differences in the apparent density or distribution of MSNs between NC and TS [8].

Consistent with these preliminary results, in a subsequent more comprehensive study involving 5 cases of TS and matched NCs we found a 55.7% decrease in the density of PV^+ interneurons in the striatum of TS individuals (Figure 11.2) [9].

In this study, we also examined two other classes of interneurons, the medium sized CR^+ and the large size $ChAT^+$ cholinergic interneurons, as well as DARPP-32^+ MSNs and cresyl violet staining to assess the total number of neurons. There was no difference in the number of medium-sized CR^+ interneurons (10-20 μm) between NC and TS individuals, whereas the density of large-sized $ChAT^+$ cholinergic interneurons was decreased by 49.4% in the TS striatum (Figure 11. 2) [9]. The density of cresyl violet-stained neurons in the striatum was not significantly different between NC and TS, indicating that this combined decrease in PV^+ and cholinergic interneurons in the striatum of TS patients is not attributable to a generalized neuronal loss. This specificity of changes in PV^+ and cholinergic neuron density was also confirmed using DARPP-32 antibodies, demonstrating no significant change in MSNs.

We analyzed the distribution of cholinergic interneurons in the three main functional subdivisions of the striatum, namely the associative, sensorimotor, and limbic regions, to evaluate physiological implications of the above changes for cholinergic circuitry [9]. The associative region showed the most pronounced decreases in cholinergic neuron density (60.0% decrease, $p < 0.0005$) followed by the sensorimotor region (44.7%

decrease, $p = 0.008$) whereas the limbic region was not significantly different between TS and NC individuals ($p = 0.402$).

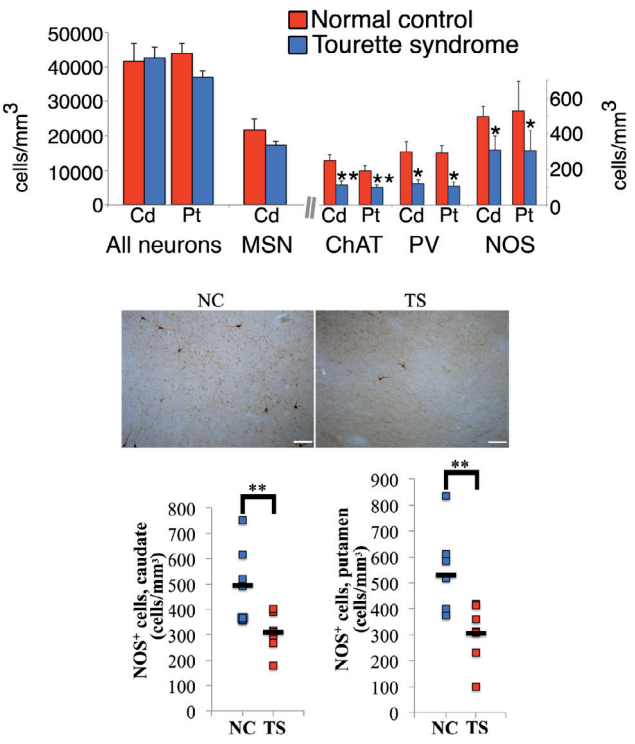

Figure 11.2. Cellular abnormalities In TS. Top, summary of stereological counts in postmortem brain tissue of TS and matched controls (NC). Note different scale of the Y axis for MSN and interneurons. Bottom, representative immunostaining and cell density counts of NOS+ interneurons in the CD. Each dot represents an individual subject. PV= Parvalbumin interneurons; ChAT= choline-acetyltransferase interneurons; NOS= nitric oxide synthase interneurons. For more details see Kataoka et al (2010) and Lennington et al (2016).

The remaining class of GABAergic interneurons, containing SST+/NOS+/NPY+ interneurons, was assessed using NOS immunostaining and stereology. In the Kataoka et al. study there was a strong trend toward a decrease of NOS+ cell densities in the striatum of TS individuals (-45.4 % in the CD, -53.2 % in the PT), but this did not reach a statistically significant difference (p = 0.0556 in the CD, p = 0.0542 in the PT). In a subsequent study [10] we were able to show a significant decrease in this class of interneurons at both the RNA and protein levels (Figure 11.2). This decrease is noteworthy since the SST+/NOS+/NPY+ interneurons are mediating the cholinergic-

induced suppression of striatal activity, and thus act downstream of cholinergic cells in the striatal circuitry [170].

Transcriptome analyses

Transcriptome profiling was carried out in bulk CD and PT tissue of nine TS and NC matched for sex and age [10]. A total of 1,131 genes were found to be differentially expressed between TS and NC, including 309 that were down-regulated and 822 that were up-regulated (FDR<0.05). For the 309 down-regulated genes, the top IPA BioFunctions were related to nervous system development and function, neurological disease, and cell signaling.

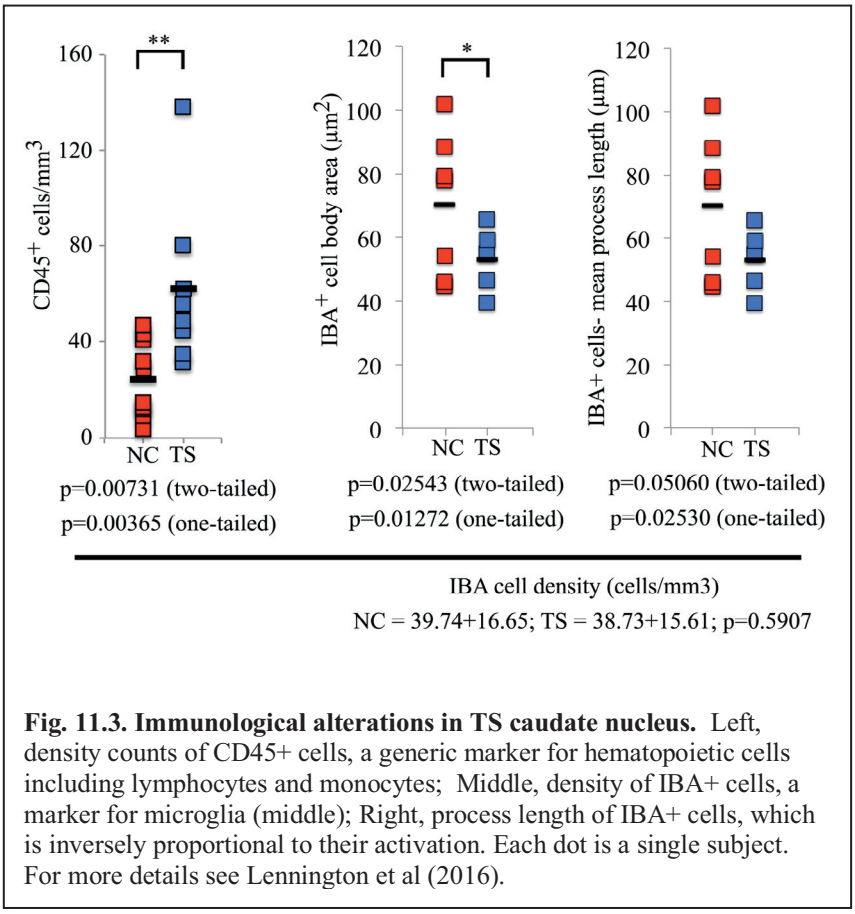

Fig. 11.3. Immunological alterations in TS caudate nucleus. Left, density counts of CD45+ cells, a generic marker for hematopoietic cells including lymphocytes and monocytes; Middle, density of IBA+ cells, a marker for microglia (middle); Right, process length of IBA+ cells, which is inversely proportional to their activation. Each dot is a single subject. For more details see Lennington et al (2016).

These categories included genes involved in G-protein coupled receptors and GABAergic receptor signaling (**Table 1**). Several of the down-regulated genes in TS were involved in neurotransmitter biosynthesis, transport, and function of several classes of basal ganglia interneurons, including ChAT, the vesicular acetylcholine transporter SLC18A3, the choline transporter SLC5A7, and LHX8, a gene critical for forebrain cholinergic neuron development [171, 172] (**Table 2**). Additionally, the data revealed reduced expression of NOS1, NPY, and SST, which together are co-expressed in a class of GABAergic interneurons of the basal ganglia (Table 2). This was paralleled by a decrease in density of NOS immunoreactive cells in both in the CD (-37.6%, p=0.012) and PT (-42.2%, p=0.012) of TS versus NC (Figure 11.2). These results confirmed the decreases in cholinergic and GABAergic interneuron-specific transcripts in the striatum.

Top biofunctions for the up-regulated genes were inflammatory response and immune system-related genes [10] (Table 1). Upregulated genes included major histocompatibility complex class II molecules; immunoglobulin chains; Toll-like receptors; and components of cytokine signaling (several CCL and CCR1,2,3 and 5; CSF1R and CSF3R; several interleukins and their receptors). We also found an increase in several transcripts related to the microglial/macrophage lineage, such as IBA (+2.39, FDR 5.63E-07) and CD68 (+2.03, FDR 6.11E-06). Immunolabeling of IBA in the caudate revealed no change in the density of IBA^+ cells; however, we found

significant decreases in IBA$^+$ cell body size and decrease in IBA$^+$ cell projections, indicative of microglia activation in TS basal ganglia (Figure 11.3) [10]. In addition, cells positive for CD45/PTPRC, a transcript expressed by all differentiated hematopoietic cells including lymphocytes, monocytes, and macrophages, were significantly increased in density in TS (Figure 11.3). Previous analyses have suggested dysregulated immune response in TS, including alteration in TNFa in the blood [57]. All together these changes indicated chronic inflammatory changes, whose cause or cellular origin is presently unknown.

In summary, combined immunocytochemical and transcriptome analyses identified two potential abnormal pathophysiological events in TS: loss/dysfunction of striatal interneurons, and striatal inflammation. However, their relative contribution to disease mechanism and to the clinical manifestations of TS is presently unclear, and it is unknown if they have a reciprocal relationship (i.e., is one a secondary consequence of the other, or are they independent?). For example, striatal inflammation could lead to a host of secondary phenomena including oxidative stress and neuronal death, leading to the observed neuronal loss and the ensuing dysfunction of neuronal activity in the TS striatum. Alternatively, these could represent independent phenomena, as suggested by the lack of a correlation between the two sets of transcripts across subjects [10]. In addition, microglia/immune dysregulation has been observed in postmortem brain tissue of many other neuropsychiatric disorders including schizophrenia and autism spectrum disorders [173-177].

Potential role of the cerebral cortex in tic behavior

In primates, somatosensory cortical areas, encompassing Brodmann areas 1-3 (BA 1-3) and motor/premotor cortical areas (BA 4-6) send projections primarily to the PT, whereas the dorsolateral prefrontal cortex (PFC) projects to the CD [178-181]. There are at least four motor areas in humans, the primary motor (MI), premotor (PM), supplementary motor area (SMA) and cingulate motor areas (CMA) (for a review, see Roland and Zilles, 1996). All these areas are activated bilaterally during the planning and execution of simple and complex movements, with the exception of primary motor cortex (BA4) which is predominantly activated contralaterally. It is thought that while MI is involved in movement execution, the PM is activated by planning movements and in general whenever movements are guided by sensory information, as in tracking or manipulating objects. In contrast, the SMA is activated by the performance of self-paced, over-learned (automatic) motor sequences such as fluent speech as well as by imagining or planning learned sequences; moreover, stimulation of the SMA can arrest movement.

Activity in this distributed sensorimotor circuit and in an additional set of territories was detected by positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) before and during the occurrence of tics (see also Chapter 12). Tic-correlated activity was seen in MI, PM, SMA, rostral part of dorsolateral prefrontal cortex, the Broca area and frontal operculum, anterior cingulate, the insula, claustrum,

putamen and caudate [52, 182-184]. While activity was stronger in TS in some of these regions, it appeared to be weaker in portions of the circuitry that exert negative control over motor pathways, notably the caudate and anterior cingulate cortex; furthermore, less activity in these regions accompanied more severe tic symptoms [184]. The tic-associated activation of the phylogenetically older insular cortex, which is involved in the integration of internal motivational states with executive functions, has been hypothesized to contribute to the uninhibited behavior of TS [52, 182]. Increased excitability of primary motor cortical areas has also been detected in a transcranical magnetic stimulation study of TS individuals [113] (See also Chapter 11). Abnormal excitability of these cortical regions could be attributed to decreased intracortical inhibition or to increased excitability of thalamic afferents. Together, these studies suggest that increased activity in motor, somatosensory and paralimbic areas may be involved in tic generation and may account for the irresistible urge that accompanies tic behavior, whereas lower activity in prefrontal circuits subserving executive functions may result in poor tic control. A neurochemical evaluation of frontal cortical regions from post-mortem tissue in 3 cases of TS have revealed neurotransmitter system dysfunctions in TS, notably dopamine, dopamine type 1 and 2 receptors, dopamine transporter, adenosine type 2 receptor and vesicular monoamine transporter [185, 186]. However, given the low number of subjects, the significance of these findings for TS remains to be established.

Stern et al (2000) suggested the intriguing hypothesis that “tics may represent a paradoxical state in which brain regions …normally associated with a subjective experience of volition as they initiate action, are not operating under the volitional control of the patient.” Although the neural substrate for the subjective experience of ‘lack of control’ experienced by the patients is unclear, a reasonable hypothesis is that there is an abnormal integration between premotor and other brain regions in TS, particularly the prefrontal cortex (PFC), a heterogeneous set of areas that are important for the voluntary control of motor routines [187, 188]. Dorsal PFC areas respond to temporal contingencies, evoke memories and emotions, monitor errors, and correct motor sequences (executive functions). This is consistent with the strong engagement of wide regions of the PFC during tic suppression [110], and with observations that increased alpha frequency band coherence (a measure of functional connectivity) was detected between prefrontal and sensorimotor areas in patients with TS in connection with the successful inhibition of unwanted movements [189].

To assess whether the decrease in PV^+ cells was specific to the striatum, we estimated their density in the insular and cingulate regions of the cerebral cortex, two areas that are prominently activated during tic behavior and are implicated in TS. We found a trend toward a decrease in the insular cortex, but overall no statistically significant decrease (not shown). The low number of samples again precludes a definite conclusion; however, we note that in contrast to a clearly visible trend in the insular cortex, no such trend was observed in the cingulate cortex. These data suggest the intriguing concept

that TS individuals might exhibit differences in the number of inhibitory cortical interneurons in specific regions of the cortex. This is consistent with several transcranial magnetic stimulation (TMS) studies in which TS individuals have shown reduced short-interval cortical inhibition (SICI), a paradigm which assesses the functional recruitment of intracortical interneurons (see Chapter 12). Future analyses involving these as well as other cortical regions in a larger number of samples are required to test this possibility.

Functional Implications

The neuroimaging analyses, our postmortem brain analyses, and the neurophysiology of the thalamo-cortical-striatal feed-forward circuit, all implicate the basal ganglia and particularly the PV and cholinergic neurons in the pathophysiology of tic behavior.

Cortico-striatal feed-forward inhibition and PV$^+$ GABAergic neurons.

Neurophysiological studies in striatal slices and whole-animal recording support the idea that PV+ striatal interneurons exert a strong “no go” inhibitory influence upon motor activity in response to cortical activation. PV$^+$ cells receive direct, strong cortical synaptic input [33] and respond with shorter latency than MSNs to cortical stimulation driving powerful feed-forward inhibition throughout the striatum [36, 190] (Figure 10.1). PV$^+$ cells also drive the synchronous oscillations in membrane potential and electrical activity involving the cortex and striatum [191]. However, behavioral evidence for their implication in TS-related behaviors is still lacking. These cells are crucial for overall

regulation of excitatory activity in multiple regions of the CNS, and thus animals with widespread loss of PV^+ neuron activity develop seizures, confounding the overall picture.

Developing mice with selective PV^+ neuron inactivation or loss is a promising strategy that may eventually yield insights into the roles of PV^+ neurons in the basal ganglia and in TS. For example, the dt(sz) hamster, harboring an autosomal recessive genetic mutation which results in episodes of dystonia and choreoathetosis, develops paroxysmal dystonia at 30-40 days of life, concomitant to an increase in the activity of MSNs which has been hypothesized to be based on a deficit of striatal (but not cortical) PV^+ interneurons [192]. Interestingly, there is a spontaneous remission of paroxysmal dystonia in older dt(sz) hamsters, coinciding with a normalization of the density and increased arborization of striatal PV^+ cells [193]. The data suggest that abnormal maturation or survival of striatal PV^+ interneurons plays a critical role in the development of paroxysmal dystonia, but interestingly, this can be reversed later in life. While the data are still inconclusive given the possibility of other intervening factors in these mutants, a recent report suggests that a novel compound, selectively inhibiting the firing of fast-spiking PV^+ neurons infused into the sensorimotor striatum, induces dystonic-like movements in mice [37]. Together with the physiological evidence, these animal models strongly suggest that a dysfunction of PV^+ cells may cause a hyperkinetic movement disorders and that these cells represent a therapeutic target for TS. In general, these

findings support the conclusion that PV cells are essential to avoid reverberant activity and obtain selective activation of MSNs in the striatal neuronal network.

Thalamo-striatal feed-forward inhibition and ChAT neurons

Cholinergic neurons of the striatum are activated by prominent projections from VA/VL and centromedian-parafascicular (intralaminar) complex of the thalamus [194, 195] (Figure 10.1). The VA/VL thalamic nuclei process sensorimotor information and, in turn, receive inhibitory projections from the GPi [196, 197]. GPi projection neurons exhibit high frequency tonic and oscillatory activity. Cholinergic neurons are also the main target of thalamo-striatal afferent fibers originating from the intralaminar nuclei, which are the rostral component of the reticular activating system [198]. Thus, cholinergic neurons receive information about sensory stimulation and ongoing activity as well as the arousal state of the animal. In turn, cholinergic interneurons regulate the firing of the PV interneurons acting through nicotinic and muscarinic receptors [39] and also excite SST/NOS/NPY interneurons through nicotinic receptors. Excitation of these inhibitory striatal interneurons results in a secondary prolonged GABA-mediated inhibition of MSNs [170, 199, 200]. Other actions that have been attributed to cholinergic cells are a transient presynaptic inhibition of corticostriatal terminals impinging on MSNs of both pathways, and a direct facilitation of activity of MSNs of the indirect pathway via postsynaptic M1 class muscarinic receptors [199-201]. Hence, cholinergic interneurons exert complex actions on the striatal circuitry and their role is not completely understood. It would be interesting to know whether the inhibition of MSNs affects

preferentially those of the *direct pathway*; if true, an inhibition of the direct pathway together with a facilitation of the *indirect pathway* would profoundly excite the GPi/SNr output neurons and thus inhibit motor behavior. Based on the findings of the reduction of cholinergic interneurons in postmortem human striata [9, 10], Xu et al [202] developed a mouse model studying the effect of 50% reduction of $ChAT^+$ interneurons, induced by diphtheria toxin, in the dorso-lateral striatum (DLS, corresponding to the human putamen) and dorso-medial striatum (DMS). The reduction of $ChAT^+$ interneurons neither impacted GABAergic cells nor PV^+ interneurons. However, after acoustic startle stimuli, the DLS, but not DMS injected mice displayed tic-like stereotypy, which was not observed at baseline. Mice with $ChAT^+$ neuron deficiency in the DLS performed significantly worse in a motor learning rotorod behavioral paradigm, but drastically improved over time. D-amphetamine administration caused enhanced locomotion in $ChAT^+$ interneuron deficient mice as well as in the control group. Interestingly, only mice with a loss of $ChAT^+$ cells in the DLS displayed repetitive grooming behavior. Although these data imply a role of cholinergic neurons in the DLS in tic-like behavior in mice, it is still unclear how abnormalities in other cell types and observed inflammation contribute to the development of TS and cause an imbalance of the corticostriatal circuitry.

Possible Causes

Given the retrospective nature of our data, it is impossible to discern whether the observed changes in inhibitory and cholinergic interneurons are causally related to TS,

represent long term consequences of a remote pathophysiological event, or represent a secondary effect totally unrelated to the mechanism of disease. However the defects in interneuron number is likely to compromise the overall ability of the striatum to organize ongoing behavior. The pattern of activation of striatal cholinergic and PV interneurons by cortical and thalamic afferents, respectively, may have an important role in the suppression of unwanted motor activity. For example, as discussed above, cholinergic cells within the striatum receive a “copy” of corticothalamic projections conveying sensorimotor information to the cerebral cortex, therefore a decrease in cholinergic neuron number will inevitably decrease the integration of multimodal information within the striatum. We hypothesize that a decrease in integrative ability may create a “disconnect” that facilitates tic behavior. This is dramatically demonstrated by neurosurgical experiments in which patients with intractable TS found partial relief from their symptoms after lesions of the intralaminar thalamic nuclei, or, more recently, when these nuclei were implanted with deep brain stimulation (DBS) electrodes [203]. As discussed above, the decrease in inhibitory and cholinergic striatal interneurons is likely to be attributable to a loss of cells rather than a downregulation in levels of specific transcripts or proteins. Potential causes of the striatal interneuron loss include developmental misspecification of neurons, or toxic, degenerative, and inflammatory conditions affecting the survival of these cells in the postnatal period.

Developmental defects

Inhibitory interneurons are generated within the basal telencephalon during embryonic development. This region encompasses three ganglionic eminences, the medial, lateral, and caudal eminence (MGE, LGE, and CGE, respectively). Cholinergic neurons are generated in the preoptic area, a more medial and caudal region adjacent to the MGE. Neurons generated in these ventral regions migrate widely to the cerebral cortex and basal ganglia, in addition to the olfactory bulbs, amygdala, and hippocampus to reach their final destinations by the first postnatal week in rodents [1, 204, 205]. In humans, a portion of the cortical GABAergic interneurons might have a dorsal cortical origin, in addition to the common derivation from the basal telencephalon found in lower mammalian species [206].

Genetic programs directed by a series of divergent homeobox genes, including *Nkx2.1*, *Dlx1,2,5,6*, *Gbx1,2* and *Lhx6* control the specification, differentiation and migration of GABAergic neurons [207]. Ventral GABAergic fate is also instructed in part by the basic helix-loop-helix gene *Mash1* (Long et al., 2009). This cascade is induced early in embryogenesis in ventral regions of the telencephalon by Sonic hedgehog (*Shh*), a secreted morphogen emanating from the notochord [208]. The homeobox gene *Nkx2.1*, the earliest gene required for interneuron specification, is an immediate downstream target of *Shh* signaling in the prospective MGE [209]. *Nkx2.1* is an essential fate determinant, supported by the findings showing that mice lacking *Nkx2.1* do not form the MGE or later the globus pallidus, lack basal forebrain cholinergic neurons, and have reduced numbers of cortical GABAergic cells, including PV and SST neurons, that

migrate from the MGE into the cortex. Interestingly, haploinsufficiency of *Nkx2.1* (also called TTF1) in humans has been reported in association with a dystonic movement disorder [210]. *Dlx* genes, which largely serve as ventral inhibitory interneuron progenitors, are downstream of *Nkx2.1*, expressed widely within both the LGE and the MGE, and are essential for several aspects of interneuron and subpallial development. For example, *Dlx5* and *Dlx6* are required for the migration, maturation, and function of PV cortical interneurons [211], whereas *Dlx1* is required for CR, SST, and NPY-positive interneurons [212]. All *Dlx* mutants have disrupted striatal differentiation and epilepsy (Long et al., 2009). In the absence of *Dlx1&2*, there is an over-expression of *Mash1*, suggesting its compensatory ability in restoring aspects of striatal development and the potential for *Mash1* signaling to be parallel, overlapping and/or redundant to that of *Dlx*. The analyses of triple mutant mouse (*Dlx1&2*$^{-/-}$;*Mash1*$^{-/-}$*)* reveal their combined function in promoting complete striatal differentiation and establishing distinct dorsoventral domains (Long et al., 2009). It is interesting to note that there has been some debate around the initiation of *Dlx/Mash1* signaling in the ventral telencephalon, and whether or not *Shh* is truly the triggering molecule for their signaling cascade. This debate stems in part from evidence revealing that while *Fgf8* hypomorphs show a complete loss of both *Nkx2.1* and *Shh* expression, *Dlx* expression persists (Storm et al., 2006). Further work untethering the complexities of these dynamic signaling pathways and their control over whole regional development is necessary to further define disorders compromising the basal ganglia, like TS.

While *Nkx2.1* and *Dlx* genes mostly control MGE and cortical interneuron development, the homeobox genes *Gsh1* and *Gsh2* are required for the development of striatal interneurons by specifying LGE identity [213, 214]. Accordingly, *Gsh1* and *Gsh2* double mutant mice have severe hypoplasia of the striatum. In addition, Gsh genes are required for the development of olfactory bulb interneurons and telencephalic dopaminergic neurons [214]. In contrast, the LIM-homeobox gene *Lhx8* is required for the development of cholinergic interneurons; *Lhx8* mutants lack the nucleus basalis, a major source of the cholinergic input to the cerebral cortex, and exhibit reduced number of cholinergic neurons in several other areas of the subcortical telencephalon including the caudate-putamen, medial septal nucleus, nucleus of the diagonal band and magnocellular preoptic nucleus [171]. Interestingly, *Lhx8* expression is reduced in the basal ganglia of TS individuals (see section 2.4), suggesting a possible developmental origin for the cholinergic defects.

Degenerative and neuroinflammatory conditions

The role of neuroinflammation in TS is not fully understood (see also Chapters 9 and 14). In the normal brain, in addition to contemporary immune infiltration there are resident macrophages that enter the brain during embryonic development, and remain in a resting form until activated during infection or inflammation events [215-217] (for review see Engelhardt and Coisne, 2011; Saijo and Glass, 2011). Immune cells may also play a critical role in cell clearance and synaptic pruning during normal brain development [218]. Prenatal exposure to pathogens, and the resulting maternal immune

activation (MIA) during pregnancy, has been linked to increased risk for ASD and schizophrenia [219, 220]. It is unknown if *in utero* infections increase the risk of developing TS.

In TS there is some evidence that the immune system can trigger an autoimmune reaction involving the basal ganglia, a condition called pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection (PANDAS). The PANDAS traditionally stemmed from the hypothesis that an infection with Group A β-hemolytic streptococcus (GABHS) [63, 221] can trigger TS in childhood (for a review of the autoimmune hypothesis of TS, see Chapter 15). In support for such a hypothesis, TS individuals have displayed indices of immune activation, including increased anti-neuronal and anti-nuclear antibodies [51, 222], decreased regulatory T cells [58], increased IL-12 and TNFα serum levels, that were also found to positively correlate with tic severity [57, 223] as well as increased IL-2 and monocyte chemotactic factor -1 (MCP-1), a marker of chronic inflammation, in brain tissue [224]. These findings, however, have not always been replicated and remain controversial. Furthermore, there was a striking increase in the number of activated B-lymphocytes among a group of unselected adult patients with TS compared with healthy subjects [225]. Finally, there is a significant increase in maternal self-reported history of autoimmune disorders in TS/OCD, and in a subset of PANDAS subjects, compared to the general population [221]; and allergic comorbid symptoms were described to be significantly stronger in

patients with a dual diagnosis of tic disorders and ADHD, than in patients suffering from only one of those disorders [226].

Brain transcriptome analysis revealed that expression of genes involved in several immune system pathways are upregulated in TS subjects, including genes involved in inflammatory response and immune cell trafficking [10]. Some of the immune pathway-associated genes found to be upregulated in the brain of TS individuals have previously been found to be upregulated in the blood of these patients [227, 228], suggesting that changes in lymphocytes as well as myeloid-derived cells (monocytes/macrophages/microglia) may occur in this disorder. Interestingly, upregulation of several immunoglobulin genes were detected in the brain transcriptome of TS individuals, which agrees with evidence of abnormal oligoclonal bands of IgG in the cerebrospinal fluid of TS patients, suggestive of increased intrathecal IgG production [229].

How might these findings interface with the neuropathological findings? It is possible that the loss of interneurons in TS may be secondary to neuroinflammation. If aberrant immune cell access to the brain is causal in TS, the noted decreases in PV^+ and $ChAT^+$ interneurons may be resulting from immune cell reactivity; however, such auto-immune–mediated cell loss is in contrast with the absence of increased loss of interneurons with age. Alternatively, the immune system may be acting in a stereotyped way, following some causal trigger that leads to a cascade of cues and ultimately to increased immune

cell adhesion and infiltration into the striatum. Moving forward, the rapid onset of TS symptoms following immunological challenge in PANDAS cases provides an interesting subset of subjects in which to further explore the role of the immune system in TS.

Therapeutic implications

Discovering the neurophysiological substrate for tics and other symptoms of TS is of paramount importance for treatment. Even in the case of developmental alterations, where the causes are likely to be remote, there is still hope that we could find treatments that compensate for brain circuitry dysregulation. In the case of cell losses or dysfunctions induced by immunological alterations, potential treatments could be tried similar to those employed in autoimmune disorders of the CNS. Finally, comparable to what has been successfully tried in other basal ganglia disorders such as Parkinson's disease, neurotransmitter or cellular replacement therapy (locally delivered) represents a difficult but open area for therapeutics. It has been recently demonstrated in mouse models of seizures that transplantation of GABAergic interneuron precursors in the early postnatal mouse cortex results in diffuse cortical engraftment of exogenous GABAergic cells, and reversal of epileptic symptoms [230, 231].

BOX 1. Key points

- In TS there are decreases in parvalbumin+ GABAergic interneurons and cholinergic interneurons in the CD and PT.

- There is also loss of the normal distribution (highest in associative regions, intermediate in sensorimotor, and lowest in limbic regions) of cholinergic interneurons.
- There are no evident changes in ChAT distribution in striosomes vs matrix.
- There may also be changes in another interneuron population, the NOS-containing interneurons.

BOX 2. Questions for future research

- Developing mice with selective PV or cholinergic neuron inactivation or loss in the basal ganglia may allow studying direct and indirect behavioral and physiological consequences of this manipulation and eventually yield insights into the roles of these interneurons in the basal ganglia and in TS.
- Regarding the neuropathological changes that have been noted based on post-mortem tissue assays (western blot, qPCR, RNAseq), homogenization of tissue was done to isolate the RNA or protein. Future analysis of the candidate targets, e.g., immune system related genes, will help to determine if there are specific cells or regions where these markers are differentially expressed. Studying spatial gene expression in postmortem brain tissue slices was developed as a powerful tool to understand the impact and interconnections of transcriptional changes within and/or between brain regions. This strategy enables to study the transcriptome on a single cell level without losing the valuable information of exact location. This will ultimately reveal brain circuitry that is abnormal in this disorder.

- Three-dimensional cell structures, called organoids, enable to study early timepoints of human development *in vitro*. Using TS patient derived iPSCs to generate organoids mimicking the cortico-striatal circuitry will be crucial to understand the underlying cause of TS.

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Table 1.

DOWN-REGULATED			
Top IPA Biofunctions		**p-Value**	**# Molecules**
Nervous System Development & Function	neurotransmission	1.65E-23	40
	synaptic transmission	1.22E-21	35
Behavior	behavior	2.70E-22	57
	learning	3.08E-19	34
	anxiety	2.11E-18	35
Neurological Disease	Schizophrenia	1.22E-15	36
	Psychosis	2.25E-12	14
Top IPA Canonical Pathways		**-log(p-value)**	
cAMP-mediated signaling		4.96E00	
G-Protein Coupled Receptor Signaling		4.95E00	
GABA Receptor Signaling		3.65E00	
Top IPA Upstream Regulators		**p-value of overlap**	
NGF	growth factor	1.01E-12	
HTT	transcription regulator	9.19E-12	
BDNF	growth factor	4.93E-10	

UP-REGULATED			
Top IPA Biofunctions		**p-Value**	**# Molecules**
Cell-To-Cell Signaling & Interaction	activation of cells	9.18E-77	193
	activation of blood cells	1.48E-74	166
Hematological System Development & Function	quantity of blood cells	1.71E-74	199
	quantity of leukocytes	6.22E-71	182
Immune Cell Trafficking	leukocyte migration	7.85E-67	170
Inflammatory Response	activation of leukocytes	3.02E-68	150
	inflammatory response	5.27E-61	148
IPA Canonical Pathways		**-log(p-value)**	
Granulocyte Adhesion and Diapedesis		2.13E01	
T Helper Cell Differentiation		2.03E01	
Dendritic Cell Maturation		1.8E01	
IPA Upstream Regulators		**p-value of overlap**	
lipopolysaccharide	chemical drug	3.14E-87	
IFNG	cytokine	1.71E-78	
TNF	cytokine	1.32E-65	

Table 2. Hand-curated subset of differentially expressed genes. Genes are listed under the category they represent, along with log2 fold change (log2 FC) and FDR corrected p-values (FDR)

Cholinergic		**FC**	**FDR**
CHAT	choline O-acetyltransferase	-2.31	1.39E-07
CHRM2	cholinergic receptor, muscarinic 2	-1.99	4.32E-04
SLC18A3	solute carrier family 18 (vesicular acetylcholine), member 3	-2.17	4.04E-05
SLC5A7	CHT1; solute carrier family 5 (choline transporter), member 7	-2.55	6.65E-08
LHX8	LIM homeobox 8	-1.90	1.39E-03
NTRK1	neurotrophic tyrosine kinase, receptor, type 1	-1.91	1.17E-03
GABAergic		**log2 FC**	**FDR**
GAD1	glutamate decarboxylase 1 (brain, 67kDa)	-1.64	1.21E-02
GABRA1	gamma-aminobutyric acid (GABA) A receptor, alpha 1	-1.47	2.31E-02
GABRA3	gamma-aminobutyric acid (GABA) A receptor, alpha 3	-1.96	5.85E-04
GABRG2	gamma-aminobutyric acid (GABA) A receptor, gamma 2	-1.50	2.61E-02
GABRQ	gamma-aminobutyric acid (GABA) A receptor, theta	-1.82	1.11E-02
NOS1	nitric oxide synthase 1 (neuronal)	-1.58	6.40E-03
NPY	neuropeptide Y	-1.66	8.15E-03
NPY2R	neuropeptide Y receptor Y2	-2.81	2.33E-04
SST	somatostatin	-1.59	4.42E-03
Dopaminergic		**log2 FC**	**FDR**
DRD1	dopamine receptor D1	-1.50	3.20E-02
DRD5	dopamine receptor D5	-2.10	2.47E-03

Chapter Two:

SHH patterns human stem cells towards ventral forebrain fates

Introduction: modeling of CNS development and disease in vivo and in vitro

Development of the human central nervous system (CNS) is a complex series of sequential molecular events that guide cellular proliferation, differentiation, and migratory patterns to build the organization of what are the most advanced neural circuits in the animal kingdom. These processes generate a vast cellular diversity integrated into networks that ultimately produce higher-order human functions, from cognition and motor coordination to consciousness.

The inherent complexity of the human CNS has historically complicated scientists' ability to model and understand all of its parts, thereby making the study of developmental and degenerative abnormalities equally challenging. While animal models have long served as tools to uncover human neurobiology, their limitations have continued to emphasize the need for a reliable human model system. For instance, the anatomical and cellular differences across species impact the degree to which healthy and disease pathophysiology can be convincingly captured. Due to the essential role that the basal ganglia plays in both learning [232, 233] and motor processes [234], as well as its implication in a broad range of neuropsychiatric disorders, this subpallial region and its circuitry has been investigated in many animal models, from rodents to primates. While general cortico-basal ganglia regional architecture is maintained across rodents and humans, the globus pallidus pars interna, termed entopeduncular nucleus in rodents, and the frontal cortex are both comparatively immature structures in rodents [235]. Basal ganglia output also varies across humans and rodents, with extensive output to the

thalamus versus midbrain and brainstem regions, respectively [236]. Further, certain cell types, such as outer radial glia of the dorsal telencephalon, that are essential to human cortical development are substantially lacking in rodents [237]. Additionally, disease modeling in animals often requires genetic engineering to mimic human dysfunction that does not naturally occur, and leads to animal behaviors that are sometimes difficult to characterize in the context of human phenotypes. Higher order processing achieved by the human brain also suggests the possibility for equally complex and intricate abnormalities, and yet another reason why a human-specific model system has become an empirical necessity.

Human induced pluripotent stem cell (iPSC)-derived organoids are tridimensional multilayered aggregates with the intrinsic capability to capture human embryonic development. For this reason, organoids present a tremendous opportunity to model both human development and disease to further not only our biological understanding but also our clinically relevant investigations, from diagnosis to therapeutics. Human iPSC technology came during a time of great ethical distress over the use of embryonic stem cells, which gained empirical traction after the use of murine pluripotent cell lines [238, 239]. Instead of sacrificing a human embryo, scientists found that pluripotent stem cells could be generated from the initial harvest of a simple adult human fibroblast. The exposure of fibroblasts to a cocktail of reprogramming factors reverts their development back to pluripotency, from which point the cells can be differentiated into any cell of the human body [240, 241]. Organoids take the lead as the model system with the greatest potential to resemble the complexity of human development, and the iPSCs from which these aggregates are derived endow the system with the ability to develop into all 3 germ layers – the endoderm, mesoderm or ectoderm. For the scope of this review, however, we will focus on ectoderm-derived neural organoids. Another defining feature of the organoid system that elevates its potential beyond animal models is its subject-specific design. From a simple

non-invasive skin biopsy [241], or more recently urine [242, 243] or blood samples [244-246], the patient's cells are reprogrammed to iPSCs and cultured to become neural organoids while maintaining the patient's specific genetic background. This subject-specific nature carries the potential to reveal brain cytoarchitecture that resembles that of the individual from which the cells originally came. This opens another door of possibility, especially when considering disease modeling and medical treatment plans, paving a way towards precision medicine. An additional technical benefit is that, once reprogrammed, the iPSCs can be cultured into perpetuity, essentially creating an immortal system that can generate an unlimited reserve of cells.

At the same time, there are limitations of the organoid system to consider, as well. Among them, the struggle of organoid reproducibility has sparked substantial efforts towards protocol optimization; similarly, variability of cellular expression can be observed across preparations – both realities that raise questions about system efficiency. For additional detailed information on these limitations, please refer to the cited reviews [247, 248]. While organoid cultures can be maintained long-term for several months, achieving widespread organoid health for the same potential lifespan has been equally challenging. Further, some critical complexities of neurodevelopment have yet to be distinguished in the organoid cultures – including, but not limited to, vascularization of the tissue, and organization of all six human cortical layers, which is typically guided by the meninges (for reviews on these limitations, REF [249-252]) Given that the organoids capture embryonic-stage development best, deciphering whether organoids are applicable to both neurodevelopmental and neurodegenerative disorders is an ongoing investigation. Although the organoid system is still young and developing, it has vast potential and its methodology is ever-evolving.

Culturing human neural organoids begins first with the single layered iPSCs. The cells are placed in a physical environment that fosters their assembly into a tridimensional aggregate. Guided by a set of morphogens, the aggregates become embryoid bodies – named for their ability to become any of the 3 germ layers. However, with further culturing, these aggregates can be pushed to adopt a neural fate and express a diverse array of progenitors mimicking early regional patterning of brain tissue [253]. Once the regional identities are specified, withdrawal of mitotic factors from the media fosters post-mitotic differentiation of various cell types that reside within said regions. These aggregates are self-organizing and their morphological growth occurs spontaneously. However, unmodified organoid patterning only goes so far. Studies have found that neural aggregates inherently adopt a cortical (dorsoanterior) telencephalic fate by default [11-14], similar to the developing cerebral cortex. We can capture a broader range of human anatomy, however, by introducing critical factors that endogenously pattern the CNS into various regions.

SHH signaling during embryogenesis

The basal ganglia arise from the ventral telencephalon, an anterior extension of ventral neuraxis, which is patterned by the Sonic Hedgehog (SHH) signaling pathway In normal vertebrate development. This dynamic protein serves a multitude of functions in a context-dependent manner, both as a mitogen to promote cellular proliferation, and a morphogen to direct cellular and regional specification towards ventral fates [254-257]. Embryological investigation has exposed many of the multifaceted roles that SHH plays in development, adding insight both empirically and clinically due to the disorders observed with deficits in SHH expression [255, 258-260]. The most notable role for this dynamic morphogen is in neural patterning of the central nervous system.

Before we focus on the varying ways that SHH has been used to pattern stem cells, the command that this ligand has on instructing neural diversity and cellular organization must first be understood. One of the first SHH-expressing tissues during mammalian embryogenesis is the notochord, which begins its developmental influence in early neural plate stages and crescendos upon neural tube formation **[FIGURE 1.2]** [261-263]. SHH emanates from the notochord, and its role as a chief ventralizing signal has been originally discovered from transplantation experiments in chick embryos [264-266] and *in vitro* experiments with neural plate tissue explants [262, 263, 267]; these works were further complemented by genetic experimentation for both gain- [267, 268] and loss-of-function [258] SHH signaling. The notochord has immediate contact with the floor plate at the ventral midline of the neural tube, and induces these nearby cells to express SHH **[FIGURE 1.2B]** [269]. The capacity of SHH to program a variety of cellular identities is explicated in a graded fashion, as indicated by *in vitro* studies **[FIGURE 1.2B]** [270]. The cells of the floor plate secrete SHH and establish a concentration gradient within the CNS with the most elevated protein levels found ventrally; the degree to which different cell types emerge, therefore, is concentration-dependent. There is a unique 'release and response' duality of the neural tube that is essential for determining the positioning of cell types along the main coordinates of the neuraxis, whereby a proportion of neural tube cells, found ventrally, express and secrete SHH, while another large portion of neural tube cells respond to the ligand in a concentration-dependent fashion. The SHH concentration gradient ultimately governs the patterning of the central nervous system along the dorsoventral domains. SHH has antagonistic interactions with the dorsally-expressed bone morphogenic proteins (BMP) and WNT signaling proteins which will fully define the limits of the range of each domain. Herein lies the initial guide to the central nervous system's rise to cellular diversity.

Cell types requiring SHH signaling in vertebrate development and their role in diseases of the CNS

Along the rostrocaudal axis, SHH, in conjunction with other morphogens, induces cell types at in ventral domains at various levels in the forebrain, midbrain, hindbrain and spinal cord **[FIGURE 1.2A,C]**. In the forebrain, SHH promotes the differentiation of the ventral telencephalic ganglionic eminences, which contribute to adult basal ganglia formation [271]. In addition, the ventral telencephalic ganglionic eminences are the site of origin of essentially all telencephalic inhibitory interneurons [272]. Therefore, SHH is essential for kick-starting the cascade of signaling that leads to the development of many classes of inhibitory interneurons that eventually populate the forebrain, modulating excitatory neurotransmission and are integral components of an optimally functioning cortico-basal ganglia circuitry that directs motor coordination and cognition. Additionally, SHH governs dopaminergic and serotonergic neuronal fates in the midbrain and hindbrain regions, essential for motor and emotional control, respectively [264, 273-275]. Ye and colleagues showed that achieving such neuronal diversity is directed by the distinct combination of molecular positional cues, assembled in an informational "grid" system of morphogens [275-277]. The developmental grid system of morphogens and fate determinants, first described by Wolpert [276] and further defined by Rubenstein and colleagues [277], organizes regional neural patterning and cell fate essentially creating a topography. Extracellular molecules determine the grid and serve as signaling centers during development. As cells mature, cell type specificity is acquired based on the cells' precise location within this developmental grid. Ye et al further investigated this grid system concept in rat ventral midbrain and hindbrain explants [275]. By manipulating exposure to SHH and FGF8, blocking functionality of the morphogens, and controlling timing of morphogen exposure, Ye and colleagues showed that dopaminergic induction and neuronal commitment throughout the

midbrain and forebrain required signaling from both SHH and FGF8. More caudally, introduction of FGF4 signaling pre-patterns the rostral hindbrain, such that subsequent signaling of FGF8 and SHH pushes progenitors to adopt a serotonergic fate instead of dopaminergic [275]. Extending caudally from the hindbrain to the spinal cord, SHH promotes the differentiation of motor neurons and ventral interneurons. Later in development, SHH plays an essential role in oligodendrocyte development at many levels of the neuraxis [278]. These cell types make myelin which is critical for the speed of neural signaling and integration of sensory information [270, 279, 280].

Understanding the neurodevelopment of these important cellular lineages provides the necessary foundation from which we can decipher perturbations that yield neurological disorders, of both a neurodevelopmental and neurodegenerative nature. For instance, altered development of dopamine neurons, originating in the substantia nigra of the midbrain and projecting to the basal ganglia of the ventral forebrain, has been linked to both movement and neuropsychiatric disorders, from Tourette Syndrome [281, 282] to Schizophrenia [283-285]. Additionally, dysfunction and fluctuations in serotonergic neuron numbers, originating in the hindbrain but whose axonal projections innervate areas throughout the entire brain, are directly implicated in many neuropsychiatric disorders such as anxiety and depression [286-288]. In the forebrain, abnormal development of the striatum in the basal ganglia contributes to other movement and psychiatric disorders, from Huntington's Disease to Obsessive Compulsive Disorder (OCD).

Elucidating the SHH signaling pathway paves the way for application in *in vitro* patterning

The fundamental role of SHH and other morphogens, revealed by gain-of-function and loss-of-function studies, has paved the way for scientists to try to identify these complex processes in human models [254, 289, 290]. Pioneering genetic studies in *Drosophila* identified several key genes that are integral components of SHH signaling [291], most notably Patched, Smoothened, and three separate Gli genes (*cubitus interruptus* genes in Drosophila). Patched (ptc) and Smoothened (smo) are both transmembrane protein receptors, but mediate SHH signaling in opposing ways, in that ptc inhibits SHH signaling while smo activates it [292, 293]. In the absence of SHH, ptc binds smo and constitutively inactivates it, preventing signaling of the pathway. However, in the presence of SHH, the ligand binds ptc, which alters the receptor's conformation; ptc then becomes physically untethered from smo, relieving its inhibition such that the SHH pathway can become active [294] **[FIGURE 2.2]**. Further examination of ptc and smo in vertebrates has suggested their similarly important roles in mediating SHH signaling to pattern the neural tube [292, 293].

As will be discussed in the next section, the SHH signaling pathway has been exploited as a vital component of many novel human *in vitro* strategies to uncover the neurobiology of developmental disorders and to discover novel therapeutic technology. The remainder of this review will discuss how the community has utilized SHH as a tool to promote neuronal differentiation into specific neuron types and regional development, and the biological and clinical applications of these distinct methodologies. We will briefly discuss the use of SHH in various monolayer stem cell preparations and their limitations before discussing its use and potential in the iPSC-derived organoid system.

The use of SHH in *in vitro* disease modeling

To explore a potential cellular repair for Parkinson's disease [295]. Kriks and colleagues used joint molecular activation of SHH and canonical WNT signaling to enhance specification of human embryonic stem cells (hESCs) towards a dopaminergic neuron fate. They achieved engraftable hESC-derived midbrain dopaminergic neurons that thrived long-term in the striatum of three different Parkinsonian animal model systems including 6-hydroxy-dopamine-ablated mice and rats and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned rhesus monkeys. Although the behavioral recovery was limited, the survival span of the graft was 1 month, and led to robustly developed fibers positive for tyrosine hydroxylase, an enzyme required for dopamine synthesis.

Another study revealed successful generation of GABAergic medium spiny neurons (MSN) from hESCs exposed solely to SHH treatment, and investigated the potential for cell therapy as a treatment for Huntington's Disease [296]. Ma and colleagues found that treatment of hESCs with a specific concentration of SHH was sufficient to foster lateral ganglionic eminence (LGE)-like development in monolayer neuronal cultures and ultimately MSN development after extended culture. Beyond gene expression, the SHH-treated hESC cultures produced the appropriate striatal MSN morphology, sprouting dendrites with spines, and the differentiated neurons displayed functional properties in the form of spontaneous synaptic activity as well as action potentials subsequent to current injection. Transplantation of these LGE-like progenitors into mice with lesioned striatal tissue confirmed the progenitors' ability to mature and differentiate into GABAergic DARPP32-positive neurons, with concomitant recovery of volume loss and cellular concentration of the lesioned striatum as early as four months-post-transplant.

While Parkinsonian symptoms can be ameliorated by sufficient replenishment of dopamine stores, the complexity of circuitry restoration remains a barrier for developing Huntington's Disease treatments. Long range projections, characteristic of medium spiny neurons, are required to innervate various target regions of both the basal ganglia and cerebral cortex to regulate the dynamic motor circuits. Both distance and continued cellular access serve as considerable obstacles that the in vitro-generated neurons have yet to overcome. That being said, these findings convey the powerful control SHH has alone to pattern developing neurons to a quasi-mature and functional extent in an animal model.

Another study found that prolonged long-term exposure of iPSCs to SHH could ultimately yield motor neurons able to form neuromuscular connections upon co-culturing with myotubes [297]. Du and colleagues used iPSC-derived SHH-induced motor neurons to model disorders plaguing specifically motor neuron populations, including spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS). Du et al capitalized on the patient-specific nature of the iPSC technology and compared patient-derived motor neurons to healthy controls. The expression of the survival motor neuron (SMN) gene was, as expected, decreased in differentiated motor neurons cultured from hiPSCS derived from SMA as opposed to non-SMA control individuals. Further, differentiated motor neurons from ALS individuals carrying the D90A mutation in the superoxide dismutase gene (*SOD1*), revealed a downregulation of *NEFL* gene expression, encoding for the light polypeptide neurofilament protein, consistent with this particular ALS mutation. These findings highlight the ability of SHH to direct the correct generation of cell types equipped with appropriate endogenous machinery, such that this *in vitro* approach was capable of recapitulating disease phenotypes.

The potential use of the SHH agonist for discovery and application is further exemplified by yet another recent study utilizing SHH to direct the differentiation of functional serotonin neurons of the raphe nucleus [298]. Using both hESCs and hiPSCs, Lu et al. showed that transient exposure to SHH was sufficient to bias hindbrain progenitors towards a serotonin fate. SHH signaling, along with WNT activation, were the essential modulators used to enrich the cultures towards tryptophan hydroxylase-positive, electrophysiologically functional serotonin neurons.

Important to note, while both motor neuron [297] and serotonergic neuron [298] generation were obtained under the influence of similar morphogenic cocktails, achieving these distinct neuronal populations was possible by adjusting timing and concentration of these morphogens. For both preparations, it was imperative to first caudalize the cultures with WNT agonists to achieve a posterior fate, before ventralizing the system with SHH. While this is true for both motor and serotonergic neuron generation, motor neurons required 1μM of the synthetic SHH agonist, purmorphamine, for 6 days [297], while serotonergic neurons required 29.3μM for 1 week [298]. The motor neuron preparation also coupled the SHH treatment with retinoic acid to assist in ultimate differentiation, which was absent from the serotonergic preparations. Similar to the endogenous human system, interactions among these differing factors play significant roles in the developmental trajectory and ultimate differentiation of these neuronal populations.

After ascertaining the appropriate gene expression patterns consistent with serotonin neurons [298], the cultures were utilized for pharmacological screening to reveal the potential of the *in vitro* methodology as a system to test drug candidates for disease treatment. Two classes of serotonin-targeting drugs were tested, including activators of serotonin release and selective serotonin reuptake inhibitors. The treatment of the hiPSC-derived hindbrain serotonin neurons with either class of drug resulted in increased serotonin concentration in both a dose- and time-

dependent manner, validating the known pharmacology of the drugs. Therefore, the differentiated serotonin neurons present a potential experimental approach for examination of drug therapies for a number of neuropsychiatric disorders, including, but not limited to, a variety of eating, sleep and mood disorders.

While these studies show the broad range of applications for SHH in *in vitro* systems, there are many limitations of the 2D system still to consider. The over-arching theme is that the 2D system's does not closely recapitulate the physiological complexity and developmental biology of the human system. The 2D system cultivates homogeneous cellular populations, unlike *in vivo* physiology, and this biological simplicity significantly limits cellular interactions thereby disrupting the overall development of the cells. The variety of functions that a single cell type possesses is a combined result of its environment, the cellular network it resides within and the other cell types with which it communicates. The impairment in cell-to-cell interactions which are inherent to a 2D system, coupled with excessive contact with extracellular matrix, result in biological deficiencies which are seen not only in these cells' morphologies and survival, but also their ability to differentiate. As discussed in the Lu et al study, many serotonin-expressing cells in the 2D system lack the cellular mechanics and equipment to function properly. As described in the Du et al study, motor neuron progenitors cultured in the 2D system have a short lifespan and limited differentiation potential, lasting a mere 2-5 passages only before losing their potency. These differentiation impairments, in addition to difficulties in replicability of the preparations, can also arise from the lack of cell-to-cell contacts and Notch signaling among precursors and daughter cells [299]. These limitations make the use of the cells for disease modeling, drug screening and cellular replacement unpredictable.

Furthermore, the aforementioned studies have had few or no follow-up investigations, which raises the question of additional complications in reproducibility, reliability and applicability of these systems. Important to note, serotonergic neuronal generation was followed up by additional characterization of the serotonergic neurons post-transplantation in the mouse brain [300]. While the transplanted neurons were found to project to various regions of the hindbrain, their projections were limited and not seen to reach the anterior regions of the brain. Thus, although this transplantation survival shows promise, future follow-up studies are necessary before the 2D model systems can be applied to human therapeutics.

The potential of organoids to accurately capture the diversity of the endogenous cellular milieu comes from its ability to mimic the cytoarchitecture of the human brain by self-organizing into a multilayer aggregate where different cell types interact and signal to each other through close intercellular contacts **[FIGURE 3.2]**. For example, ventricular radial glial cells communicate via N-cadherin and Notch signaling [301] and outer radial glia through LIFR-STAT3 [302] and mTOR [303] signaling, all of which profoundly influence their differentiation. Additionally, unlike the 2D system, organoids can be cultured for months at a time and even up to a year, maturing into electrophysiologically functional and biologically robust neural aggregates. This improved differentiation and maturation allow the development of long-range projections after organoids transplantation in mice, overcoming an obstacle that was of great consideration for the aforementioned medium spiny neuron development [304, 305]. Indeed, Mansour et al were able to show subpallial innervations following cortical transplantation of organoids in mice, with axonal projections to not only the striatum but to rostral regions as well. Additionally, projections spanned both hemispheres, with evidence of projections crossing the corpus callosum [304]. These works provide support and promise for the future of the organoid system. The remainder

of this review will focus on the use and potential of SHH in the organoid system towards development and disease modeling.

The future of *In Vitro* technology

Scientists have continued to push the boundaries of empirical possibility, discovering strategies that more realistically render neural networks and neuronal circuitry. Beyond any previous *in vitro* system, organoids have better reproduced the range of cell phenotypes that reside in a specific brain region, and offer an assay to model the developmental interactions of two or more brain regions, recapitulating dynamic and complex migration patterns, regional crosstalk and brain circuitry. These single-region organoids or multi-region *assembloids* present remarkable new opportunities to examine circuit dysregulation and inhibitory/excitatory imbalances plaguing many neurodevelopmental disorders.

The organoid system reproduces the embryonic-fetal window of development, a unique and essential time span that is difficult to thoroughly investigate in many other model systems [13, 15]. Researchers have used these multilayered diverse cellular aggregates to grow various brain regions and model many diseases, from Autism Spectrum Disorder [18] to Rett Syndrome [306] and Timothy Syndrome [125]. Individually cultured dorsal and ventral forebrain regional organoids were fused to achieve whole forebrain-like organoids, complete with migrating DLX2+ inhibitory populations in the ventral-dorsal direction, mimicking *in vivo* neuronal behavior [16, 125]; these experiments required the use of SHH agonists to stimulate development of the ventral forebrain and its migrating progenitors. With a recent influx of methods to foster ventral telencephalic-like organoids, the potential to study neurodevelopmental disorders affecting the basal ganglia, such as OCD and Tourette Syndrome, is perceivably high.

The union of independently generated regional organoids into assembloids achieves active cross-regional communication via processes such as cell migration; however, the cohesive nature of whole forebrain development and fluid transition of gene expression and cellular diversity is lost in assembloid strategies. As described earlier in this review, multi-axis neural patterning – dorso-ventral, medio-lateral, and anterio-posterior – is instructed by the SHH, WNT and BMP gradients endogenously established during neurogenesis **[FIGURE 1.2B]**. The gradient fosters a precise topographic map that specifies regional identity within the human brain. Cederquist et al. recognized that current organoid technologies lack such holistic topography, although it is an essential feature of neurodevelopment. To mimic *in vivo* spatial organization of the human forebrain and achieve distinct positional domains, Cederquist et al developed an inducible SHH-expressing hPSC line (iSHH) to initiate an inducible (rtTA-dependent) SHH gradient at a single pole of an organoid [307]. To create what Cederquist and colleagues refer to as a *SHH organizer*, 1,000 iSHH cells were cultured independently to become a mini cellular aggregate, after which time a larger number of wild-type cells were added to assemble atop and fuse to the iSHH aggregate. The product created what the group refers to as a "chimeric spheroid", with a much smaller ratio of iSHH cells to wild-type cells [307]. The organoid's response to the resultant SHH protein product then becomes a function of distance: the farther away cells are from the SHH organizer, the weaker the SHH-induced gene expression, thereby creating a gradient effect within the organoid and constructing a neuraxis.

The SHH organizer successfully produced four positional identities, dorsal, ventral, anterior and posterior [307]. Immunochemistry revealed the strong ventral expression of NKX2.1 in cells closest to the organizer, while those farthest away expressed the dorsal PAX6. Additionally, cells farthest from the organizer also expressed the anterior FOXG1 while those closest expressed the posterior OTX2. The distribution of SHH as its expression gradient traversed the

organoid achieved the major subdivisions of the human forebrain, including the neocortex and ganglionic eminences MGE and LGE, and even anterior hypothalamic development [307].

The inducible system generated a single organoid representative of topographically ordered multiple cellular fates and regional identities. When comparing the 3D strategies currently available for neurodevelopment, the iSHH system has its advantages, as it more thoroughly captures the interconnectedness of the human forebrain. There are surely synchronous as well as sequential elements of neuro-pathway activation, the compound impact of which is essential for mimicking the remarkable complexities of neurodevelopment, and are likely missing from multi-organoid assembloid generation. A gradient-based system, as achieved with the SHH organizer, inches closer to *in vivo* conditions and is more capable of mimicking the dynamic cellular continuum achieved by the human forebrain. That is because morphogen's concentrations inherently have differential ability to transcriptionally control gene expression, where precise concentrations either induce or silence gene expression and related cascades of signaling events, hence the complexity that gradients can establish. Furthermore, gradient-based organoid systems can be combined with multi-regional assembloid, where benefits to each are compounded, and may help construct the most appropriate systems to use to answer the empirical question in the end.

The *in vitro* organoid system provides, at least in principle, a source for investigation of a variety of developmental periods, with the potential to model therapeutic approaches, including modulation of gene expression, gene editing, and drug design. The tridimensional design offers a strategy that can enhance the empirical study of Parkinson's and Huntington's diseases mentioned previously, as well as a wide range of other neuropsychiatric disorders. Using either the assembloid or gradient approach, the development of dopaminergic projections from the

human substantia nigra to the striatum can be investigated and their abnormalities characterized in a Parkinsonian organoid model. Long range projections of medium spiny neurons from the striatum to the other regions of the basal ganglia can be modeled and their functionality tested in a Huntington's organoid model. Tourette Syndrome and OCD can be modeled by observing the development of the basal ganglia, and cortico-basal ganglia circuitry can be tracked in these diseases as well. These are only some of the possible applications of this system, but there are many exciting doors to be opened with this technology to advance our understanding and treatment of human disorders – we are still only at the beginning.

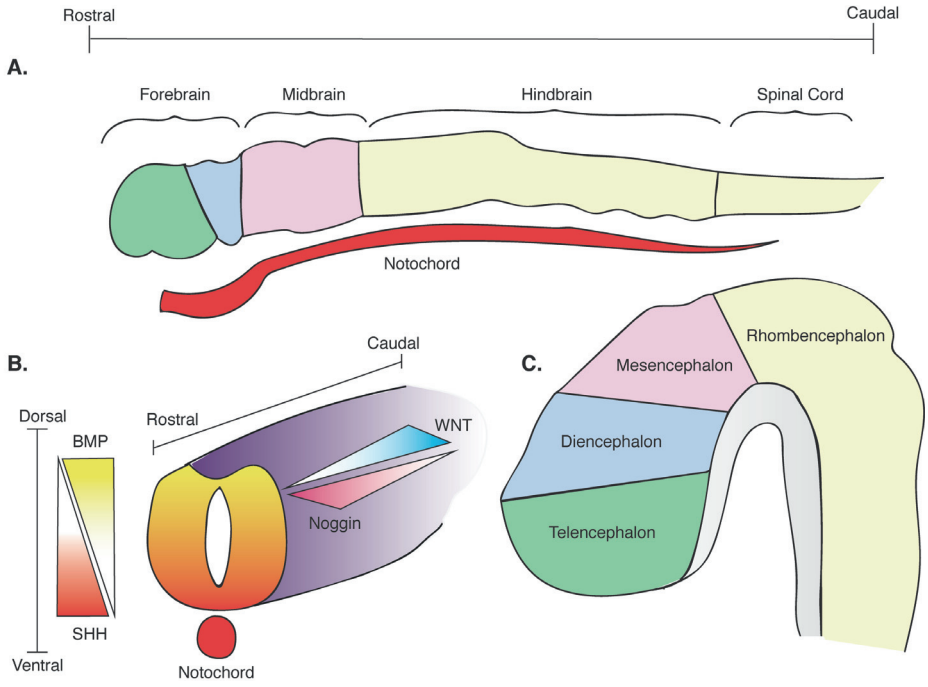


Figure 1.2. Schematic of neural tube formation and cerebral regional identity. (A) Regional definition of the neural plate with rostrocaudal axes and its contact with the SHH-releasing notochord. (B) Formation of the neural tube and its contact with the SHH-releaseing notochord. Doroventral and rostrocaudal axes are defined with corresponding critical morphogenetic gradients: Dorsal BMP; Ventral SHH; Rostral Noggin; Caudal WNT) (C) Development of the embryonic brain and divisions of the central nervous system.

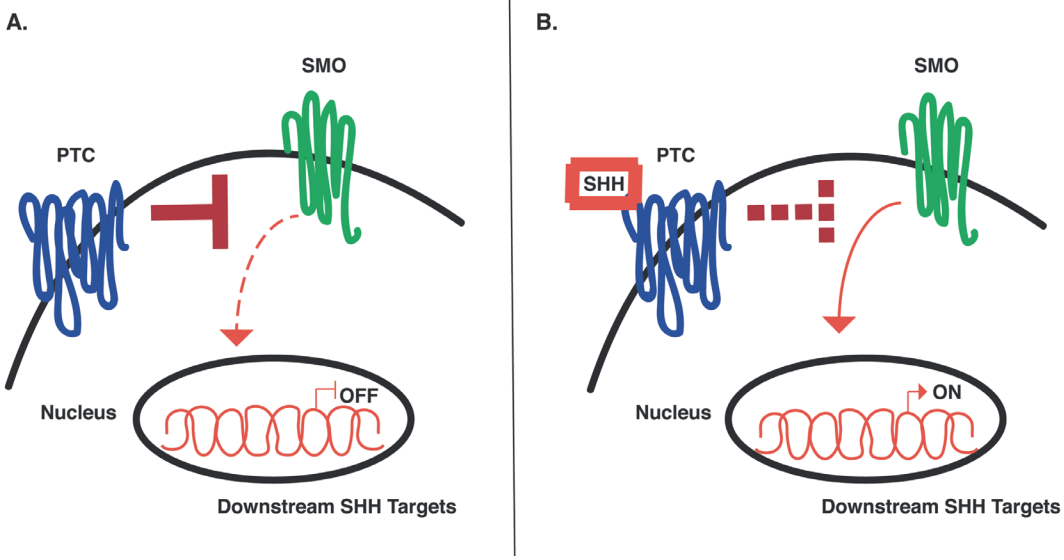


Figure 2.2: SHH signaling pathway. (A) SHH pathway in the absence of SHH ligand. PTC inhibits SMO activity, thereby inhibiting its ability to positively influence transcription (dashed lines). Nuclear transcription of downstream SHH targets is stopped. (B) SHH pathway in the presence of SHH ligand. SHH binds PTC, lifting its inhibition on SMO (dashed lines), thereby allowing SMO to positively influence transcription. Nuclear transcription of downstream targets is activated.

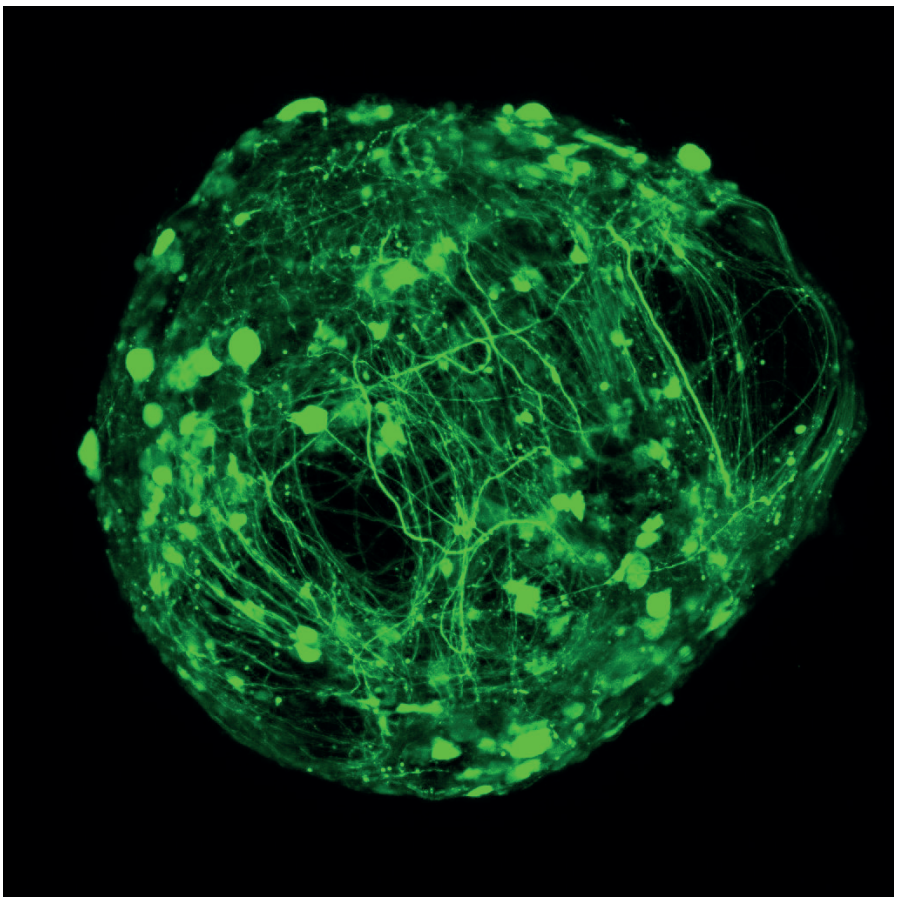


Figure 3.2: Human iPSC-derived organoid. iPSC-derived organoid was transduced at 7 months of age with SYN-GFP to illuminate neuron-specific development. Imaging was obtained by two-photon microscopy to show longevity and network complexity within these 3D aggregates.

A word about GLI proteins and SHH signaling

The following section is on GLI proteins and their significance in SHH signaling within the developing forebrain. This section is not part of the official review in this chapter; however, GLI proteins and their role in patterning dorsal and ventral regions of the telencephalon are an essential component of my thesis work. Therefore, the following section is necessary for understanding the trajectory of this thesis.

Continued study of SHH signaling *in vitro* could yield impactful advances both scientifically and clinically. The temporal and spatial precision with which SHH helps specify these many cell types remains unclear, and the dynamic processes still leave much to be appreciated. The mechanistic features of the powerful SHH signaling must not be overlooked. Signal transduction of the SHH signaling pathway is a primary leader of cellular commitment and network organization, and is a possible source of developmental abnormality.

In addition to the transmembrane patched and smoothened proteins, key players in SHH signaling are the Gli genes (Gli family zinc finger genes), producing the transcription factors Gli1, Gli2 and Gli3 [294, 308]. Genetic studies in mice have shown Gli1 and Gli2 to mainly promote transcription after SHH signaling, while Gli3 functions mainly to repress transcription [309]. Gli1 is not proteolytically cleaved, and therefore does not exhibit repressor activity; however, both the Gli2 and Gli3 transcription factors have the capability to function as activator or repressor, and in fact SHH signaling inhibits the proteolytic processing of Gli2 and Gli3, suppressing their repressor activities [310-312]. Given their functions, the Gli proteins occupy distinct regions of the neural tube. Gli1 **[FIGURE 4.2]** is expressed ventrally. In response to SHH signaling, Gli1 increases transcription for downstream targets, such as NKX2.1, thereby ultimately facilitating ventral cell type specification. On the other hand, Gli2 and Gli3 are found dorsally [**FIGURE 4.2]**. Gli2/Gli3 are proteolytically processed to become repressors of the SHH pathway; once processed, they antagonize SHH signaling to instead promote dorsal forebrain patterning. The

categorization of these proteins into activators and repressors stems from long-standing genetic research on mutant mice [259, 313, 314]. In *Gli3-/-* mutant embryos, the cortical plate, denoted by EMX1/2 protein expression, is reduced, due to disruption in dorsal telencephalic development; this results in a significantly smaller cortex, olfactory bulb and hippocampus in the mutant embryos. And while dorsal territory shrinks in these mice, the ventral territory was found to expand, indicated by significant increases in ventral progenitor gene expression, including NKX2.1 and DLX2 [259]. Additional studies have shown that transfection of embryos with Gli3 in its repressor form stunted development of both ventral progenitors and motor neurons, and promoted expansion of dorsal gene expression within the ventral neural tube [315]. This research indicates that Gli3 has dual functionality, to both promote dorsal specification and also inhibit ventral commitment; whether these processes stem from two distinct mechanisms remains unclear. More importantly, however, these findings suggest a predominantly cell-autonomous response to Gli signaling that can easily translate to a disruption of dorsoventral patterning of the neuraxis.

Interesting to note, while all three Gli proteins influence neural tube patterning, loss of Gli2 has been found to have the most fatal developmental consequences. Gli1 knock-out mice proceed to full term, are viable and fertile [316]; and although Gli3 loss-of-function mutations yield phenotypes mimicking overexpression of SHH signaling [317], for example, polydactyly [316], Gli3 mutants are viable nonetheless. However, Gli2 homozygous null mice are not viable and display dramatic neural tube defects [318, 319].

Furthermore, Gli2 – not Gli1 – has been genetically shown to be required for floor plate formation within the neural tube [318-320]. Loss of function Gli2 mutations leads to holoprosencephaly, including most notably deficient medial forebrain development and altered ventral neurospecification [321]. Interestingly, holoprosencephaly is also a consequence of human heterozygous loss of function SHH mutations [322-324]. Lastly, Gli1/Gli2 double mutants, but *not* Gli1/Gli3 double mutants, exhibit fatal defects, including an impaired development of spinal cord cell types and smaller lung sizes, leading to death soon after birth; and still, these defects appear less severe than the lone Gli2 mutant [316]. This research suggests that Gli2 function may be much more elaborate than we currently understand, and certainly, the SHH pathway mechanisms mediating increased Gli2 activity, whether as an activator or repressor, are still unclear. Gli2 repressor activity has predominantly been explored in zebrafish, where Gli2 repressor activity has been shown to interfere with hedgehog signaling via Gli1 blockade [325], and disrupt differentiation and axon guidance in the ventral forebrain [326]. While the literature denotes Gli2 to function mainly as an activator in other animal models, its repressor behavior requires further investigation to elucidate the full extent of its role in neural tube development and telencephalic patterning, and especially its potential role in neurodevelopmental disease. Indeed, *in vitro* systems, such as the aforementioned organoid system, may serve to be ideal strategies to further explore these essential developmental proteins.

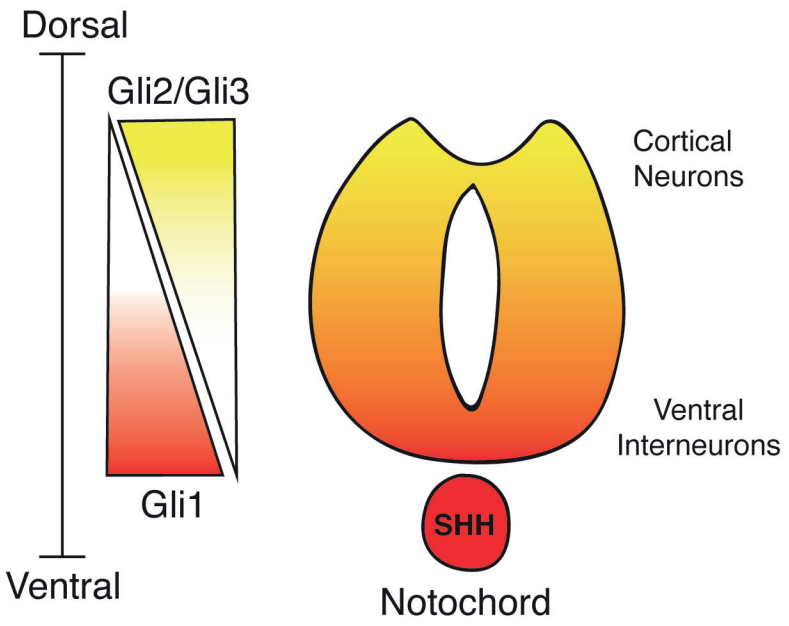


Figure 4.2: Gli Protein Expression Within the Neural Tube. Dorsal-ventral axis depicted with gradient expression of the dorsal Gli2 and Gli3, and ventral Gli1. Also illustrated is the anterior neural tube and SHH-secreting notochord. Where SHH and Gli1 are active, ventral interneurons are specified. The farther away (more dorsal) neurons are from the notochord, cortical neurons are specified.

Chapter Three:

Materials and Methods

Subject Participants

Tourette Syndrome participants in this study were recruited at the Tic and Obsessive-Compulsive Disorder Program at Yale Child Study Center by the teams of Dr. James Leckman, Dr. Robert King, and Dr. Michael Bloch. All Tourette Syndrome participants were evaluated in person or videoconference by Dr. Angeli Landeros-Weisenberger, a trained clinical researcher. Severity of symptoms was assessed using the ADSRS (Adult ADHD self Report Scale), the YGTSS (Yale Global Tic Severity Scale), the SCID (Structured Clinical Interview), the PUTS (Premonitory Urge for Tic Scale), the Y-BOCS (Yale-Brown Obsessive Compulsive Scale), the Hamilton Anxiety Rating Scale, and the LIFE-RIFT (Longitudinal Interval Follow-up Evaluation-Range of Impaired Functioning Tool). The criteria for inclusion are confirmed diagnosis of TS based on clinical evaluation and results of the diagnostic assessment. Participants were excluded if they have other psychiatric diagnoses, with the exception of OCD (Obsessive-Compulsive Disorder) and ADHD (Attention Deficit Hyperactivity Disorder), which do not represent exclusionary criteria because of frequent co-morbidity with TS.

The healthy unaffected control participants in this study were recruited from a larger pool of participants evaluated through several research projects at the Yale Child Study

Center Autism Program by the teams of Drs. Katarzyna Chawarska, Pamela Ventola, and Kevin Pelphrey.

All participants were asked to donate a small punch skin biopsy (about 3 mm) of the inner surface of the arm after topical anesthesia with lidocaine. Informed consent was obtained from each subject according to the regulations of the Institutional Review Board and Yale Center for Clinical Investigation at Yale University. See Table 1 for details.

Subject ID	Biopsy Date	Age at Time of Biopsy	Sex	Race	Ethnic Group
TS02-03	10/04/2013	519	M	White	Not Hispanic or Latino
TS03-03	10/31/2013	375	M	Many races	Not Hispanic or Latino
TS08-03	10/28/2013	348	M	White	Not Hispanic or Latino
TS10-03	06/20/2018	455	F	White	Not Hispanic or Latino
TS1064-03	10/24/2019	433	M	White	Not Hispanic or Latino
PGP1	Unknown	612	M	White	Not Hispanic or Latino
01 01	08/23/2010	570	M	White	Not Hispanic or Latino
01 02	07/12/2010	526	F	White	Not Hispanic or Latino
01 04	07/12/2010	88	F	White	Not Hispanic or Latino
07 01	02/25/2011	552	M	White	Not Hispanic or Latino
1123-01	04/14/2010	632	M	White	Not Hispanic or Latino
RDH 913-01	12/13/2017	491	M	White	Not Hispanic or Latino
RDH925-03	06/09/2017	223	M	White	Not hispanic or Latino
RDH925-01	06/09/2017	717	M	White	Not hispanic or Latino
7978-01	03/08/2016	825	M	White	Not hispanic or Latino
8090-01	12/15/2015	584	M	White	Not hispanic or Latino

Table 1: Subject Participants and iPSC Acquisition. This table outlines the Subject ID for the TS and CT individuals used in this thesis, and subject background and biopsy details. Age at the time of biopsy is in denoted in months since birth.

Primary cultures of fibroblasts were derived using standard explant procedures [327]. hiPSCs were generated using the original retroviral approach [240] or by a viral-free episomal reprogramming method [328]. See Table 2 for details. All iPSC lines used in

this study were characterized using three sets of quality control criteria: (1) morphology; (2) immuno-cytochemical expression of pluripotent markers, and (3) semiquantitative RT-PCR of pluripotency marker genes and downregulation of exogenous reprogramming factors. Whole Genome Sequencing for a subset of the iPSC lines used in this project has been previously analyzed [18, 329, 330].

The recruitment and production of iPSC lines for control subjects were supported by the following grants: MH087879, MH089176, and MH109648 from the National Institutes of Health, and by the Simons Foundation.

The recruitment and production of iPSC lines for TS subjects were supported by MH118453 from the National Institutes of Health, and by NARSAD- Brain and Behavior Research Fund.

Subject ID	Phenotype	Phen. Description	iPSC Reprogramming Method	Reprogrammed By	Details
TS02-03	TS	TS, persistent	viral-free episomal method	Yale Stem Cell Center facility	
TS03-03	TS	TS, persistent	viral-free episomal method	Yale Stem Cell Center facility	
TS08-03	TS	TS, persistent	viral-free episomal method	Yale Stem Cell Center facility	
TS10-03	TS	TS, persistent	viral-free episomal method	Yale Stem Cell Center facility	
TS1064-03	TS	TS, persistent	viral-free episomal method	Yale Stem Cell Center facility	
PGP1	CT	CT	retroviral method	Dr. In-Hyun Park	iPSC produced and donated by In-Hyun Park
01 01	CT	CT	viral-free episomal method	Yale Stem Cell Center facility	member of CT family
01 02	CT	CT	viral-free episomal method	Yale Stem Cell Center facility	member of CT family
01 04	CT	CT	viral-free episomal method	Yale Stem Cell Center facility	member of CT family
07 01	CT	CT	retroviral method	Vaccarino's Lab	father of ASD proband
1123-01	CT	CT	retroviral method	Vaccarino's Lab	father of ASD proband
RDH 913-01	CT	CT	viral-free episomal method	Yale Stem Cell Center facility	member of CT family
RDH925-03	CT	CT	viral-free episomal method	Yale Stem Cell Center facility	member of CT family
RDH925-01	CT	CT	viral-free episomal method	Yale Stem Cell Center facility	member of CT family
7978-01	CT	CT	viral-free episomal method	Vaccarino's Lab	member of CT family
8090-01	CT	CT	viral-free episomal method	Vaccarino's Lab	member of CT family

Table 2: **Subject diagnoses and details of iPSC generation**. This table identifies further descriptors of recruited subjects and derived biopsies. Subject diagnoses as well as reprogramming methods of fibroblasts and location of reprogramming facility are specified. CT=control; TS=Tourette Syndrome

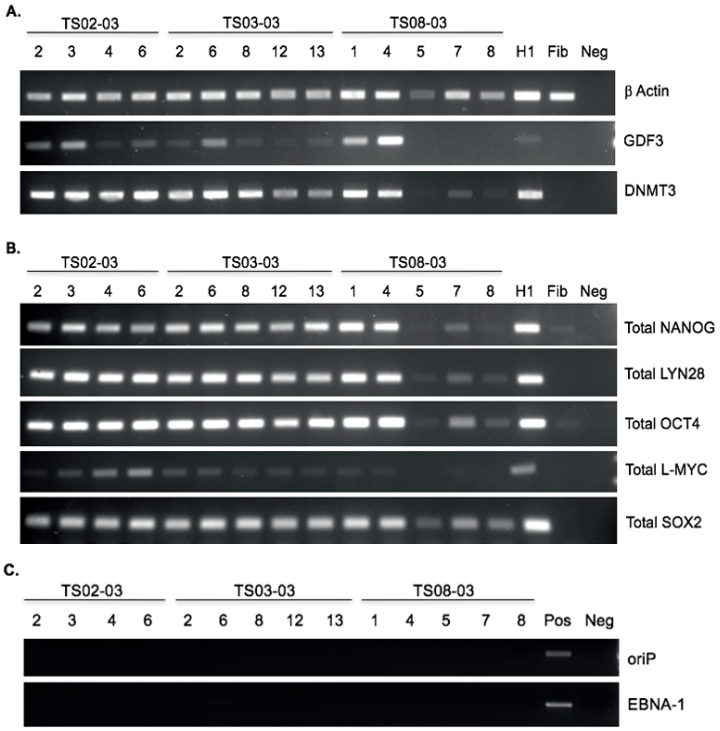


Figure 1: TS fibroblasts were successfully reprogrammed to iPSCs. RT-PCR classification of 3 iPSC lines derived from TS patients TS02-03, TS03-03 & TS08-03. H1 hESC =positive control. Fibroblasts (Fib) and PCR negative control (Neg) =negative controls. **(A)** Pluripotent markers; **(B)** Endogenous and exogenous reprogramming genes; **(C)** Absence of episomal vectors. Transfected Fibroblasts (Pos) =positive control. Transgenes OriP and EBNA-1 can detected in all five episomal plasmids.

Induced Pluripotent Stem Cells (iPSC)

Fibroblast skin biopsies were taken from each individual utilized for this study and successfully reprogrammed to iPSC lines, both as previously described [13] and by integration-free methods (See Table 2). A total of 25 iPSC lines were utilized and differentiated for the data described (Table 1). Each of the iPSC lines for both TS probands (n=5 biological replicates; at least 2 clones were used per line = 12 lines total)

and the healthy CT individuals (n=11 biological replicates; 13 lines total) were generated by using either the classical Yamanaka retroviral approach or by non-viral episomal methodology [328]. The following CT iPSC lines were previously described: PGP1 [13, 331]; 07-01 [18]; and 1123-01 [329]. The TS individuals' iPSC lines are unpublished. The iPSC characterization and quality control for TS individuals TS02-03, TS03-03 and TS08-03 are shown in Figure 1.

Neuronal Differentiation

For the investigations conducted in this thesis, I achieved telencephalic neural organoids favoring ventral forebrain development by differentiating the iPSC lines using a modified methodology for 3D organoids in suspension [13, 18]. Briefly, iPSCs are cultured to create 3D cellular aggregates called embryoid bodies. The floating embryoid bodies are maintained in suspension and stationary for one week under neuralizing and proliferative conditions. The neural aggregates form lumen-filled rosettes, which are manually harvested and cultured under potent ventralizing and growth-promoting conditions to generate basal ganglia organoids. Specifically, ventral morphogens – the SHH agonist purmorphamine (PUR, 2 μM) and fibroblast growth factor 8 (FGF8, 200ng/ml) – were added on Day 8 for 4 days (see Figure 1A for an outline). Terminal differentiation (TD) is deemed the day in which growth media are withdrawn and changed into differentiation-promoting media, which is day 12 from the beginning of culture. Upon entry into terminal differentiation, the then termed organoids are cultured for one week under ventralizing and maturation-promoting conditions. Specifically, PUR

and FGF8 are added at a reduced concentration (PUR, 1μM; FGF8, 100ng/ml), and retinoic acid (RA, 500ng/ml) and bone morphogenic protein 9 (BMP9, 10ng/ml) [332, 333] are introduced to promote commitment and maturation of basal ganglia organoids. Terminal differentiation and maturation-promoting conditions are continued up to one year. Differentiations for both TS probands and healthy CT individuals were conducted in parallel for each preparation. Please see the full basal ganglia organoid protocol in the appendix of this thesis, and the schematic illustrated in Chapter Four, Figure 1 of this thesis.

RNA Isolation, qPCR and RNA Sequencing Experiments

RNA was extracted from basal ganglia organoids on day 12 (TD0), 26 (TD14) or 38 (TD30) *in vitro,* as previously described [13]. For each time point, 16-20 organoids were harvested and pooled for material. Briefly, the PicoPure RNA isolation kit (Applied Bioscience) was utilized, according to the manufacturer's instructions, to isolate total RNA from harvested organoids. The RNA from these extractions were used for both qPCR and RNA sequencing. To perform qPCR for both culture characterization and RNA sequencing validation, cDNA was first synthesized utilizing the SuperScript III First-Strand Synthesis Kit and random hexamers (Invitrogen) using 100 ng of total RNA as starting material. The StepOnePlus Real-Time PCR System (Applied Biosystems) was used to complete qPCR experiments, using Power SYBR Green PCR Master Mix (Applied Biosystems); the samples were normalized to GAPDH housekeeping gene expression. qPCR data were analyzed using a Two-Way ANOVA and post hoc test to

establish significance of gene expression. Please see Table 3 below for the primer sequences used for qPCR amplification.

Gene	Forward Primer	Reverse Primer	Reference
SIX3	CCGGAAGAGTTGTCCA TGTT	CGACTCGTGTTTGTTGATGG	doi:10.1242/dev.084608
OTX2	TCAACTTGCCCGAGTCGAGG	CAATGGTCGGGACTGAGGTG	doi:10.1242/dev.084608
GBX2	CTCGCTGCTCGCCTTCTC	GCCAGTCAGTCAGATTGTCATCCG	doi:10.1242/dev.084608
EMX2	GGGATCCGTCCACCTTCTAC	CTCAAAGGCGTGTTCCAGCC	doi:10.1242/dev.084608
ARPP21	GTGCAAAGCGTGA TGGTTTCC	CCTTGACCTGCCTGGTTAGG	doi:10.1242/dev.084608
DLX6	TACCTCCAGTCCTACCACAAC	AATAAATGGTCCGAGGCTTCCG	doi:10.1242/dev.084608
LHX6	CGACACCATGATTGAGAACC	TTGGGTTGACTGTCCTGTTC	doi:10.1176/appi.ajp.2012.12030305
CALBINDIN	GCTCCAGGAATACACCCAAA	CAGCTCATGCTCGTCAATGT	doi:10.1093/cercor/bht254
CALRETININ	AGCGCCGAGTTTATGGAG	GGGTGTATTCCTGGAGCTTG	doi:10.1176/appi.ajp.2012.12030305
PV	CTACCGACTCCTTCGACCA	CCTTGTCCAGCATGTGAAA	doi:10.1176/appi.ajp.2012.12030305
SOX6	AGAACGCGCTTTGAGAATTT	GCCCAGTTTTCCATCTTCAT	doi:10.1176/appi.ajp.2012.12030305
EVF2 (DLX6-AS1)	AGTTTCTCTCTAGATTGCCTT	ATTGACATGTTAGTGCCCTT	dol.10.1186/s12935-015-0201-5
mGluR1	GTGGTTTGATGAGAAAGGAG	GTTGCTCCACTCAAGATAGC	doi.org/10.1073/pnas.1107304108
REST	TGTCTCGGAGCCACCTCGGG	CCTGCCGCGCCATCTCACTC	doi:10.1371/journal.pone.0095374
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA	doi.org/10.1016/j.biopsych.2014.07.018
CHAT	GGCCATTGTGCAGCAGTT	TCATTCAGCCAGTACTCAGACAC	doi.org/10.1016/j.biopsych.2014.07.018
SLITRK	TTGCGGAGATCCTGCTAGAG	GGGAGACTAGAATCCACTCGG	
NOS1	GGCTGCTGATGTCCTCAAA	CCTCATGGTATCGGTTGTCA	
NPY	CGCTGCGACACTACATCAAC	CTCTGGGCTGGATCGTTTTCC	
SST	ACCCAACCAGACGGAGAATGA	GCCGGGTTTGAGTTAGCAGA	
LHX8	GAATGACCTATGCTGGCATGT	ACCCAGTCAGTAGAATGGATGTG	
PPP1R1B (DARPP-32)	GAGCCTCAGCTGGAGATCCG	TTTCAGCGAAGGTGGTGTGT	
GAD1	GCTTCCGGCTAAGAACGGT	TTGCGGACATAGTTGAGGAGT	doi.org/10.1016/j.cell.2015.06.034
SOX1	GCAAGATGGCCCAGGAGAA	CCTCGGACATGACCTTCCA	
DLX1	TCCAGCCCCTACATCAGTTC	CCACCACCGTGCTCTTCT	doi.org/10.1016/j.cell.2015.06.034
PAX6	GTGTCTACCAACCAATTCCACAAC	CCCAACATGGAGCCAGATG	doi.org/10.1016/j.cell.2015.06.034
TBR1	ATGGGCAGATGGTGGTTTTA	GACGGCGATGAACTGAGTCT	doi.org/10.1016/j.cell.2015.06.034
NKX2.1	TGTCCTCGGAAAGTCAGCTC	GTGCTTTGGACTCATCGACA	
BCL11B (CTIP-2)	CTCATCACCCCAGAGGCTG	TGTCATAGCAGGCACCCAAG	doi.org/10.1016/j.cell.2015.06.034
FOXJ1	CAGATCCCACCTGGCAGA	CGTACTGGGGGTCAATGC	
CHRM2	AACTCCTCTAACAATAGCCTGGC	GTTCCCGATAATGGTCACCAAA	
SLC5A7	TTCCTCAGCCACCTATGCTC	CCCATATGCAGTCTGGTTCC	doi.org/10.1016/j.biopsych.2014.07.018
SLC18A3	TTCGCCTCTACAGTCCTGTTC	GCTCCTCCGGGTACTTATCG	
GABRA3	ACAAGTCACTGTTACATGACCAG	TCGTCTTGATTCCCCTTGACC	
DRD1	AGGGACTTCTCTGTTCGTATCC	GGAACCTGATAACGGCAGCA	
MASH1/ASCL1	GCTTCTCGACTTCACCAACTG	ATGCAGGTTGTGCGATCA	doi.org/10.1016/j.cell.2015.06.034
FOXG1	AGAAGAACGGCAAGTACGAGA	TGTTGAGGGACAGATTGTGGC	doi.org/10.1016/j.cell.2015.06.034
DLX2	AGCAGCTATGACCTGGGCTA	AATTTCAGGCTCAAGGTCCTC	doi.org/10.1016/j.cell.2015.06.034
ISL1	GGATTTGGAATGGCATGCGG	CATTTGATCCCGTACAACCTGA	doi:10.1016/j.stemcr.2020.12.019.
BRN2	AATAAGGCAAAAGGAAAGCAACT	CAAAACACATCATTACACCTGCT	doi.org/10.1016/j.cell.2015.06.034

Table 3: List of primers used for qPCR analysis. Forward and Reverse primer sequences are noted, as well as the publication from which they were used.

For RNA sequencing experiments, RNA was extracted for identical timepoints as with qPCR – day 12 (TD0), 26 (TD14) or 38 (TD30) *in vitro.* HiSeq paired-end, 100 bp RNA sequencing (with rRNA depletion) was conducted on a NovaSeq platform to a

sequencing depth of 40 million reads per sample. Two batches of samples were submitted, but no batch effect was observed (Figure 2).

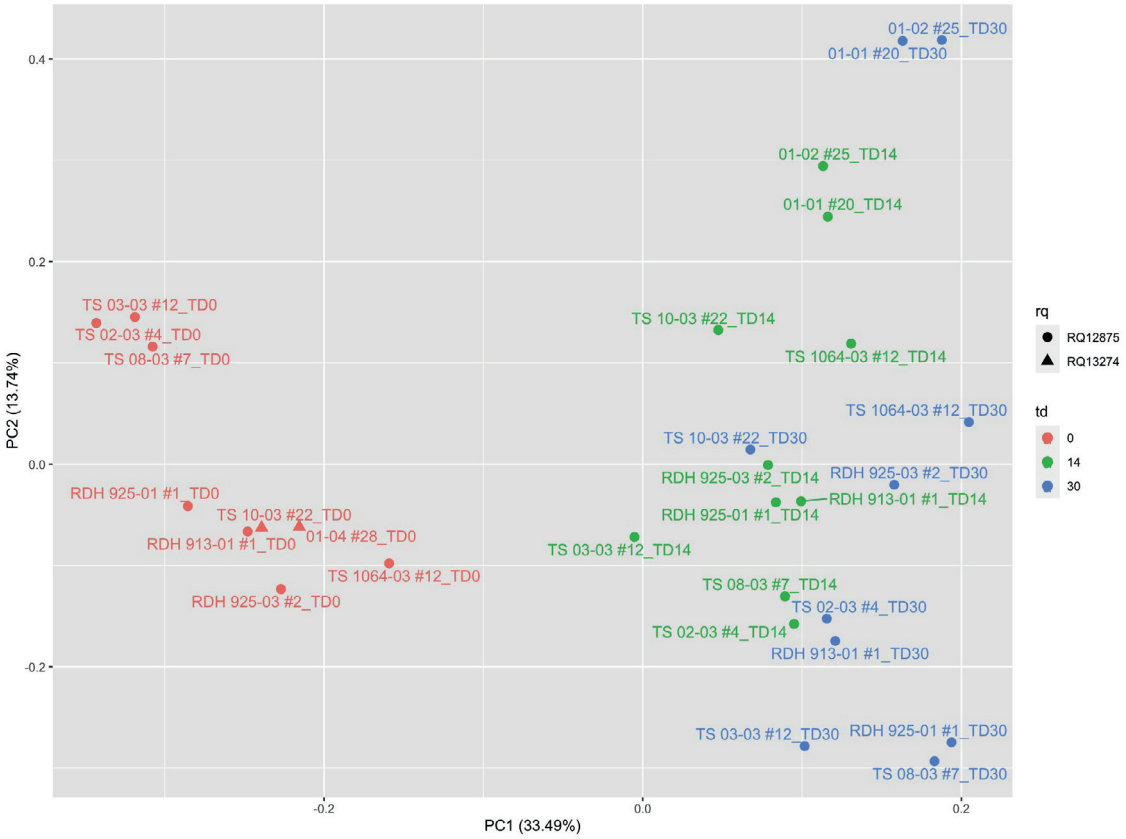


Figure 2: Principle Component Analysis (PCA) from RNA sequencing submissions shows no batch effect. Both submissions (submission 1: circle – RQ12875; submission 2: triangle – RQ13274) cluster similarly, with no obvious separation between samples attributed to separate submissions. PC1 (along X axis) shows that the strongest separation (33.49%) between samples is driven by time, as TD0 samples are sharply separated from TD14 and TD30 samples. PC2 (Y axis) shows that the next separation is attributed to individual background (TS vs CT); however, this separation is not as strong. These trends follow typical findings from patient and control PCA.

Immunostaining and Stereology

Basal ganglia organoids were harvested and visualized at day 26 (TD14) or 38 (TD30) *in vitro*. Samples were prepared as previously described [18]. For each time point, 5-10

organoids were pooled per line and fixed in 4% PFA for 3 hours. The samples were subsequently incubated in 25% sucrose overnight. After removal from sucrose solution, each organoid was immediately embedded in OCT while at room temperature and stored at -80 degrees Celsius. For further empirical use, the embedded samples were crysectioned at 12 μm. For immunostaining, the cryosections were first incubated at room temperature for 1 hour in blocking solution (10% donkey serum/0.1% Triton X-100 in PBS). Samples were treated with primary antibodies that were placed in diluted blocking buffer (5% donkey serum/0.05% Triton X-100 in PBS); sections were incubated overnight at 4 degrees Celsius. The sections were subsequently washed three times in 5 minute periods in PBS/0.1% Triton X-100. Next, sections were treated for 1 hour at room temperature with secondary antibodies (1:500, Alexa or Jackson Laboratories) in diluted blocking buffer. Primary antibody dilutions are as follows: PAX6 (mouse, BD Bioscience, #561462, 1:200); N-cadherin (mouse, BD Bioscience, #610920, 1:500); TBR1 (rabbit, Abcam, #ab31940, 1:1000); SOX1 (goat, R&D System, #AF3369, 1:20); DLX-2 (rabbit, gift of Yuri Morozov, Yale University, New Haven, CT, 1:1000); NKX2.1 (mouse, Thermo Scientific, #MA5-16406, 1:200); BRN2 (goat, Santa Cruz, #sc-6029, 1:100); Ki67 (rabbit, Vector, #VP-RM04, 1:500); pH3 (rat, Sigma, H9908, 1:1000); CHAT (goat, Chemicon, #AB144P, 1:1000).

For image acquisition, an ApoTome-equipped Axiovert 200M with Axiovision 4.5 software was used. Stereological analysis was performed to quantify immunostained

cells utilizing a Carl Zeiss Axioskop 2 Mot Plus, which was connected to a computer running Stereoinvestigator software (MicroBright-Field).

For quantification, two organoids and four sections per organoid (per individual) were used. The optical fractionator probe (100x oil-immersion objective) was utilized to count the nuclear profiles of stained cells. The percentage of positive cells were calculated relative to total DAPI+ cells.

Western Blotting

Western blot experiments were conducted on basal ganglia organoids day 12 (TD0) *in vitro,* as previously described [18]. For protein extraction, basal ganglia organoids were first washed with cold PBS before treated with complete Lysis-M, EDTA-free buffer (Roche, 04 791 964 001), which was additionally supplemented with protease (Roche, 11 836 170 001) and phosphatase inhibitor cocktail tablets (Roche, 04 906 837 001). Samples were then centrifuged for 15 minutes at 14000 rpm at 4 degrees Celsius. Next, the supernatant fraction was obtained and the total protein per sample was quantified using the Qubit™ Protein Assay Kit (Invitrogen, #Q33211). For each protein gel, 40 μg total protein per sample was loaded into TGX stain-free 4-15% precast gels (BioRad, #P4568084) and SDS gel electrophoresis was performed. The gels were subsequently transferred to nitrocellulose membranes (BioRad, #1704158). For this thesis, all membranes were visualized using SuperSignal West Pico Chemioluminescent Substrate (Thermo Scientific, 34080) and quantified using ImageJ software. Primary antibodies were diluted as follows: GLI1 (rabbit, Abcam, #ab134906, 1:500); GLI2

(rabbit, Millipore, #ABN2241, 1:500); GLI3 (rabbit, Abcam, #ab6050, 1:500); GAPDH (mouse, Millipore, MAB374, 1:1000).

Transcriptome Analysis by RNA sequencing

RNA-seq data analysis.

We used STAR [334] to map the reads to the human genome (hg38) and the GencodeV34 for transcriptome annotation. The mapped reads were sorted by coordinates in BAM file format using SAMtools [335]. Gene expression in counts was estimated using the RSubread function featureCounts [336]. Gene expression in RPKM was estimated using the edgeR function rpkm [337]. All the heatmaps were plotted using $\log_2(1+\text{rpkm})$ values scaled at row level. Differential expression analysis has been performed between controls and probands at TD0, TD14 and TD30 by using edgeR [337] functions glmFit and glmLRT and using trended dispersions as dispersion input. Lowly expressed genes have been filtered before analysis by using edgeR function filterByExpr. An FDR cut-off of 0.05 was used for all the tests. For GO term and canonical pathway enrichment analysis, we used ConsensusPATH tool [338]. Differentially expressed genes were identified using a base limit corrected P value (FDR) of $\leq$0.05 and a fold change of +/-2. Heatmaps were generated using RPKM values per gene per individual.

Chapter Four:

Results: Ventralizing the organoid system – the basal ganglia protocol

We previously developed a cortical organoid model to examine pathogenesis of ASD [13, 18], and I since have employed modifications to enrich for a basal ganglia-like cellular commitment within the organoids. Selecting for a ventral specification fosters basal ganglia-like neural organoid development and enables the study of cellular and molecular characteristics of neuronal precursors in TS. Our findings show that optimizing timing and concentration of a cocktail of ventral morphogens induce ventral telencephalic organoid development (Figure 1), triggering necessary transcriptional programming to generate BG-like organoids.

The Protocol for Differentiating Basal Ganglia-like Organoids

To obtain neural organoids enriched with ventral telencephalic precursors, manually selected free-floating neural rosettes are cultured in serum-free medium supplemented with fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) (10ng/ml each) plus the sonic hedgehog (SHH) agonist purmorphamine (PUR, 2 μM) and fibroblast growth factor 8 (FGF8, 200ng/ml) for 4 days (Figure 1A). Adding these two essential ventral morphogens during the proliferative phase of organoid development is the initial trigger to shift cellular commitment away from the default dorsal telencephalic fate [11-14] and towards a ventral one. Further, this addition activates the crucial early ventral forebrain determinant, NKX2.1, the transcription factor that pioneers ventral

telencephalic development [339] and is absent in cortical cultures (Figure 1B). After these 4 proliferative days, organoid cultures are differentiated in neurobasal media supplemented with brain-derived neurotropic factor (BDNF), glial cell line-derived neurotropic factor (GDNF), Ascorbic Acid and dibutyryl-cAMP. Upon entry into the terminal differentiation phase of organoid development, PUR and FGF8 are added at a reduced concentration (PUR, 1μM; FGF8, 100ng/ml), and retinoic acid (RA, 500ng/ml) and bone morphogenic protein 9 (BMP9, 10ng/ml) are introduced to promote commitment and maturation of the organoid's cellular composition to neuronal cell types exclusive to the ventral telencephalon (Figure 1A). Within the field of neurodevelopment, RA has been shown to play an essential synergistic role with SHH and FGF8 in anterior telencephalic patterning [340-346]. Further, retinoid signalling guides striatal differentiation in the developing basal ganglia [345], and is specifically required for the differentiation and maturation of the striatum-destined GABAergic neurons that originate from the LGE [345, 347]. Although BMP signalling is critical for dorsal patterning in the human forebrain (See Chapter 2, Figure 1), the septum-expressing BMP9 has been shown to be required for the development of cholinergic neurons that populate the ventral telencephalon [332, 333, 348], including those that migrate to the basal ganglia.

The combination of all four ventralizing factors persists in the terminal differentiation organoid media for the first seven days of terminal differentiation (Figure 1A). I found that adding these factors too early in the preparation stunts aggregate growth and rosettes fail to appear; adding factors all at once during early aggregate development

masks each factor's ability to individually influence the preparation and potent RA becomes the most influential, posteriorizing the aggregates and promoting caudal specification (data not shown). Overall, the incremental addition of factors subsequent to neural induction provides the strongest ventral specification and highest degree of differentiation into diverse cellular populations found within the ventral telencephalon.

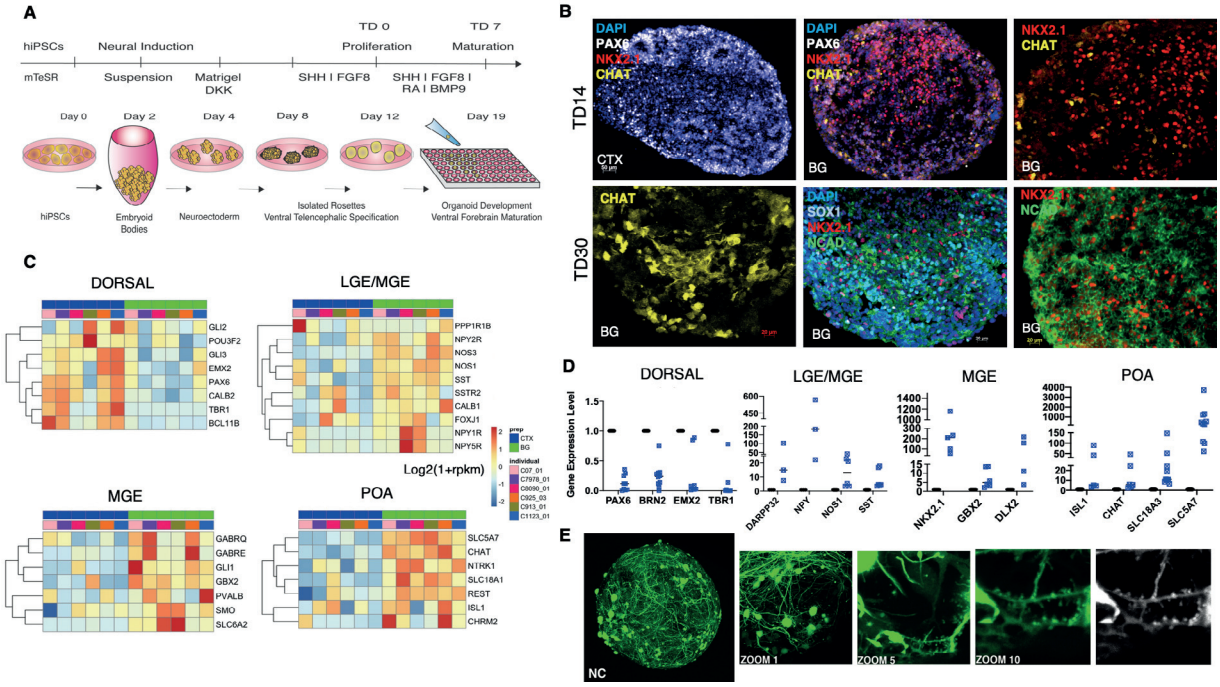


Figure 1: Organoids from iPSCs are enriched for ventral telencephalic development. (A) Schematic of the ventralized basal ganglia organoid protocol. (B) Immunostaining at terminal differentiation time points early TD14 and late TD30, showing increased expression exclusive to the ventral telencephalon. In contrast to the cortical organoids (Mariani, 2012), ventralized organoids have larger numbers of NKX2.1+/CHAT+ cells (putative MGE-type interneurons) and smaller numbers of PAX6+ cells (dorsal cortical progenitors and neurons). (C) Heatmaps of a set of DGEs from bulk RNA-sequencing analyses ($P \leq 0.05$) show the differential pattern of topographical (Dorsal, LGE, MGE, POA) gene expression in basal ganglia versus cortical organoids N=6 CTX; N=6 BG. Log2(1+rpkm) values were scaled in each row and heatmapped. (D) RNA sequencing findings are validated by qPCR. CTX samples are annotated in black; BG samples are annotated in blue. Statistics are done by Two-Way Anova analysis: Dorsal ($P < 0.0001$); LGE ($P=0.0063$); MGE ($P=0.0329$); POA ($P=0.008$). CTX is used as a reference (measuring as 1 in the graph); all quantifications were normalized to the housekeeping gene GAPDH. N=7 CTX; N=7 BG (E) Organoids were transduced at 6 months of age with SYN-GFP and visualized by two-photon microscopy to visualize overall health and spine development. (N=2)

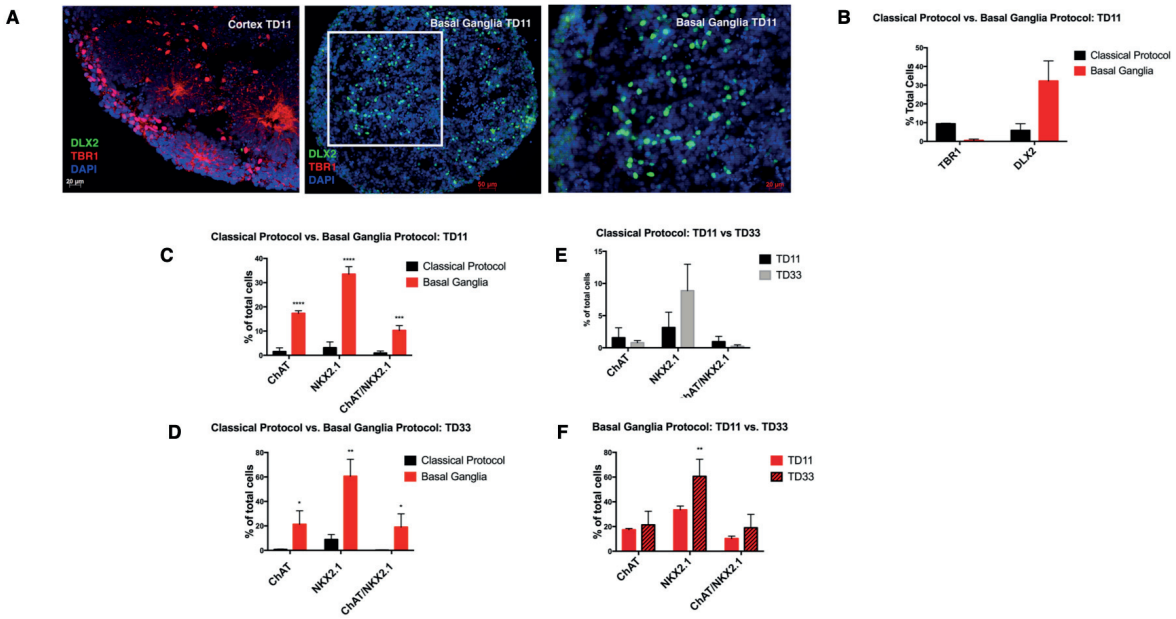
Immunostaining and Stereology Confirm a Ventral Commitment

My data show that the cocktail of ventral morphogens yields organoids consistently enriched with NKX2.1+ cells (>60% increase; p=0.03) and DLX1/DLX2+ cells at the RNA and protein levels (Figure 1B,D; Supplemental Figure S1). There is also a significant reduction in expression of the cortical progenitor transcription factors PAX6, BRN2, EMX2, TBR1 RNA and TBR1 and PAX6 protein (Figure 1B,D, Supplemental Figure S1). The majority of NKX2.1+ cells co-express the neuroepithelial marker N-Cadherin+, consistent with a medial ganglionic eminence (MGE) fate [349] (Figure 1B). Around 15-20% of the NKX2.1+ cells co-express CHAT, the enzyme choline acetyl transferase essential for acetylcholine synthesis, as quantified via stereological counts (Supplemental Figure S1). As the organoids mature, cholinergic expression at the protein level intensifies throughout the basal ganglia organoid, as evident by immunostaining at TD14 and TD30 (Figure 1B); this increase is quantified via stereological counts (Supplemental Figure 1). These findings remain consistent across three biological replicates (iPSC lines from separate individuals) and seven technical replicates.

Bulk RNA Sequencing Show Ventral Shifts in Gene Expression Profiles in Basal Ganglia-Like Organoids

To confirm on a global scale the ventral identity of our basal ganglia organoid preparation, we performed transcriptome analyses comparing dorsal cortex (CTX) (performed according to Mariani et al, 2015 [18]) and basal ganglia (BG) organoids at

TD14 differentiated in parallel from 6 biologically distinct control iPSC lines. Figure 1C shows expression levels in each biological replicate for selected marker genes of dorsal cortex, lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and pre-optic area (POA). Heatmaps were generated from manually curated gene lists based on general human neurodevelopmental literature and include only genes that meet the significance criteria for differential expression (DGE) across CTX and BG organoid preparations (FDR $\leq$ 0.05).. The data show that expression of dorsal genes is higher in cortical organoids when compared to their ventral basal ganglia counterparts (n=6 biological replicates). Instead, and comprehensively, the basal ganglia organoids show a strong bias for ventral telencephalic gene expression, including both LGE, MGE and anterior preoptic area (POA) development (Figure 1C). There is also increased expression of more caudally-derived genes representative of the caudal ganglionic eminence, as well (data not shown). As represented in the heatmaps, the basal ganglia organoids show both ventral progenitors and post-mitotic neuron gene expression, including various interneuron populations (NOS/SST/NPY, CHAT, PV) that are integral to the basal ganglia and important for TS disease modelling (Figure 1C) [8-10]. The basal ganglia organoids' transcriptome profiles show that they encompass GABAergic and cholinergic cell types, and those that ultimately respond to dopaminergic inputs, such as medium spiny neurons.



Supplemental Figure S1: Additional immunostaining comparing cortical and basal ganglia organoids and stereological counts. (A) Staining of ventral progenitor DLX and cortical TBR1 in cortical and basal ganglia organoids. (B) Stereological counts quantifying immunostaining in A. (C) Stereological counts of immunostaining for CHAT, NKX2.1 and their co-localization at TD11 between cortical and basal ganglia preparations. (D) C repeated at TD33. (E) Stereological counts quantifying immunostaining of cortical preparations comparing TD11 and TD33 for CHAT, NKX2.1 and their co-localization. (F) Stereological counts quantifying immunostaining of basal ganglia preparations comparing TD11 and TD33 for CHAT, NKX2.1 and their co-localization. Classical Protocol refers to the cortical preparation (Mariani et al, 2015)

Real Time qPCR Validate Ventral Shifts in Gene Expression Profiles observed via RNA Sequencing in Basal Ganglia-Like Organoids

Further quantification and validation of gene expression via real-time qPCR confirms appropriate ventral forebrain topography and cellular lineages in the ventralized organoids compared to the dorsal cortical organoids (Figure 1D). The expected reduction in dorsal telencephalic fate is observed with decreased expression of the cortical genes PAX6, BRN2, EMX2 and TBR1. In contrast, genes specific to LGE, MGE and POA fates are upregulated in the ventralized preparations (n=7, Figure 1D); this is

consistent across time points. Notably, genes consistent with medium spiny neuron development (DARPP32), as well as multiple interneuronal populations of both cholinergic (CHAT) and GABAergic types (SST, NOS1, NPY) are present in the ventralized organoid preparations. Additionally, cholinergic machinery is observed with the upregulation of the choline vesicular (SLC18A3) and high affinity transmembrane (SLC5A7) transporters (Figure 1C & 1D). Overall the data indicate that progenitors and neurons resembling LGE, MGE and POA are all represented within these organoids, suggesting a neuronal diversity complete with the first example of cholinergic expression in neural organoid cultures.

Two-Photon Imaging Shows that Basal Ganglia Organoids Thrive Long-term.

Additionally, organoids generated with the ventralized protocol can grow and remain healthily long-term in culture and develop functional morphology. Organoids were visualized at around six months (TD160-TD200 days) via two-photon microscopy, following transduction with a SYN-GFP lentivirus [350] to observe general neuronal development (Figure 1E). There is robust neuronal extensions throughout the BG organoids, as well as several healthy soma of various sizes. Dendritic branchings were observed throughout the organoids, as well as putative spine development (n=4 organoids, from 2 biologically different iPSC lines). Organoids survived up to 6 months in n=5 biological replicates (Figure 1E) and up to a year in n=2 biological replicates (data not shown).

Collectively, gene and protein expression in ventralized organoids are consistent with differentiation into ventral progenitors, indicative of MGE, LGE and POA development, as well as striatal post-mitotic neuronal development. Additionally, and importantly, dorsal gene expression is downregulated in the basal ganglia organoids, which is consistent with correct patterning and mimic mammalian (including human) neurodevelopment. Next, I applied this basal ganglia protocol to examine the early developmental underpinnings of TS.

Chapter Five:

Modeling Tourette Syndrome pathophysiology using a ventralized organoid strategy

While roughly 75% of TS patients' tic symptoms fade as they enter adulthood (remitted TS), 1/4 of TS patients experience persistent mild or severe symptoms of TS throughout the entirety of their lives (unremitted TS) [6, 71]. Pathogenesis of either patient category is largely unknown. Smaller caudate volumes in children with TS have served as a predictor of unremitting TS [7], and support the hypothesis that TS biologically manifests during early development. In this chapter, we utilized the BG organoid protocol described in Chapter 4 of this thesis (Chapter 4, Figure 1A) to compare the neurodevelopment of the ventral telencephalon between severe unremitting TS adult individuals (n=5) and healthy controls (CT, n=10) (Chapter 3, Table 1). To accomplish this, we recruited TS individuals from our Tourette/OCD clinic at the Child Study Center and generated iPSC lines using both integrating-non-integrating methods (See Chapter 3, Tables 1 & 2). This facilitated my subsequent experiments where these iPSC lines were differentiated into ventral telencephalic progenitors and compared to CT lines obtained from the Vaccarino lab iPSC biobank (see Table 1) using the BG organoid model (Chapter 4, Figure 1A).

Patient iPSC-Derived Basal Ganglia Organoids Show Disrupted Organization and Ventral Expression

In the following studies we used 5 biologically distinct iPSC lines for TS probands, with 2 distinct iPSC clones per individual, and 10 biologically distinct iPSC lines for CT. All lines were utilized for neuronal differentiation studies and analyzed at three time points: TD0, TD11 and TD30. All lines were used for RNA-seq studies, except that 4 biologically distinct TS iPSC lines (rather than 5) were used at TD0. For a general overview of TS vs CT organoid transcriptome profiles, samples were submitted for bulk RNA sequencing for transcriptome profiling across time for TD0, TD14 and TD30; a total of 2 submissions were made, but no batch effect was observed (See Chapter 3, Figure 2).

Overall DGE

When assessing the overall DGE profiles across patient and CT individuals there are general conclusions that can be made. The majority of differentially expressed genes that are upregulated are seen early on, at TD0 (309) (Figure 2A), while the majority of differentially expressed genes that are downregulated are seen at the latest stage, at TD30 (581) (Figure 2B). Additionally, there is little overlap between the differentially expressed genes (DEGs) identified at different developmental time points, suggesting that the changes in gene expression are generally time-specific. The data reveal that, when combining the three time points and direction of differential gene expression (up and downregulation), there are 1512 DEGs in total (Figure 2C). When disregarding time

and performing a combined DGE analysis across all data, there are only 881 DEGs, of which 705 overlap with the union of the time-specific DEGs (Figure 2C). Therefore, there are only 176 novel DGE additionally obtained from the combined analysis despite the increased power, again suggesting the time-specificity of the DGE.

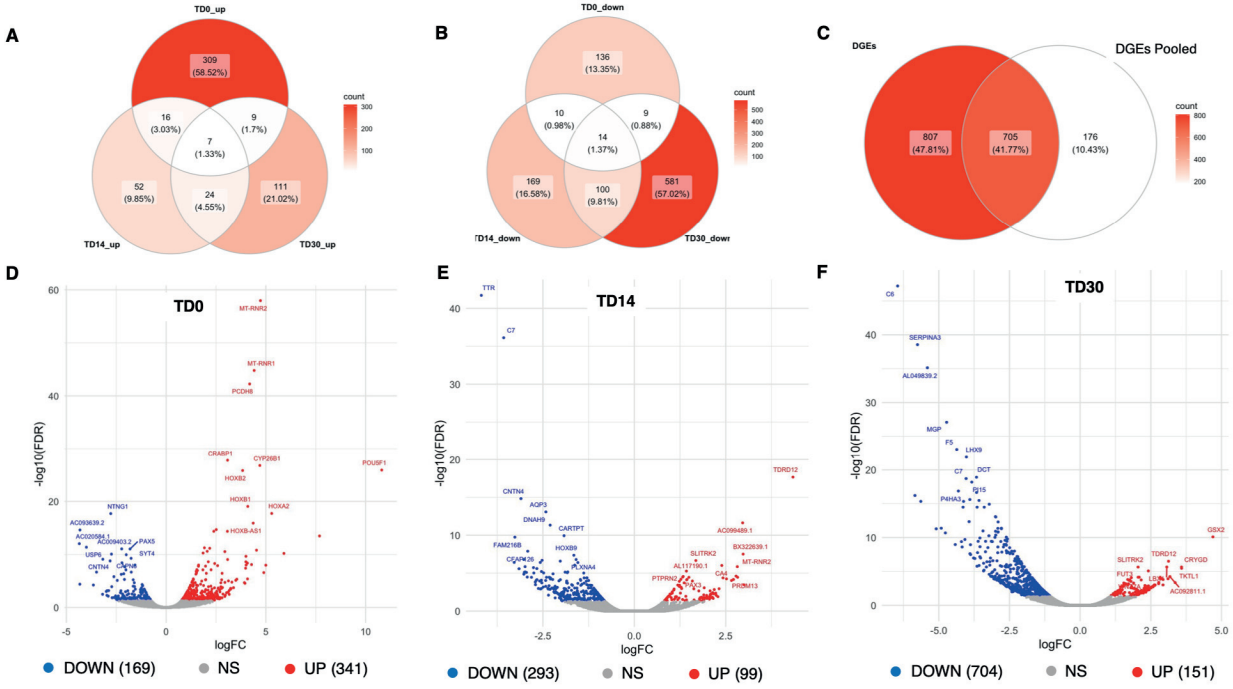


Figure 2: Bulk RNA-Sequencing analysis showing overall DGE when comparing TS vs CT across time. (A) A Venn diagram showing number of upregulated genes across TD0, TD14 and TD30. (B) A Venn diagram showing number of downregulated genes across TD0, TD14 and TD30. (C) A Venn diagram showing number of combined DGE from all 3 time points (red) vs DGE from a pooled analysis disregarding time (DGE_pooled). (D, E and F) DGEs plotted on volcano plots for TD0 (D), TD14 (D) and TD30 (F), using significance by corrected p value (Y axis, FDR) and fold change (X axis, logFC). Top most significant up and downregulated genes were labeled. For TD0, N=4 CT; N=5 TS. For TD14, N=5 CT; N=5 TS. For TD30, N=5 CT; N=5 TS.

The DEGs were then ranked by significance (y axis, -logFDR) and fold change value (x axis, logFC) and displayed in volcano plots across time, for TD0, TD14 and TD30. General conclusions can also be determined from these graphs. The trends observed from the Venn diagrams in panels A-C (Figure 2) are reiterated in the volcano plots, where the most upregulated genes in TS individuals are seen at TD0, while more downregulated genes in TS individuals are seen at TD30. Interestingly, at TD0, many of the most significantly upregulated genes by both FDR and fold change are HOX genes. These genes are mostly expressed in the brainstem and are therefore indicative of a posterior fate. At TD14, CNTN4 is among the top most significant downregulated genes in TS, a gene that has previously been linked with the disorder [23, 351]. With this foundational overview of developmental changes across TS and CT individual-derived basal ganglia organoids, the three time points are further explored in the following sections.

Functional Annotations of DGE Analyses

With general DGE lists generated across time as shown in Figure 2, we ran functional annotation analyses to bring greater understanding and context to their expression changes. Enrichment analyses among downregulated and upregulated genes were performed using the ConsensusPathDB platform from Max Planck Institute (http://cpdb.molgen.mpg.de/) for GO (Gene Ontology) terms at all three timepoints – TD0, TD14 and TD30. Focusing closely on the top 10 most significant (P value $\leq$ 0.05)

GO terms offered an unbiased approach to the understanding of developmental shifts identified in the TS-derived organoids.

TD0

Among the top 10 most significant downregulated GO terms for DGE at TD0 (Figure 3A), categories surrounding nervous system development and neurogenesis are generally evident. As current postulates in the TS field revolve around basal ganglia circuitry dysfunction, I took great interest in GO term #7, *Regulation of Trans-Synaptic Signaling* encompassing several DEGs related to synaptic function (Figure 3B), which will later be revealed as a consistent downregulated category across time. When looking at the top 10 most significant upregulated GO terms for TD0 (Figure 3C), additional neurodevelopmental categories are noted. In line with the observations seen in the volcano plot at TD0 (Figure 2D), GO term #9 identifies *Anterior/Posterior Pattern Specification*; when compiling the list of DEGs for this category, the lists reveal many HOX and WNT genes more highly expressed in TS-derived basal ganglia organoids than in CT (Figure 3D). This suggests an early preference for a posterior fate in the TS-derived organoids. Further categories include the manually curated *SHH signaling* for downregulated genes, and the additional GO term category of interest, while not among the top 10 most significant, *Midbrain patterning* (P Value = 3.23E-07) for upregulated genes (Figure 3E). Enrichment in these categories and expression trends of the related DEGs reiterate the potential decreased ventral commitment in the TS-derived basal ganglia organoids, with alternate fate preferences.

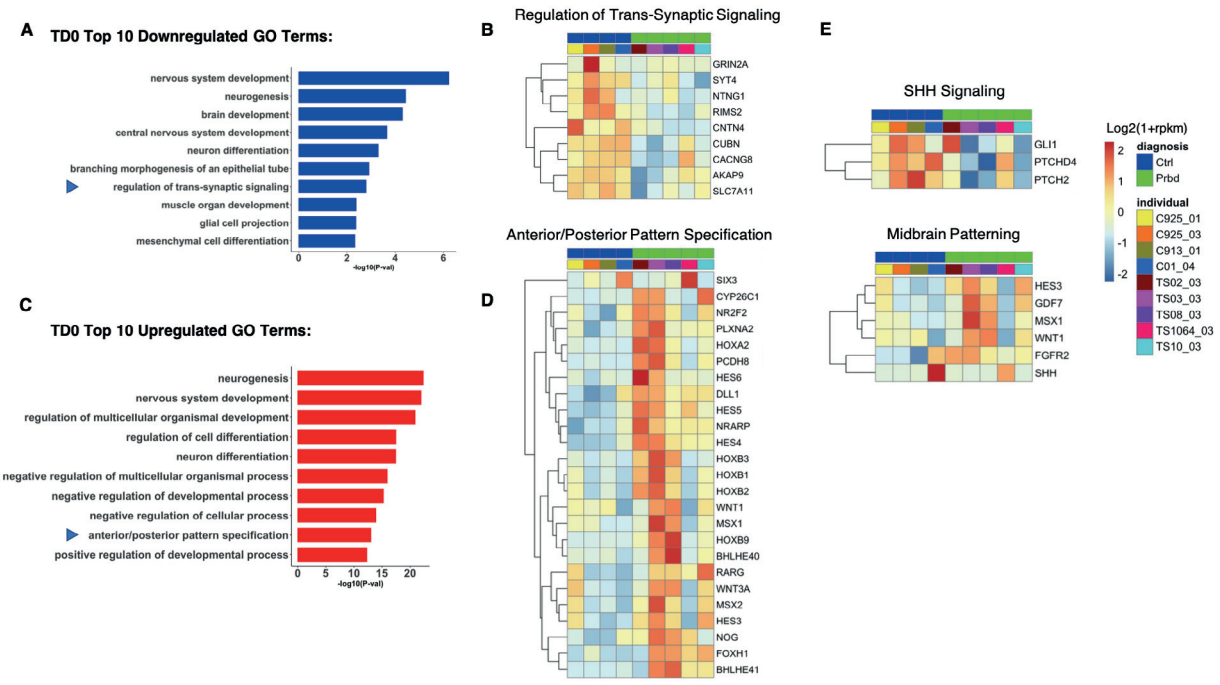


Figure 3: GO term analysis at TD0. (A) Top 10 most significantly downregulated GO terms that meet significance ($P \leq 0.05$). (B) Heatmap of the DGEs associated with the GO term #7 from top 10 from panel A. (C) Top 10 most significantly upregulated GO terms that meet significance ($P \leq 0.05$). (D) Heatmap of DGEs associated with the GO term #9 from top 10 from panel C. (E) Additional heatmaps of DGEs associated with relevant patterning categories from GO term analysis, SHH and Midbrain. For all TD0 RNA sequencing, N=5 TS; N=4 CT were submitted for analysis. All heatmaps show log2(1+rpkm) values were scaled in each row.

TD14

For TD14, experimental analysis was expanded to include three separate assays – immunocytochemistry, qPCR and RNA sequencing analyses. In line with a potentially compromised ventral telencephalic commitment seen at TD0 (Figure 3), my data reveal a strong cellular phenotype, with decreased NKX2.1 gene expression in TS basal ganglia organoids via real-time qPCR, confirmed by representative images of immunostaining (Figure 4A,B). To determine a potential consequence of this loss and identify disruptions in post-mitotic gene expression, we investigated the specific

cholinergic interneuron population via immunostaining to reveal an absence of CHAT expression in TS organoids (Figure 4A). I expanded our analyses to target the same topographical areas that I focused on during protocol optimization (Chapter 4, Figure 1C &1D) to reveal that significant patterning abnormalities are apparent at TD14 (Figure 4B & 4C).

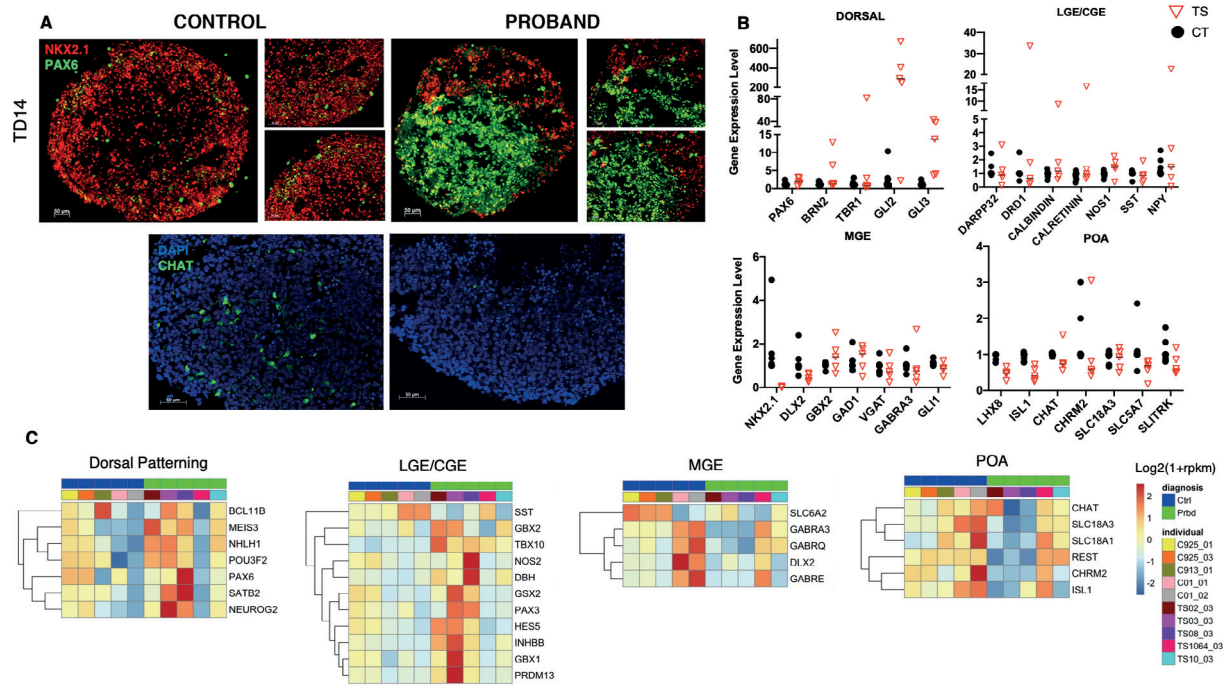


Figure 4: TD14 TS Basal Ganglia organoids show a decreased ventral patterning and increased dorsolateral commitment. (A) Representative immunostaining shows reduced NKX2.1 and CHAT expression in TS-derived basal ganglia organoids. Experiments were repeated with similar results (N=3 TS; N=3 CT). (B) Validation via qPCR analysis show TS organoids (red triangle) exhibit loss of NKX2.1+ expression, GABA and cholinergic interneurons, and increased expression of LGE and dorsal cortex markers as compared to CT (black circle). Statistics are done by Two-Way Anova analysis: Dorsal ($P < 0.0001$); LGE ($P=0.0108$); MGE ($P=0.0171$); POA ($P=0.0003$). N=5 TS (2 clones each = 10 lines); N=10 CT. (C) Heatmaps of a set of DGEs from bulk RNA-Sequencing analysis ($P \leq 0.05$) show topographical (Dorsal, LGE, MGE, POA) gene expression of basal ganglia organoids in comparison to cortical organoids. N=5 TS; N=5 CT. Log2(1+rpkm) values were scaled in each row and heatmapped.

Analyses of RNA via real-time qPCR show overall reduction in medioventral gene expression, disrupting both MGE and POA identities. Essential genes expressed in progenitors of these regions, including NKX2.1, DLX, LHX8 and ISL1, show reductions consistent across all 5 TS individuals (Figure 4B), along with post-mitotic neuronal machinery of both GABAergic and cholinergic interneurons which are derivatives of the MGE and POA and observed via both qPCR (Figure 4B) and RNA sequencing (Figure 4C). Remarkably, the medioventral loss is accompanied by an upregulation of dorsolateral gene expression, enhancing dorsal and LGE identities within the TS basal ganglia organoids, and observed via both qPCR (Figure 4B) and RNA sequencing (Figure 4C).

With the developmental deficits apparent so strongly at TD14, suggesting mispatterning and topographical shifts in telencephalic growth, I wanted to investigate further at the RNA sequencing scale. Functional annotation analyses were completed for TD14 (Figure 5) to explore the biological significance of the transcriptional alterations in the TS individuals. The top 10 most significant downregulated GO terms (Figure 5A) revolve heavily around cellular development and signaling components, such as cilia. I first focused on GO term #7, which is *Extracellular Matrix Organization* (Figure 5B). Many of these DEGs are collagen and laminin-related, all of which are significantly reduced in expression in TS-derived basal ganglia organoids. Although the significance of this finding is not yet fully clear, these downregulated genes could suggest a potential reduction in cellular stability and support, an altered cell polarity (which has been

postulated in TS [82] as well as a hindrance of complex cellular signaling [264, 352, 353]. A deeper look into the GO terms reveal additional signaling categories, including *Trans-Synaptic Signaling* (P Value = 0.00833) (Figure 5C) and *Serotonergic Signaling* (P Value = 0.00668) (Figure 5D), both of which show significant enrichment among downregulated genes. The Trans-Synaptic Signaling gene list is more elaborate now at TD14 than what was seen at TD0, and the consistency in this category across time is important to note. Additionally, serotonergic neurons traverse the human brain, extending from the hindbrain and innervating various regions of the cerebrum, including the basal ganglia. Deficits in serotonergic expression have previously been described in TS transcriptome [10] and serotonergic signaling has been previously implicated in TS [354-357], making this category of particularly high interest. When examining the top 10 significantly upregulated GO terms (Figure 5E), additional neurodevelopmentally-related categories appear. Focusing on both *Embryonic Axis Specification* (GO term #3) (Figure 5F) and *Nervous System Development* (GO term #7) (Figure 5G), posterior WNT and PAX3 genes are upregulated in TS, as well as the LGE-specific GSX2 gene. These enrichments further signify a mispatterning away from the typical anterior-ventral telencephalic fate in the TS-derived basal ganglia organoids. Further, another upregulated GO term interesting to note is the *Glycosphingolipid Synthesis* gene category (Supplementary Figure S2). While there are only two genes identified for this category, the expression trend is strong and consistent across all 5 TS-derived basal ganglia organoids, with very little variability. This category is interesting due to its past linkage to Parkinson's Disease, both a movement disorder and one whose etiology is

rooted in the basal ganglia [358]. These parallel with TS emphasizes Glycosphingolipid Synthesis as a strong contender for future investigation.

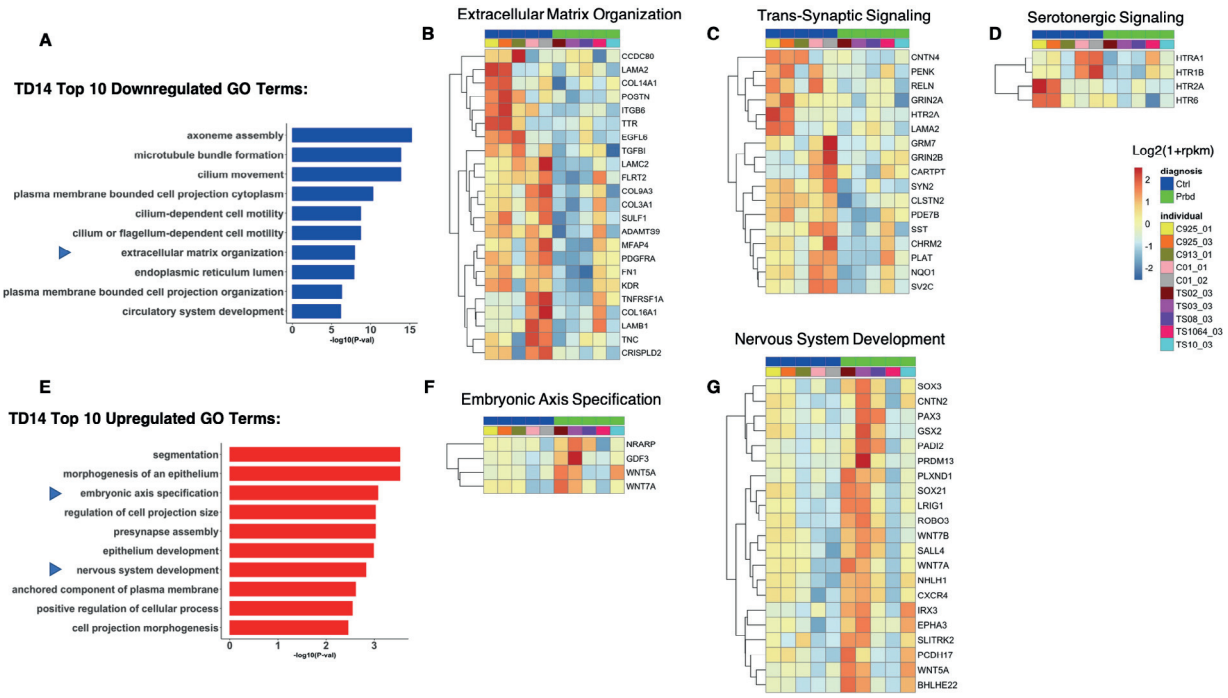


Figure 5: GO term analysis at TD14. (A) Top 10 most significantly downregulated GO terms that meet significance ($P \leq 0.05$). (B) Heatmap of the DGEs associated with the GO term #7 from top 10 from panel A. (C) Heatmap of the DGEs associated with the additional GO terms for relevant signaling disruptions, Trans-Synaptic and Serotonergic Signaling (D) Top 10 most significantly upregulated GO terms that meet significance ($P \leq 0.05$). (E and F) Heatmap of the DGEs associated with the GO term #3 (E) and GO term #7 (F) from top 10 from panel C. (G) Heatmap of the DGEs associated with the additional GO term for gene category previously linked with movement disorders, Glycosphingolipid Synthesis. For all TD14 RNA sequencing, N=5 TS; N=5 CT were submitted for analysis. All heatmaps show log2(1+rpkm) values were scaled in each row.

TD30

For TD30, for emphasis on consistent expression trends and categories, I will begin with the description of the top 10 most significant upregulated GO terms (Figure 6A). Immediately apparent, categories signifying caudal expression in the TS-derived basal ganglia organoids are plentiful even among only the top 10. There are two different spinal cord-related categories, and the #1 most significant GO term is *Spinal Cord*

Development (Figure 6B), where the list of DEGs in intersection further indicates a posterior preference. The #2 GO term is *Dorsal/Ventral Pattern Formation* (Figure 6C), where the listed upregulated DEGs in TS favor a dorsal fate. These two gene sets reiterate the previously suggested fate shifts within the TS-derived basal ganglia organoids whereby the cells show a preference for a dorsal and posterior fate, instead of a ventral-anterior one.

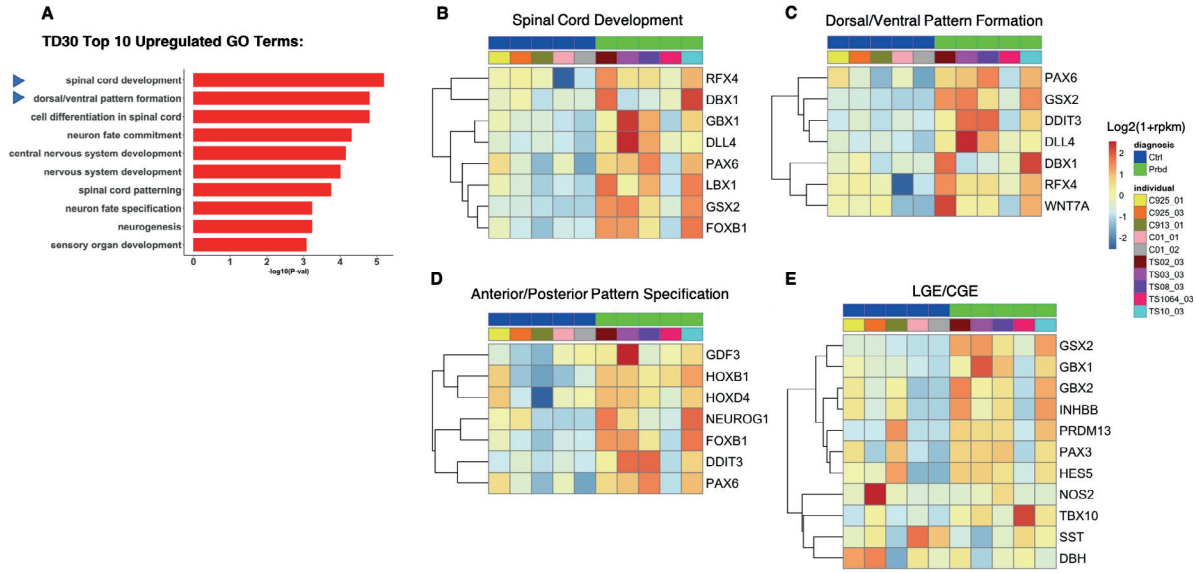
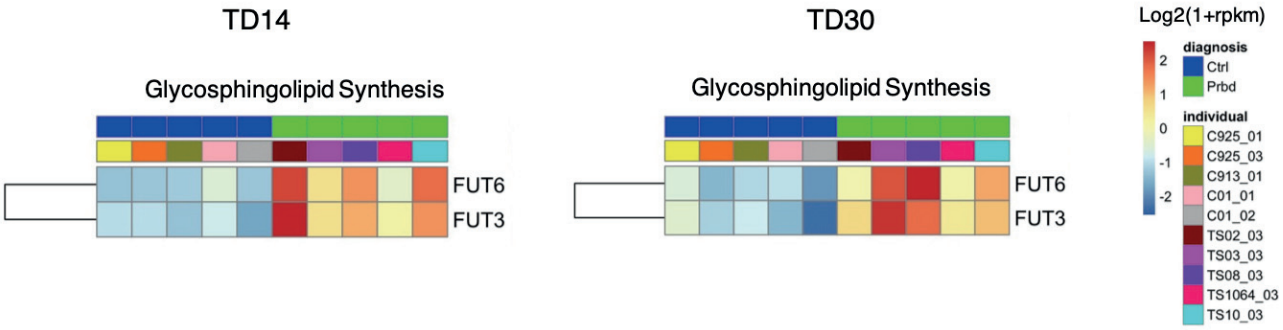


Figure 6: Top Upregulated GO term analysis at TD30. (A) Top 10 most significantly up-regulated GO terms that meet significance ($P \leq 0.05$). (B) Heatmaps of the DGEs associated with the GO terms #1 and (C) #2 from top 10 from panel A. (D) Heatmaps of the DGEs associated with the additional GO term for relevant patterning disruptions, Anterior/Posterior Pattern Specification. (E) Heatmap of the manually curated DGEs associated with the LGE emphasizing addition patterning shift. For all TD30 RNA sequencing, N=5 TS; N=5 CT were submitted for analysis. All heatmaps show log2(1+rpkm) values were scaled in each row.

Additional support for this comes from the *Anterior/Posterior Pattern Specification GO term* (P Value = 4.43E-05) (Figure 6D), which is not in the top 10 Go terms, but where the genes favor a posterior fate with consistent upregulations in HOX genes. A curated gene list for *LGE/CGE development* (Figure 6E) further confirms an apparent fate shift

away from a ventral-anterior fate with upregulations in the lateral GSX2, and the caudal GBX2, PAX3 and TBX10 among the list. Additionally, *Glycosphingolipid Synthesis* appears as another GO term not in the top 10 (Supplementary Figure S2), with the same two genes expressed with similar strikingly evident trends of upregulation in the TS-derived basal ganglia organoids. The consistency across these categories, spanning from early organoid formation to later more mature time points, brings greater emphasis to the altered transcriptional programming and mispatterning evident in TS-derived basal ganglia organoids.



Supplemental Figure S2: Glycosphingolipid Synthesis. Enrichment for the GO terms Glycosphingolipid Synthesis across TD14 and TD30 show consistent upregulation across all 5 TS samples. For all TD14 and TD30 RNA sequencing, N=5 TS; N=5 CT were submitted for analysis. All heatmaps show log2(1+rpkm) values were scaled in each row.

To begin the examination of downregulated genes at TD30, I am highlighting among the top significant GO terms (Figure 7A) GO term #1, *Extracellular Matrix* Organization, which is an extension of what was defined at TD14 (Figure 7B) and GO term #6, *Regulation of Cell Motility* (Figure 7C). These two categories are related in their roles of cellular development, stability and signaling, and also in their expression trends, where

the downregulation observed in TS samples is driven largely by two control samples with strong expression of the listed genes. However, for the consistency in the conceptual relationship to this thesis, I felt these unbiased gene lists were important to describe.

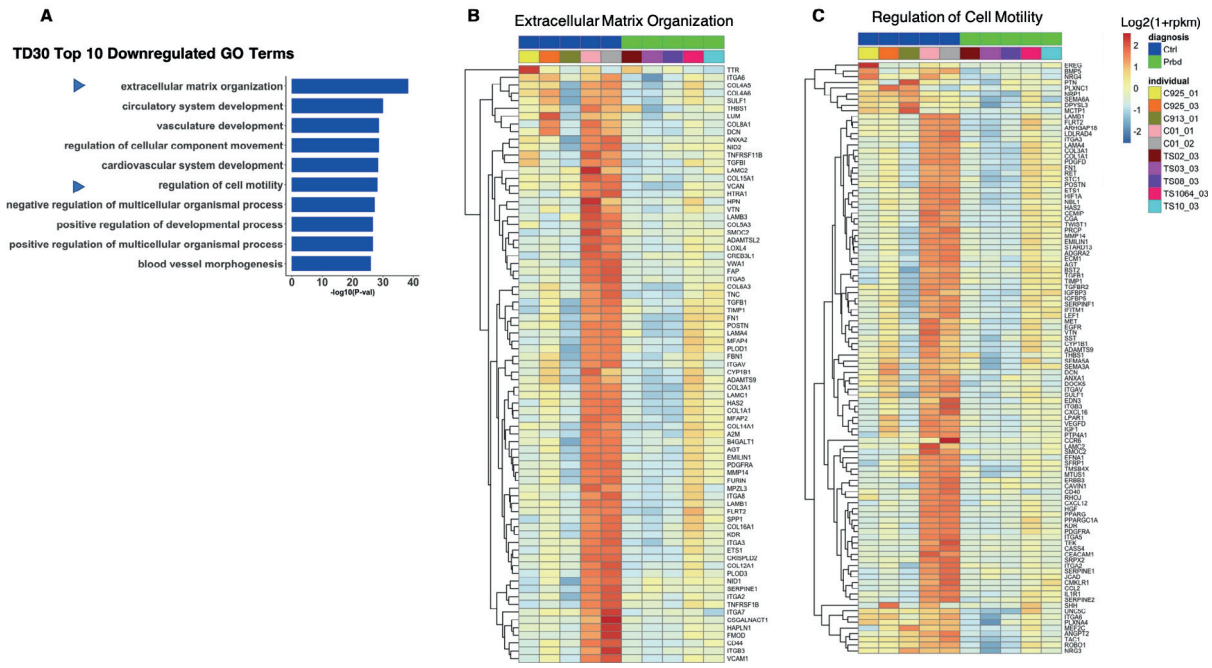


Figure 7: Top downregulated GO term analysis at TD30. (A) Top 10 most significantly downregulated GO terms that meet significance ($P \leq 0.05$). (B) Heatmaps of the DGEs associated with the GO terms #1 and #6 from top 10 from panel A. For all TD30 RNA sequencing, N=5 TS; N=5 CT were submitted for analysis. All heatmaps show log2(1+rpkm) values were scaled in each row.

When looking deeper at more clinically relevant downregulated GO term categories, several are integral to TS etiology, even though they are not among the top 10 categories. Beginning with *Tran-Synaptic Signaling* (P Value = 3.91E-08), what can be appreciated from this list (Figure 8A) is its abundance in comparison to that at TD14 or TD0. The consistency not only across time but also in this category's potential role in the TS-derived basal ganglia organoids' development is remarkable. Building off of the

conceptual significance noted at TD0, deficiencies in synapse development and hence trans-synaptic signaling potentially have a profound role in TS.

With basal ganglia developmental dysregulation at the forefront of the field's general understanding of disorder causality, synaptic signaling becomes an important piece of the TS story when determining the ability of multiple brain regions within the cortico-striatal-thalamic circuit to cross-talk effectively, especially in view of the hypothesized patterning disruption and ensuing differences in neuronal subtypes.

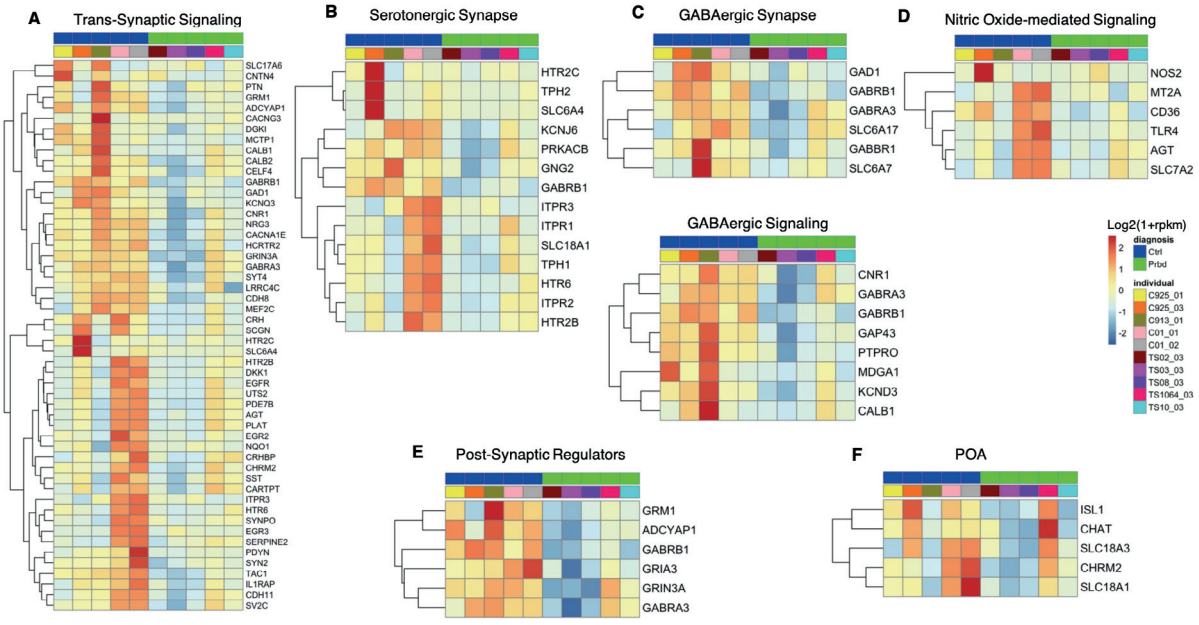


Figure 8: Additional downregulated GO term analyses at TD30. (A) Heatmap of the DGEs associated with the Trans-Synaptic Signaling (B) Heatmap of the DGEs associated with the Serotonergic Synapse (C) Heatmap of the DGEs associated with the GABAergic Synapse and Signaling (D) Heatmap of the DGEs associated with the Nitric Oxide-mediated Signaling. (E) Heatmap of the DGEs associated with the Post Synaptic Regulators (F) Heatmap of the manually curated DGEs associated with POA. For all TD30 RNA sequencing, N=5 TS; N=5 CT were submitted for analysis. All heatmaps show log2(1+rpkm) values were scaled in each row.

Further support of these points are that multiple types of synapses are downregulated in the TS-derived basal ganglia organoids. For instance, the *Serotonergic Synapse* term (P Value = 0.00313) (Figure 8B) is another large gene list, much expanded from TD14. Additionally, *GABAergic Synapse and Signaling* (P Value = 2.30E-05) (Figure 8C) are both perturbed in TS-derived basal ganglia organoids. *Nitric Oxide-mediated Signaling* (P Value = 0.00134) (Figure 8D) is also downregulated across TS individuals, which agrees with deficiencies in number of NOS-containing interneurons found in adult basal ganglia of TS individuals (Lennington et al; Chapter 1B, Fig. 11.2). *Post-Synaptic Regulators* (P Value = 1.78E-05) is another GO term aligning with these classifications (Figure 8E). Finally, genes specific for *POA* development continue to be significantly downregulated as represented in a curated list and corresponding heatmap (Figure 8F). In conclusions, the data support that signaling of serotonergic, GABAergic and specific interneurons such as the cholinergic and NOS types is perturbed in TS-derived basal ganglia organoids. These developmental disruptions affect both what is produced inherently by the basal ganglia (GABAergic; nitric oxide; cholinergic), and what is received by it (serotonergic). Finally, revisiting the overall DGE analyses from Figure 2, it is relevant to reiterate that the overlap of DGE across time is minimal. Therefore, while several of the gene categories are consistent over the three time points – TD0, TD14 and TD30 – the gene lists vary substantially and further underscore their functional convergence even if there is minimal intersection across time.

Expression Trends Align with Adult Human TS Post-Mortem Data

Previously published transcriptome data of adult human TS post-mortem brain tissue [10] has served as a foundation and motivation for this study, the experimental trajectory and the data described in this thesis. I referenced the previously published DEGs from post mortem TS tissue [10] with those obtained from the TS-derived basal ganglia organoids to determine overlap between them.

	Gene	Gene Name	Strategy	FC	FDR	P Value
Cholinergic	CHRM2	Cholinergic receptor, muscarinic 2	Postmortem	-1.99	4.32E-04	
			Organoid - TD14	-1.49	0.038147	0.000577
			Organoid - TD30	-1.44	0.007725	0.000158
	NTRK1	Neurotrophic tyrosine kinase receptor, type1	Postmortem	-1.91	0.00117	
			Organoid - TD14	0.17	0.999993 - NS	0.676473
			Organoid - TD30	-1.66	0.050879	0.00185
GABAergic	GAD1	Glutamate decarboxylase 1 (brain, 67 kDa)	Postmortem	-1.64	0.0121	
			Organoid - TD14	0.14	0.999993 - NS	0.722477
			Organoid - TD30	-1.61	0.002681	0.0000425
	GABRA3	Gamma-aminobutyric acid A receptor, alpha 3	Postmortem	-1.96	0.000585	
			Organoid - TD14	-0.93	0.128552 - NS	3.26E-03
			Organoid - TD30	-1.32	0.015885	0.000412
	SST	Somatostatin	Postmortem	-1.59	0.00442	
			Organoid - TD14	-0.93	0.021343	0.000268
			Organoid - TD30	-2.07	0.000000669	2.88E-09
Serotonergic	HTR2C	5-hydroxytryptamine (serotonin) receptor 2C, G protein-coupled	Postmortem	-1.62	0.00744	
			Organoid - TD14	-0.51	0.999993 - NS	2.90E-01
			Organoid - TD30	-2.31	0.000122	0.00000107

Table 1: Expression trends overlap with adult human TS post mortem Data. A manually curated gene list from TS post mortem brain tissue was compared with DEG from TS basal ganglia organoids to find overlap in gene categories. Strategies listed for comparison include postmortem tissue analysis and two organoid time points – early TD14 and late TD30. FC, fold change; FDR corrected p value, false discovery rate.

From a total of 1131 DEGs obtained from post mortem TS tissue, 20 were manually curated and highlighted as particularly interesting in our previous publication (see Table 1 in Lennington et al [10]); of these 20 manually curated DEGs, 6 were found to overlap

in the same direction with DEGs obtained from the TS organoids (Table 1). The gene categories also follow both the post mortem classifications as well as the aforementioned sequencing GO term analyses. These include cholinergic, GABAergic and serotonergic genes. This table offers further support for the findings and interpretations outlined in this chapter.

Summary

These data suggest a potential shift away from an anterior-medial-ventral fate in TS patient-derived organoids. Concordant with the hypothesis of an MGE deficit in TS organoids, these data reveal an increase in some cortical and LGE genes, while also revealing a preference for a posterior fate within the TS BG organoids as compared to healthy CT-derived basal ganglia organoids, differences that are consistent across all three time points. Multiple assays at both RNA and protein levels further confirm this cellular signature. The gene categories affected a range of different cell types both present within the basal ganglia and those that, in the human brain, send projects to this subpallial region. The gene trends also follow previously published trends seen in adult human TS post mortem brain tissue. Together, these data show that deficits observed at the adult level are also seen at the embryonic/fetal level, and possibly arise from patterning disruptions during neurodevelopment.

Chapter Six:

Mechanism of mispatterning in Tourette Syndrome

Altered SHH Pathway and Primary Cilium Development in Basal Ganglia Organoids from TS Patients

Given the significant deficit in ventral telencephalic development observed within the TS-derived BG organoids, I next wanted to investigate possible contributors to the detected developmental deficit. I focused on the SHH pathway because of its central involvement in basal ganglia development and its alterations evident in organoid transcriptomes. SHH signaling transduction takes place within microtubular organelles called primary cilia [260, 359, 360]. During embryogenesis, primary cilia protrude from nearly every vertebrate cell, conducting signal transduction. Because of their connection with SHH signaling, primary cilia are critical for facilitating dorsoventral patterning within the telencephalon [361]. The SHH pathway exists in two states with opposing functions (Figure 9A). In the dorsal telencephalon, the SHH pathway is in its inactive state. The SHH ligand is minimally expressed dorsally [362]; in its absence, the 12-transmembrane SHH receptor, Patched 1 (PTCH1), inhibits the 7-transmembrane G-protein coupled receptor Smoothened (SMO), thwarting its accumulation at the cilium surface [362, 363]. With SMO repressed, two intracellular GLI transcription factors, GLI2 and GLI3, are phosphorylated and then proteolytically lysed to generate truncated repressor forms of both proteins, GLI2R and GLI3R [362]. Subsequently, these repressor proteins translocate to the nucleus of the cells where they inhibit transcription of downstream

SHH target genes (Figure 9A). The inhibition of the SHH signaling pathway in the dorsal telencephalon allows cortical gene expression to thrive by default, resulting in dorsal specification of this domain. Instead, within the ventral telencephalon, SHH ligand is potently expressed. In the presence of the SHH ligand, the PTCH1 receptor relieves its inhibition of SMO [5]. With SMO now active, GLI1 is the dominant intracellular transcription factor, which ultimately translocates in its full-length form into the nucleus, where it activates transcription of downstream SHH genes (Figure 9A). The activation of the SHH signaling pathway in the ventral telencephalon activates the NKX2.1 gene within ventral progenitors that give rise to the post-mitotic neurons that are exclusive to the basal ganglia [5].

I focused on these important proteins to determine if potentially disrupted SHH signaling in the TS patient-derived BG organoids could explain the patient organoid mispatterning phenotype observed. To move forward with these analyses, I concentrated on the early culture time point, TD0. To briefly review, in the development of the organoids, TD0 marks the 4th day post-exposure to SHH and FGF8 (Figure 1A), an incubation period responsible for triggering a ventral telencephalic cellular commitment and regional identity. It is only after prolonged exposure to high concentrations of these morphogens that downstream SHH targets begin to be transcribed [4]. With this reasoning, TD0 was the logical time point to investigate potential differences in the proteins that orchestrate downstream SHH signaling, which could potentially underlie the phenotype observed at the later TD14 time point.

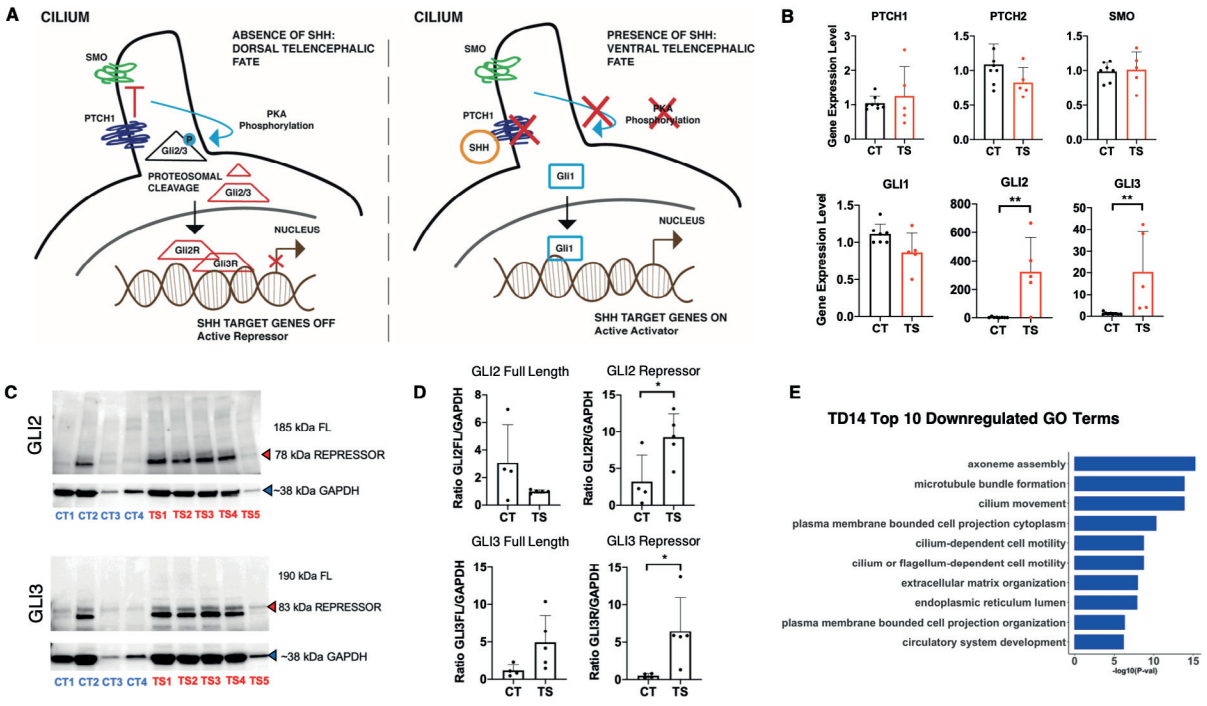


Figure 9: Upregulation of GLI genes and disruption of cilia in TS-derived basal ganglia organoids. (A) Schematic of SHH signaling in dorsal and ventral contexts. (B) qPCR from TD0 examining expression levels of key SHH signaling players. (C) Western blots to determine expression of GLI Repressor proteins in TS-derived basal ganglia organoids. (Please see Supplemental Figure 3 for additional western blots with Full Length (FL) expression) (E) Protein bands quantified by ImageJ software (N=4 CT; N=5 TS) (E) Revising top 10 most significantly downregulated GO terms that meet significance ($P \leq 0.05$) at TD14 showing ciliary disruptions. For all TD14 RNA sequencing, N=5 TS; N=5 CT were submitted for analysis.

By real-time qPCR, while expression level of the surface SHH receptors were not significantly altered, I found altered GLI protein expression. Across all 5 TS patients, their BG organoids showed significantly increased expression of the GLI2 and GLI3 genes (Figure 9B), both of which can act as repressors of the SHH pathway (Figure 9A). However, RNA expression cannot determine whether or not the truncated repressor form of the protein is expressed. Therefore, to examine levels of the potential repressor forms of the GLI2 and GLI3 proteins, I performed western blots on organoids

from the TS and NC cohort (Figure 9C). Remarkably, prominent bands for the truncated repressor forms of GLI2 (~78 kDa) and GLI3 (~83 kDa) were observed and significantly increased in expression across the TS patients when compared to the control individuals (Figure 9C). The expression was quantified and first internally normalized to the GAPDH expression per individual prior to comparing CT and TS expression overall (Figure 9D quantified via ImageJ software; For additional blots, see Supplementary Figure S3). The expression of GLI repressor proteins in controls are expectedly low, in line with typical human ventral telencephalic development. The increase in GLI repressor protein expression in TS is in line with the evident dorsal and posterior fate preference observed in organoids from these individuals.

To explore this further, I revisited the TD14 RNA sequencing data, where the GO pathway analyses revealed that the top 10 downregulated categories were largely of a ciliary nature (Figure 9E). Given the significance of cilia in SHH signaling, and the potential alteration in downstream effectors of SHH signaling with upregulated GLI repressor protein expression in the TS organoids, the genes listed across all of the cilia-related GO terms were pooled to create one single heatmap (Figure 9 Continued, F & G). These genes tend to be downregulated across the TS-derived basal ganglia organoids. While some of the genes are largely driven by two CT individuals, the elaborate list also shows greater distribution across all 5 CT individuals overall as well.

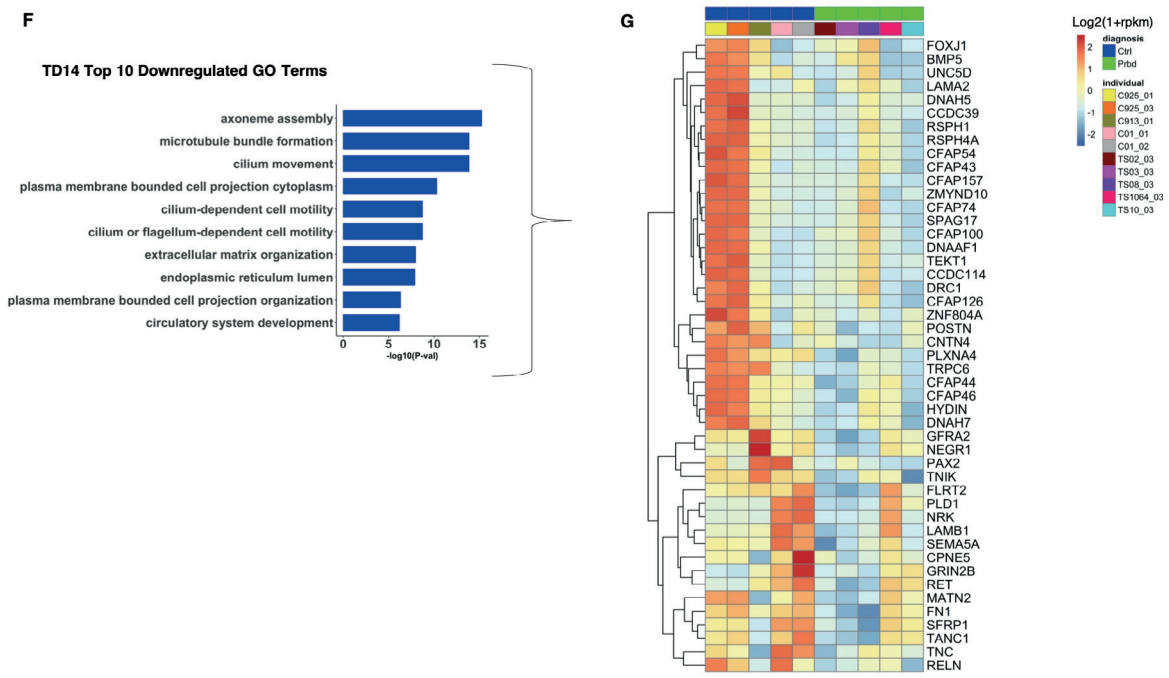
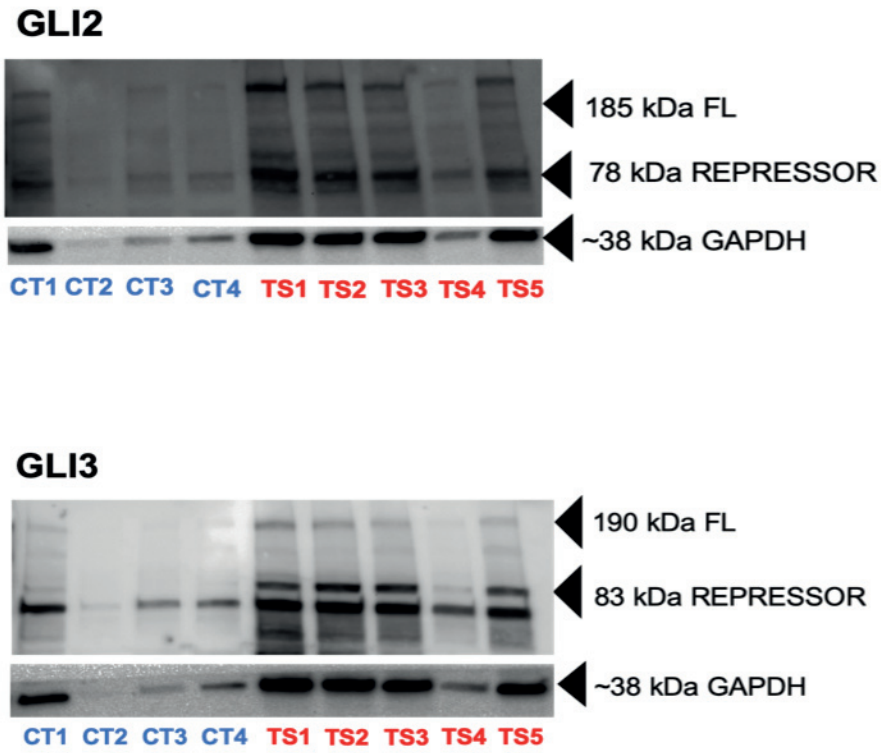


Figure 9 Continued: Upregulation of GLI genes and disruption of cilia in TS-derived basal ganglia organoids. (F) Top 10 most significantly downregulated GO terms that meet significance ($P \leq 0.05$) at TD14. (G) Heatmap of the DGEs associated with the union of all ciliary GO terms from top 10 in panel A. All heatmaps show log2(1+rpkm) values were scaled in each row. For all TD14 RNA sequencing, N=5 TS; N=5 CT were submitted for analysis.

Taken together, these data suggest that cilia formation and development is altered in TS-derived basal ganglia organoids, with related SHH signaling alterations. The TS-derived basal ganglia organoids show increased GLI Repressor protein expression for both proteins, GLI2 and GLI3, which are typically exclusive to a dorsal fate. In the presence of GLI Repressors, SHH signaling is inhibited, which can explain the mispatterning observed across all 5 of the TS individuals, where the derived basal ganglia organoids favor a dorsolateral and posterior neural development rather than the expected medioventral anterior neural development seen in the CT individuals. Further,

the ciliary disruptions observed are concentrated at TD14, suggesting the long term alterations of this critical cellular component. Finally, with their roles in cellular signaling, the ciliary disruptions – in both cilia development and SHH signaling capability – also support the observed trans-synaptic deficits seen across all three developmental time points within the TS basal ganglia organoids.



Supplemental Figure S3: **Additional GLI western blots**. These western blots for GLI2 and GLI3 show the full length band present.

Chapter Seven:

Discussion

This thesis offers a new bioassay to model basal ganglia development and provides the first investigation of TS in organoids. This work details a system for studying both normal and abnormal neurodevelopment. Utilizing iPSC technology, I generated basal ganglia organoids that show transcriptional programming distinct from cortical organoids (Chapter 4, Figure 1) [18]. The basal ganglia organoid protocol was then applied to examine TS. The recruited adult TS patients were diagnosed with persistent unremitting TS, with severe tic symptoms. Unremitting TS accounts for a subset of TS patients that correspond to approximately only 25% of TS diagnoses. Although TS is considered a heterogenous neurodevelopmental disorder with few linked mutations (See Chapters 1 & 2), I identified neuronal deficits at both RNA and protein levels during an early developmental period that showed consistency across the five TS patients, as well as over three separate time points. Transcriptome analyses uncovered alterations in gene expression indicative of early telencephalic patterning disruptions that were seen across various stages of organoid development. Further, gene expression for proliferating progenitors and post-mitotic neuron types specific to the basal ganglia were reduced. Additionally, synaptic development appeared to be decreased in the TS-derived basal ganglia organoids, as well.

When examining transcriptome-based functional annotations, I found differences in early regional specification between the CT and TS-derived basal ganglia organoids. In contrast to the CT organoids, the TS-derived basal ganglia organoids favored dorsal-posterior patterning, a phenotype observed at all three time points analyzed. These changes come from an apparent upregulation in both HOX and WNT signaling gene expression. The observed early preference for dorsal-posterior patterning – away from a ventral-anterior commitment – culminates in an enrichment for spinal cord development among upregulated DEGs in TS organoids at TD30. Importantly, these data are also consistent with the topographical shift observed by qPCR and RNA-sequencing, showing enhanced dorsal and LGE expression, and reduced MGE and POA expression in TS organoids. The deficit in ventral commitment is at least partially attributable to an early downregulation of SHH signaling genes seen by transcriptome analyses, and concordant loss of NKX2.1 expression seen at both RNA and protein levels.

In addition to significant and consistent patterning alterations, I also found the expression of several neuronal subtypes to be downregulated in the TS-derived basal ganglia organoids. Most notably, deficiencies were seen across GABAergic, cholinergic and serotonergic genes. Expression of nitric-oxide (NOS) -related genes were also downregulated; NOS is a category of striatal interneurons, and this observed deficit in the organoid transcriptome reiterates findings from post mortem TS tissue [10]. Further, a broad but elaborate list of trans-synaptic signaling genes were also downregulated in the TS-derived basal ganglia organoids, indicating potential functional consequences in

these cultures. Previous transcriptome analyses of post mortem TS tissue show decreases in gene expression fitting GABAergic, cholinergic and serotonergic neuronal types. I found an interesting convergence of DEGs from the TS-derived organoids with the post mortem data. I did not expect to find a complete convergence of genes given the two distinct timepoints at the time of analysis – one representing early development while the other is adult post mortem. However, what can be appreciated from this data is the physiological relevance of the bioassay utilized for this study. Through this work, we see not only a portion of what is true in the human brain (and what is seen to be true for TS individuals) recapitulated in organoids, namely, decrease in markers for SST, NOS and cholinergic neurons, and a number of synaptic signaling related genes, but we also see an expansion of these findings to implicate early patterning genes that are instead not expressed in the adult brain. The two findings may be related to each other in the sense that the patterning defects may in part explain interneuron deficit—although further experiments are necessary to prove this point. In conclusion, the in vitro data are consistent with known roles for GABAergic, cholinergic and serotonergic genes in adult human TS, as well as insightful with the introduction of new biomarkers of interest.

Given the patterning disruptions observed in the TS-derived organoids across time in conjunction with the deficits in the differentiation and maturation of particular cell types, I investigated a potential mechanism for the regional and cellular phenotypes. When examining critical components of SHH signaling transduction, two abnormalities were found: alterations in cilia development and function, as well as upregulation of GLI2 and

GLI3 repressor proteins in the TS-derived basal ganglia organoids. There is much to be considered with these findings, most notably the fact that ciliary disruptions are upstream of SHH signaling, while GLI production is a downstream component. While further investigation is required, I can offer postulates for the described data. The field details that, in the dorsal telencephalon, active PTCH inhibits the ability of SMO to accumulate in the cilium [362, 363]. Upon receptor binding, SHH inhibits PTC therefore allowing SMO cilia sequestration, which in turns triggers downstream components of the SHH pathway, including inhibition of PKA and GLI1 transcription (Chapter 6, Figure 9A). While SMO is not seen to be altered when comparing the CT and TS organoids at the RNA level, this does not confirm that SMO is ultimately appropriately sequestered to the cilium upon SHH treatment. Although it may be sufficiently transcribed, an insufficient sequestering of SMO receptor protein to the cilium in response to SHH in TS organoids could be a potential consequence of cilia malformation and malfunction. Continuing with this theory: a reduced concentration of SMO at the cilia may impair reaching a threshold of activation to ultimately induce GLI1 production and signaling, as my data also reveal early downregulation of GLI1 at the transcriptome level in TS organoids. Without sufficient cilia sequestration/activation of SMO, PKA phosphorylation of GLI2/3 proteins can still occur, flagging the GLI2/3 proteins for proteolytic lysing, as demonstrated by Western blot analyses in TS organoids. Post-translational modifications, such as phosphorylation, are known to be feedback regulators of cellular processes [364]. As such, PKA phosphorylation of GLI2 and GLI3 may initiate a positive feedback loop to engage further transcription of GLI2 and GLI3, which can explain the upregulation in

gene expression levels seen by qPCR further feeding the increased GLI repressor production for both GLI2 and GLI3 show in TS organoids. The field has a long history of evidence to show that GLI repressor activity inhibits ventral telencephalic development, and overexpression of GLI2 and GLI3 repressors lead to an expansion of dorsal commitment in ventral territory [315]. My data show a patterning shift in the TS-derived organoids away from a ventral-anterior commitment and toward a dorsal-posterior one, consistent with the field's understanding of GLI repressor activity. Therefore, my data suggest an overall alteration in forebrain topography with an increase in dorsal-posterior patterning at the expense of ventro-anterior patterning (Figure 10A2, B2), (Figure 10A3, B3 respectively), in line with altered SHH signaling and increased GLI repressor generation. These data, in combination with these postulates, offer a potential developmental mechanism to explain the observed phenotype in the TS-derived basal ganglia organoids. For future investigation, protein studies can be performed to elucidate SMO protein production, appropriate transmembrane localization and activity. Additionally, PKA activity levels and phosphorylation can be investigated to determine intracellular processing of GLI proteins.

The data presented here indicate new pathophysiological findings for TS and offer a new implication for SHH signaling. This work further reveals the suitability of a human in vitro tissue preparation for disease modeling, and offers a bioassay for investigation of other disorders affecting the basal ganglia. A potential mechanism for TS disorder pathogenesis is explored uncovering new significance for SHH signaling. Despite

disorder heterogeneity, these data illustrate the importance for a human model system that targets early development to explore and detect convergent pathophysiology. With new biomarkers identified, further investigation for therapies and advancement of clinical diagnoses can be pursued.

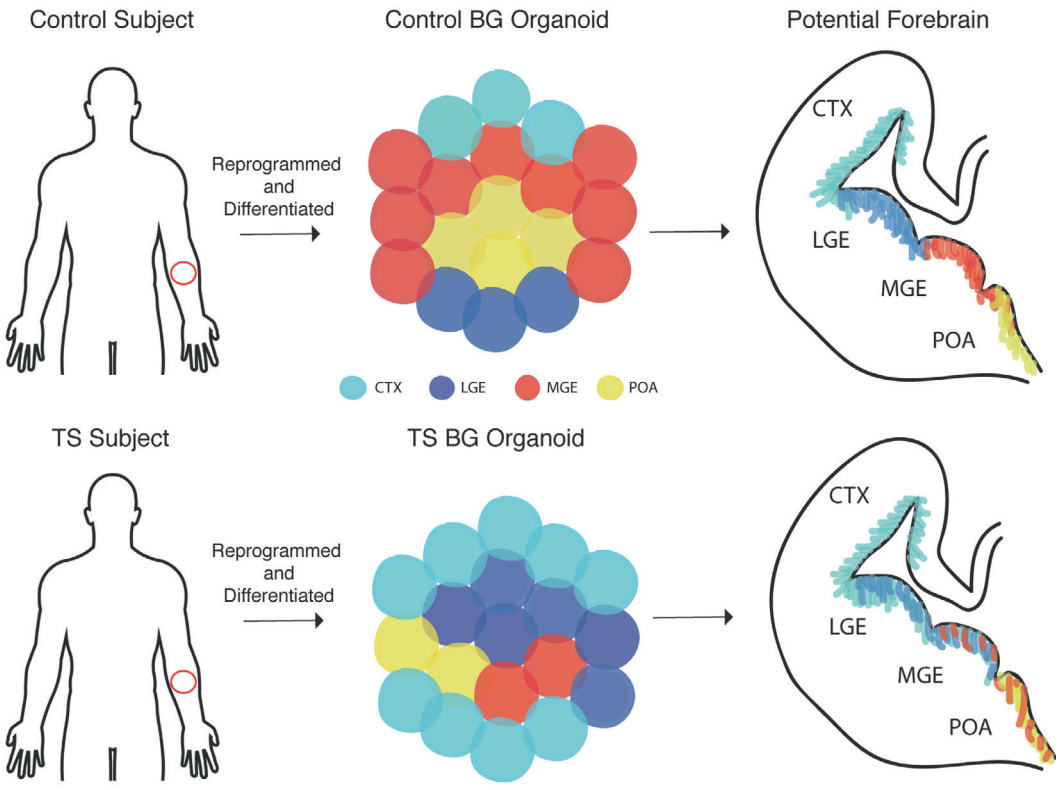


Figure 10: Schematic of cellular phenotype in organoids and prospective forebrain. Reading figure horizontally and from left to right – (A1) CT and (B1) TS subjects are biopsied, and their fibroblast cells are reprogrammed into iPSCs and differentiated into basal ganglia organoids (A2,B2). When assessing lineage topography across CT (A2) and TS (B2) organoids, the distribution of regions are disrupted in TS (B2). Based on these results: In contrast to the control brain (A3), the prospective forebrain for TS individuals may possibly show an expansion of dorsolateral expression and less of a ventromedial expression (B3) profile during neurodevelopment.

Appendix

Basal Ganglia Protocol

DAY 0:

1. Pre-treat cells with 5 uM ROCK inhibitor (Y-27632) in mTeSR for 45 minutes, to increase EB efficiency
2. Wash media off cells with PBS, then add warmed Accutase (5 mL for each 10 cm dish) directly to cells (diluted 1:2 with PBS). Incubate at 37 degrees for about 10-15 minutes
3. Add 5 mL of mTeSR to dilute Accutase. Transfer the cells to a clean 50 ml tube. Add another 5 mL of PBS to gently wash off cells from dish
4. Centrifuge cells at 1200 rpm for 4 min
5. Aspirate supernatant and gently resuspend cells in 2 mL of neural EB medium growth factors free, without Vitamin A, but containing 5 uM Y-27632
    a. **NB medium**:
        i. DMEM-F12 with Glutamax base
        1% N2
        4% B27 (without Vitamin A)
        1% P/S
        55 uM BME
6. Count cells in hemocytometer. Need **3 x 10^6 cells** for each well of AggreWell
    a. Spin down again at 1200 rpm for 4 min
    b. Resuspend cells in 1.5 mL of NB medium supplemented with 5 uM Y-27632 and 200ng/ml recombinant mouse Noggin
    c. Plate cells into AggreWell [See Rinsing Instructions for New AggreWells on TC desktop]
        i. Rinse each intended well with 1 mL of DMEM-F12 and aspirate to remove
        ii. Add 0.5 mL of NB complete medium (with Noggin & Y-27632) to each intended well
        iii. Centrifuge plate at 2000 x g for 5 minutes to remove air bubbles
        iv. Without removing the well's media, add the **3 x 10^6 cells** resuspended in 1.5 mL of NB complete medium and pipette gently up/down to distribute cells in well
        v. Centrifuge the AggreWell plate at 100 x g for 3 minutes and return cells to the incubator

DAY 1:

7. **SKIP THIS DAY** – leave in incubator

DAY 2:

8. Collect EBs with a strainer* and move them to a 10 cm petri dish in 10 mL NB medium (**from now on the NB medium uses REGULAR B27**) + 5 uM Y-27632 and 200 ng/mL Noggin. Disperse by shaking the plate before putting in incubator. 1 well = 1 10 cm dish
    a. Strainer: collect EBs using 2 mL pipette over an inverted 40 uM-cell strainer on top of a 50 mL falcon tube and wash well 2-3 times with 2 mL DMEM-F12 to obtain more EBs from well. The EBs should be collected on the bottom side of the strainer. Then put the cell strainer right side up on the petri dish and spray the EBs with 10 mL NB medium + 5 uM Y-27632 and 200 ng/mL Noggin.]

DAY 3: **SKIP THIS DAY**

DAY 4:

9. Transfer the EBs to a 15 mL conical tube and let the EBs settle by gravity. Replate EBs using NB medium + 200 ng/mL of Noggin (no more Y-27632) on Tissue Grade plates coated with 2x growth factor-reduced Matrigel. Neural EBs should attach and spread out the next day.

DAY 5:

10. Change medium and supplement with 20 ng/mL FGF2, 200 ng/mL Noggin and 200 ng/mL rhDKK1. Over the next few days, rosettes should appear. Change media every other day. May take up to 3 days to appear.

~DAY 7:

11. Harvest rosettes by extracting central area of each spreading EB using a pipette tip and the dissectorscope. Successful plates should have ~90-95% of EBs turned into rosettes. Collect all of them and replate the rosettes in suspension in bacterial grade petri dishes **for 4 full days**. Plate in NB medium + FGF2 & EGF (10 ng/mL each) **AND** 200 ng/mL FGF8 & 2 uM SHH (Purmorphamine).

DAY 12:

Terminal Differentiation Day 0

12. Manually place individual organoids in 1 well of 96 well plate using large orifice pipette tips and dissector scope
13. Replenish well media with TD medium + ventral morphogens
    a. **TD medium**: most preps will need volumes of 100 mL
       Neurobasal medium base
       1% N2
       2% B27
       1.5% HEPES
       1% P/S
       1% DMEM-F12 + Glutamax
       1% NEAA
       55 uM BME

    b. Add **growth factors FRESH** to whatever volume of TD medium is necessary to replenish the media on that day. Add these factors into perpetuity:
       10,000x GDNF
       2,500x BDNF
       500x Ascorbic Acid
       200x cAMP

    c. **AND** Add **ventral morphogens FRESH** to whatever volume of TD medium is necessary to replenish the media on that day.
       100 ng/mL FGF8
       1 uM SHH
       500 ng/mL (light sensitive) RA
       10 ng/mL BMP9

14. Change media 2x/week (I change media on Tuesdays and Fridays)
    ** I only expose the organoids to the ventral morphogens for 7 days. This allows for 2 media changes with fresh ventral morphogens before changing the TD media to only having growth factors. Even still, I never remove the media fully. When the media is added to the 96 wells on TD0, I add 200 ul of TD media. Every time I refresh the media, I remove and discard 150 ul of old media from the well and add a fresh 150 ul to the well. After 7 days, the remaining ventral morphogen solution in the well will continue to be naturally titrated out with each following media change. Time points I harvest are TD0, TD14 and TD30

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