



Functional Evaluations of Genes Disrupted in Patients with Tourette's Disorder

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Tourette's disorder (TD) is a highly heritable neurodevelopmental disorder with complex genetic architecture and unclear neuropathology. Disruptions of particular genes have been identified in subsets of TD patients. However, none of the findings have been replicated, probably due to the complex and heterogeneous genetic architecture of TD that involves both common and rare variants. To understand the etiology of TD, functional analyses are required to characterize the molecular and cellular consequences caused by mutations in candidate genes. Such molecular and cellular alterations may converge into common biological pathways underlying the heterogeneous genetic etiology of TD patients. Herein, we review specific genes implicated in TD etiology, discuss the functions of these genes in the mammalian central nervous system and the corresponding behavioral anomalies exhibited in animal models, and importantly, review functional analyses that can be performed to evaluate the role(s) that the genetic disruptions might play in TD. Specifically, the functional assays include novel cell culture systems, genome editing techniques, bioinformatics approaches, transcriptomic analyses, and genetically modified animal models applied or developed to study genes associated with TD or with other neurodevelopmental and neuropsychiatric disorders. By describing methods used to study diseases with genetic architecture similar to TD, we hope to develop a systematic framework for investigating the etiology of TD and related disorders.

Keywords: Tourette's disorder, functional studies, genes, neurobiology, induced pluripotent stem cells

INTRODUCTION

Tourette's Disorder (TD) is a childhood-onset neurodevelopment disorder characterized by the presence of both motor and vocal tics. Prevalence ranges from 1–3% and is found across many ethnic groups around the world (1). However, a recent meta-analysis of previous TD prevalence studies re-estimates the population prevalence of TD to be 0.3–0.9% (2). Males are affected three to four times more often than females (3–5). A high percentage of TD patients have comorbid conditions, most commonly attention-deficit/hyperactivity disorder (ADHD) and obsessive-compulsive disorder (OCD) and to a lesser extent autism spectrum disorders (ASDs).

Consistent evidence from family and twin studies suggest a significant genetic contribution to TD, most likely the result of complex and heterogeneous inheritance involving both common and rare variants, though most of specific findings still require replication. The neurobiological basis of TD is not well understood, but appears to involve alterations in the development, structure, and/or

functioning of cortico-striato-thalamo-cortical (CSTC) circuits (6). Specific genes have been found to be associated with TD. It is unclear if mutations in these genes cause TD and, if so, how these alterations affect the function or structural development of the nervous system. Our focus is to review the neurobiology of TD, describe the biological functions of those genes previously associated with TD, and discuss the various functional analyses that are required for evaluating and establishing the pathogenicity of these putative genetic causal variants for TD.

NEUROBIOLOGY OF TOURETTE'S DISORDER

Alterations of the CSTC circuits are considered as the neuropathological basis of tic generation (6, 7). These alterations are apparent in functional and structural imaging studies (8), histopathological studies of specific neuronal populations (9), and defective inhibition in various electrophysiological experimental paradigms (10, 11). In addition to the male predominance, the developmental features of TD pose an explanatory challenge, with tics usually not appearing till 4–6 years of age and most often, but not always, improving spontaneously by late adolescence.

Neurotransmitter pathways that modulate the activity and the output of the CSTC circuits in the basal ganglia have been the focus of intensive investigation, driven in part by the quest for more effective pharmacological interventions. The most supported neurotransmitter dysregulation hypothesis in TD involves the hyperactivity or imbalance of the dopamine signaling in the striatum (12). Within the basal ganglia, dopamine is released to the striatum by dopaminergic neurons originating from the substantia nigra. In the striatum, the effect of dopamine on subsequent neural signal transmission is modulated by the striatal medium spiny neurons expressing either D1 or D2-like dopamine receptors (13). The dopamine hypothesis is supported by the clinical observation that dopamine D2 receptor antagonists effectively reduced tics in some TD patients (14, 15). Also, dopamine pathway dysregulation was reported in post-mortem TD brain samples (16, 17) and in living TD patients' brain (12, 18).

Due to their excitatory and inhibitory effects within CSTC circuits, glutamatergic and GABAergic pathways have also been studied in TD. In post-mortem samples, lower levels of glutamate in subcortical brain regions were reported (19). However, it is unclear whether TD is associated with hyper- or hypo-glutamate levels (20). For the GABAergic pathway, an altered number and distribution of striatal GABAergic neurons were described in TD post-mortem brain samples (9, 21).

Disrupted serotonin signaling has been implicated in OCD, a common comorbid condition among TD patients. Selective serotonin reuptake inhibitors (SSRIs) have proven effective in reducing OCD symptoms (22) and are also used to treat TD patients with comorbid OCD (23). Interestingly, sequence variants at the serotonin transporter (SERT) gene were found in both OCD and TD patients (24), suggesting alterations in the serotonin pathway as one possible mechanism in the etiology of TD.

Until the recent discovery of a dominant negative non-sense mutation in the *HDC* gene co-segregating with TD in a large family, histaminergic (HA) neurotransmission was not considered a top candidate for TD etiology (25). However, other findings provide additional support for the involvement of HA neurotransmission in TD. For example, single nucleotide polymorphisms (SNPs) within the *HDC* gene region showed association with TD (26). Also, rare copy number variants (CNVs) found in TD patients were enriched in chromosomal regions harboring HA pathway genes (27). Furthermore, mice lacking the *Hdc* gene exhibited tic-like behaviors (28). Even though no evidence showed direct actions of serotonin and histamine on movements, it is proposed that serotonin and histamine pathways might indirectly regulate movements by modulating the dopamine system in the substantia nigra. In particular, both serotonergic and HA innervations are observed in the substantia nigra (29, 30). Also, serotonin and histamine receptors are expressed on nigrostriatal dopaminergic neurons (30, 31).

Aside from the neurotransmitter dysregulation hypothesis in TD, developmental and neuroimmunological findings also provide a context for assessing the relevance of potential gene findings. Altered distribution of parvalbumin interneurons (21) and reduced numbers of parvalbumin and cholinergic interneurons in basal ganglia were observed in the post-mortem brain samples of TD patients (9), suggesting another possible, perhaps developmental, mechanism for alterations of CSTC circuits. Additionally, a dysregulated brain-immune system involving microglia cells was suggested to contribute to TD (32). Gene expressions of inflammatory factors were examined using post-mortem basal ganglia samples from TD patients and controls (33). An elevated expression of the *CD45* gene was observed in TD patients even though the elevation was not statistically significant. CD45 is a surface marker of microglia and its expression is increased due to the activation of microglia. In another study, transcriptome analysis of post-mortem striatum of TD patients and controls revealed upregulation of microglia-related genes (34).

GENES DISRUPTED IN PATIENTS WITH TOURETTE'S DISORDER

In this section, we will review 15 genes that have been associated in TD (Table 1); to suggest how we might move beyond association to establish a role in TD pathogenesis, we will examine what is known about the biological effects of these genes. We group these genes into several categories: (1) neurite outgrowth: *SLITRK1*; (2) histamine pathway: *HDC*; (3) serotonin pathway: *SERT*, *HTR1A*, *HTR2B*; (4) glutamate pathway: *SLC1A3*; (5) synaptic signal transduction and cell-adhesion pathway: *NLGN4*, *CDH2*, *CNTNAP2/CASPR2*, *DPP6*; (6) mitochondrial functions: *IMMP2L*, *MRPL3*; and (7) genes associated with other diseases: *DNAJC13* [Parkinson's disease (PD)], *OFCC1* (orofacial clefts), and *HCRT2* or *OX2R* (excessive daytime sleepiness). The diverse functions of these genes – ranging from neurotransmitter synthesis, neuronal migration, synaptic plasticity, cell adhesion, and protein transportation and synthesis – highlight the complexity of unraveling the pathogenesis of TD. However, in addition to the

TABLE 1 | Genes disrupted in TD.

Biological pathways	Gene name	Disruption(s) found in TD patients and references
Neurite outgrowth	<i>SLITRK1</i>	Inversion, frameshift variant, Var321 (38) Synonymous variant: 708C > T (39) 3'-UTR variant: 3383G > A (43) 3225 T > C (41) D397G (44)
Histamine	<i>HDC</i>	W317X (25) Intronic transition and synonymous variants: 426C > A, 1743G > A (55)
Serotonin	<i>SERT</i> <i>HTR1A</i> <i>HTR2B</i>	I425V and 5-HTTLPR (24) R219L (78) M63R, R449Q (79)
Glutamate	<i>SLC1A3/EAAT1</i>	E219D (88)
Synaptic signal transduction and cell adhesion	<i>NLGN4/NLGN4X</i> <i>CDH2</i> <i>CNTNAP2</i> <i>DPP6</i>	Deletion across exon 4, 5 and 6 (99) N706S, N845S (106) Intronic insertion (121) Microdeletion at exon 1 (128)
Mitochondrial functions	<i>IMMP2L</i> <i>MRPL3</i>	Translocation (141) <i>De novo</i> duplication (142) Translocation and cryptic deletion eliminated the exon 1–3 (143) Intragenic deletions (144) S75N (150)
Genes associated with other diseases	<i>DNAJC13</i> (Parkinson's disease) <i>OFCC1</i> (orofacial clefts) <i>HCRT2/OX2R</i> (excessive daytime sleepiness)	A2057S (150) R129G and a novel variant at 5'-UTR (150) P10S (162)

genetic disruptions discussed here, large structural variations, for example copy number variations (CNVs), have also been investigated in TD patients. Genes disrupted by these structural variants have been discovered and indicated as potential TD associated genes (35–37).

Neurite Outgrowth

SLIT and NTRK-Like Family, Member 1 (*SLITRK1*)

In a TD patient with comorbid ADHD, a *de novo* chromosome 13 inversion was identified (38). The *SLITRK1* gene was mapped close to the breakpoints. Targeted sequencing of the *SLITRK1* gene identified a non-coding variant (var321) and a frameshift mutation (38) in another 174 unrelated TD patients but not in a large control sample. The frameshift mutation led to impaired dendrite growth in neurons and the var321 variant may cause reduced *SLITRK1* protein expression (38). While the var321 and additional novel variants within *SLITRK1* were found in other TD patients, these associations were not replicated in subsequent studies (39–49).

Members of the *SLITRK* protein family are transmembrane proteins. They are structurally homologous to the *SLIT* and the *TRK* proteins, which are involved in axon guidance pathway and neurodevelopment (50). The *SLITRK1* gene is highly expressed in developing and mature neuronal tissues and promotes neurite

outgrowth in culture (51). The *SLITRK1* protein is localized to the post-synaptic densities and has been hypothesized to affect synapse formation at excitatory synapses through interactions with the pre-synaptic cellular adhesion molecule LAR-RPTP (52, 53). In *Slitrk1* knockout mice, although stereotypic behaviors were not observed, the mice exhibited anxiety-like and depression-like behaviors, which were attenuated by chemicals modulating noradrenergic neurotransmission (54). Neurochemical abnormalities were also detected in *Slitrk1* knockout mice: norepinephrine and its metabolites were significantly increased in the prefrontal cortex and the nucleus accumbens while choline and acetylcholine levels were significantly lower in the striatum (54). Taken together, the evidence suggests that the *SLITRK1* gene might play a role in neurochemical modulation.

Histamine Pathway Histidine Decarboxylase

Histamine neurotransmission was first linked to the etiology of TD when a rare non-sense mutation within the *HDC* gene was discovered in a multiplex family in which the father and all eight children were diagnosed with TD. The mutation resulted from a G to A transition at the ninth exon of the *HDC* gene and led to a premature stop codon (W317X) (25). The heterozygous W317X mutation co-segregated with all affected individuals in this family. The W317X mutation was not found in 3360 unrelated individuals unaffected with TD or another 720 TD patients, suggesting this is a very rare cause for TD. *In vitro* enzymatic assay demonstrated that the truncated protein produced by the mutation lost histidine decarboxylase (HDC) activity and had a dominant negative effect on the activity of wild-type *HDC* protein. After the initial finding, more TD patients were screened for mutations in the *HDC* gene in different studies. Only an intronic variant and two synonymous variants were identified in a study involving 120 TD patients (55). However, an association of the *HDC* gene and TD phenotypes was reported in a study including 520 TD nuclear families (26). Also, rare, genic CNVs identified in 460 TD patients were enriched for HA pathway genes (27), supporting the potential involvement of the histamine pathway in TD etiology.

In the adult human central nervous system, the *HDC* gene is exclusively expressed in the soma and axon varicosities of HA neurons mostly originating from the tuberomammillary nucleus in the posterior hypothalamic region of the brain (30). The *HDC* homodimer converts L-histidine into histamine in the soma of HA neurons. Histamine-containing neuronal fibers are seen in many brain areas in rodents and human including cerebral cortex (56, 57). Therefore, loss-of-function mutations at the *HDC* gene will likely cause a lack of histamine in the widespread brain regions receiving HA innervation. In addition to serving as a neurotransmitter, histamine is a neuromodulator, inhibiting dopamine release by striatal dopaminergic neurons through binding to the H3 receptors expressed on these neurons in mice (58). Given the hypothesis that hyperactivity of nigrostriatal dopaminergic neurons is responsible for tic generation (12), it is reasonable that histamine dysregulation may contribute to TD.

Since *HDC* protein functions as a homodimer (59), individuals harboring the W317X mutation have approximately 25% residual *HDC* activity remaining compared to the healthy

controls. Therefore, the *Hdc* knockout mice may recapitulate the behavioral outcomes caused by the W317X mutation in humans. As expected, the *Hdc* knockout mice show tic-like stereotypic behaviors after psychostimulant administration and reduced prepulse inhibition (28). Interestingly, the striatal dopamine level was increased in the *Hdc* knockout mice during the dark cycle, which could be decreased by administration of histamine in the knockout mice. Also, higher levels of dopamine D2 receptor occupancy were found in the basal ganglia of TD patients carrying the W317X mutations, the *Hdc* knockout mice and the *Hdc* heterozygous mice, indicating that the dopamine release in the basal ganglia of the brain might be disinhibited due to histamine depletion (28). Taken together, parallel studies in TD subjects and mice demonstrated that lack of histamine results in dopamine dysregulation, providing a potential mechanism for the proposed role of dopamine disruption in TD (12).

Serotonin Pathway

Serotonin Transporter (*SLC6A4* or *SERT*)

Given the effectiveness of the SSRIs in reducing OCD symptoms, the *SERT* gene has been studied as candidate gene for OCD (60–64). Serotonin-transporter-linked polymorphic region (5-HTTLPR) polymorphisms and a gain-of-function missense mutation I425V have been associated with OCD (65–69). Sequence variants of the *SERT* gene were first associated with TD in a two-generation pedigree (24). In this family, the heterozygous “long” 5-HTTLPR variant and the I425V mutation perfectly segregated with TD individuals in a dominant pattern. The “long” 5-HTTLPR produces higher *SERT* mRNA level compared to the “short” 5-HTTLPR (70). The I425V mutation results in constitutive activation of the SERT protein whose activity is regulated by cGMP-dependent protein kinase (71). Therefore, carrying both “long” 5-HTTLPR and the I425V mutation is expected to have a synergistic effect that increases the expression of *SERT* mRNA and increases the amount of activated SERT protein.

In the mammalian central nervous system, the *SERT* gene is primarily expressed in the serotonergic neurons that originate from the raphe nucleus in the hindbrain and project widely to other parts of the nervous system, descending to the spinal cord and ascending to the forebrain (72). The human SERT protein is a transmembrane protein (73, 74). Cell surface expression of SERT protein can be regulated by SERT antagonists and substrates (75). The serotonergic axons can innervate and regulate other neurotransmission systems. For example, serotonin can facilitate or inhibit dopamine release in the striatum in a direct or indirect manner (76). Therefore, dysregulation of SERT expression on the plasma membrane may affect dopamine transmission (77). So far, no other sequence variants in the *SERT* gene have been associated with TD and no corresponding transgenic mice are available for *in vivo* studies.

Serotonin Receptor 1A (*HTR1A*) and Serotonin Receptor 2B (*HTR2B*)

In addition to the *SERT* gene, other serotonin pathway genes have been examined in TD patients. A missense mutation causing an amino acid change from arginine to leucine was identified in the serotonin receptor 1A gene (*HTR1A*) in one TD patient. However,

the mutation was not predicted to change the receptor activity (78). Two novel non-synonymous missense variants and three known SNPs in the serotonin receptor 2B (*HTR2B*) gene were also found in 132 Caucasian and 128 Chinese Han TD individuals, though the associations were not statistically significant (79).

There are currently 14 known serotonin receptors and these are categorized into seven classes (80, 81). The *HTR1A* and *HTR2B*, both of which are G protein-coupled receptors, belong to class I and class II, respectively (81). The *HTR1A* receptor is highly expressed in the brain. Lower brain expression of the *HTR1A* receptor has been associated with mood disorders in humans. *Htr1a* knockout mice exhibit depression- and anxiety-like behaviors and have been used for antidepressant drug screening (82, 83). The *HTR1A* receptors are located at both pre-synaptic and post-synaptic neurons in the CNS. Activation of the pre-synaptic *HTR1A* receptors on the serotonergic neurons leads to inhibition of serotonergic neuron firing and reduced serotonin release whereas activation of the post-synaptic *HTR1A* can modulate the release of other neurotransmitters (84). Compared to the *HTR1A* receptor, the role that *HTR2B* plays in the CNS is not well understood. However, there is evidence suggesting that *HTR2B* may regulate SERT activity by phosphorylating SERT protein (85). In mice, the *HTR2B* receptor may be involved in modulating serotonin release from the serotonergic neurons. (86). Taken together, dysfunction of the *HTR1A* or the *HTR2B* receptor might lead to abnormal serotonin release in the CNS.

Glutamate Pathway

Excitatory Amino Acid Transporter 1 (*SLC1A3* or *EAAT1*)

Altered cortico-striatal-thalamo-cortical (CSTC) circuitry is believed to provide the neurobiological basis for TD (7, 87). Glutamate is the major excitatory neurotransmitter in CSTC circuitry. A missense mutation (E219D) in the glutamate transporter gene (*SLC1A3*) was associated with TD in a case-control study (88). In the same study, cells transfected with the E219D mutant glutamate transporter gene exhibited increased glutamate uptake activity compared to cells transfected with the wild-type gene. The proposed mechanism for the increased glutamate uptake activity was elevated glutamate transporter expression at the plasma membrane due to the E219D mutation. However, whether TD might be associated with hypo- or hyper-glutamate activity is still controversial.

One of the five subtypes of glutamate transporters, EAAT1, is responsible for the reuptake of the excitatory neurotransmitter, L-Glutamate, from the synapses back into cells. Dysfunction of glutamate transporters may lead to imbalanced extracellular glutamate levels, further affecting downstream glutamate neurotransmission or causing glutamate excitotoxicity to neurons (89, 90). EAAT1 is primarily expressed in glial cells. Regionally, EAAT1 proteins are found in neocortex, striatum, cerebellum, and spinal cord (91). *Eaat1* knockout mice showed hyperactivity and reduced acoustic startle response compared with the wild-type mice (92) but did not exhibit the altered prepulse inhibition behavior, which has been found in TD patients (93). No *Eaat1* gain-of-function mutant mice are available to test the “hypo-glutamate activity” hypothesis in TD.

Synaptic Signal Transduction and Cell-Adhesion Pathways

Neuroigin 4, X Linked (*NLGN4* or *NLGN4X*)

Mutations in the neuroigin (NLGN) family members have been identified in patients with neuropsychiatric disorders such as ASD (94–97) and schizophrenia (98). A small deletion in the *NLGN4* gene was detected in a mother and her two sons (99), one of whom was diagnosed with autism while the other was diagnosed with TD and ADHD. Their mother, who also carried the deletion, had a learning disability, depression, and anxiety. The deletion spanned exon 4, 5, and 6 of the *NLGN4* gene, resulting in a truncated protein. No other known genes were affected by the deletion.

Neuroigins are cell adhesion molecules located on the plasma membrane of the pre-synaptic and post-synaptic neurons. By interacting with neuexins, another family of cell adhesion proteins, NLGNs modulate proper signal transmission between pre-synaptic and post-synaptic neurons (100). Reduced expression of NLGNs in cultured neurons or mice cause deficits in synaptic maturation and plasticity (101, 102). *Nlgn4* knockout mice have been studied at both the behavioral and cellular levels. Because *Nlgn4* gene disruptions had been associated with ASD, the *Nlgn4* knockout mice were tested for ASD-like behaviors. As expected, *Nlgn4* knockout mice exhibited deficits in social interactions and reduced ultrasonic vocalizations compared to the wild-type mice (103). In another study, the *Nlgn4* knockout mice displayed reduced neural network response upon stimulation in both excitatory and inhibitory circuits (104). More interestingly, the *Nlgn4* knockout mice also showed stereotypic repetitive behaviors and increased obsessive compulsive like behaviors (105), supporting the possibility that disruption of the *NLGN4* gene might play a role in TD or related disorders.

Cadherin 2, Type1, N-Cadherin (*CDH2*)

Cadherin 2 (*CDH2*), also known as N-Cadherin, is another cell adhesion protein that has been associated with TD. *CDH2* participates in neuron–neuron communication and in synaptogenesis. In a recent study, exons of the *CDH2* gene were sequenced in 160 OCD probands and 160 controls (106). Four variants in the *CDH2* gene were identified in subjects with OCD or TD. Two mutations were of particular interest: the N706S and the N845S variants. N706S is a rare and novel mutation close to the predicted proteolytic cleavage site of the *CDH2* protein while the N845S variant is located at the β -catenin interacting region. Both mutations reduced the *CDH2* protein level by more than 50% in transfected HEK293 cells (106), suggesting possible adhesion deficits in cells.

Cadherin 2 is a calcium-dependent cell–cell adhesion glycoprotein. *CDH2* primarily mediates neurite outgrowth and axon guidance of neurons on myotubes (107) and on the surface of astrocytes (108, 109). The cytosolic domain of the *CDH2* protein forms complexes with catenin proteins (110), and these complexes regulate synaptogenesis in both pre- and post-synaptic neurons (111, 112). Additionally, the cleaved C-terminal domain of the *CDH2* is a repressor of CBP/CREB-mediated transcription whose target genes are critical in neural development and

plasticity (113). The complete knockout of the *Cdh2* gene is an embryonic lethal in mice whereas *Cdh2* heterozygous null mice do not exhibit obvious morphological defects during development (114). Conditional knockout of the *Cdh2* gene in the cerebral cortex of mice caused disrupted cortical structure (115). Thus far, behavioral analyses in the *Cdh2*^{+/-} mice or *Cdh2* conditional knockout mice have not been conducted.

Contactin-Associated Protein-Like 2 (*CNTNAP2*/*CASPR2*)

Variants in the *CNTNAP2* gene have been associated with ASD in several family based studies (116–119). Also, putative deleterious mutations were found in the *CNTNAP2* gene in ASD patients (120). In one family, an insertion on chromosome 7p35–p36, disrupting intron 8 of the *CNTNAP2* gene, was shared by a father and his two children, all three of whom were diagnosed with TD (121). However, a translocation disrupting intron 11 of *CNTNAP2* did not cause TD phenotypes in another three generation family (122).

CNTNAP2, a transmembrane protein, belongs to the neuexin superfamily and is highly expressed in neurons and localized to the axonal membrane of the juxtaparanodal region. The *CNTNAP2* protein interacts with clustered Shaker-type potassium channels and plays an important role in the axon-glia septate-like junction (123). It is required for normal action potential propagation along myelinated axons of the neurons (124), and it has been hypothesized that malfunctions of the *CNTNAP2* protein leads to deficits of electric signal transduction between neurons (121, 125, 126).

Behavioral assessments of *Cntnap2* knockout mice led to stereotypic motor movements (127). Interestingly, a reduced number of GABAergic interneurons were reported in the striatum of the *Cntnap2* knockout mice, which is consistent with previous post-mortem studies showing a reduction in the number of striatal GABAergic interneurons in TD patients (9, 21). Therefore, understanding the molecular mechanism of neuronal loss caused by disruption of *CNTNAP2* may help to pinpoint biological pathways altered in TD.

Dipeptidyl-Peptidase 6

A heterozygous microdeletion at the first exon of the *DPP6* gene was identified in a boy with TD as well as the boy's father and paternal uncle both of whom were diagnosed with tic disorder and ADHD (128). The microdeletion led to decreased *DPP6* mRNA level in the boy's blood cells. *DPP6* has also been associated with other neuropsychiatric disorders such as ASD (95, 129) and schizophrenia (130).

Dipeptidyl-peptidase 6 (*DPP6*) is a transmembrane protein belonging to a family of serine proteases. However, *DPP6* does not have protease activity (131). *DPP6*'s expression is enriched in the brain and different isoforms have different distributions in the brain. (132). *DPP6* proteins interact with A-type voltage-gated potassium channels, specifically the Kv4 subunit (133, 134). The A-type potassium channels participate in the modulation of dendritic signal transmission (135, 136). Moreover, the A-type potassium channel was reported to control the tonic dopamine release

by substantia nigra dopaminergic neurons (137). Even though DPP6 has no peptidase activity, it may have novel functions and play essential roles in Kv4 intracellular trafficking, membrane expression, and proper function of the A-type potassium channels (138). *Dpp6* knockout mice show abnormal synaptogenesis (139), and knocking down the *Dpp6* gene specifically in the mouse brain caused impaired learning and memory abilities (140).

Mitochondria Functions

Inner Mitochondrial Membrane Peptidase 2 Like

A familial translocation segregating with TD or tics (141) and a *de novo* duplication in a TD patient with other developmental and mental phenotypes implicated *IMMP2L* as a possible TD candidate gene (142). This was the first mitochondria-related gene in TD. Subsequently, a cryptic deletion eliminating exons 1–3 of the *IMMP2L* gene and 21 other genes was identified in a TD patient with learning and speech difficulties (143). Also, a case-control study of copy number variations reported intragenic deletions at the *IMMP2L* gene in seven TD patients (144). Among the seven, three deletions at intron 3 led to production of a shorter *IMMP2L* mRNA transcript due to alternative splicing. In the same study, the expression of both the short and the long transcripts was examined by reverse transcription PCR in 19 regions of the human brain. While the long transcript was ubiquitously expressed in all 19 brain regions, the short transcript was selectively expressed at a lower level in only 10 brain regions, suggesting that the short transcript might have a more specific role in the human central nervous system (144).

The human inner mitochondrial membrane peptidase 2 like (*IMMP2L*) protein is one of the catalytic subunits of the inner mitochondrial membrane peptidase (IMP) complex (145, 146). The IMP complex participates in the cleavage of the inner mitochondrial membrane targeting signal sequence from its protein substrates, allowing maturation of the substrates. Loss of any subunit will cause the decomposition of the whole complex (147). Expression and function of the mammalian *IMMP2L* protein have been studied in animal models and human tissues. *Immp2l* knockout mice exhibit mitochondrial dysfunction, increased ischemic brain damage, and infertility (148, 149). The human *IMMP2L* mRNAs are ubiquitously expressed in various tissues except for adult liver and lungs. Unlike other TD associated genes, there is no enriched *IMMP2L* mRNA expression in the brain compared to other tissues (142). However, linking mitochondrial dysfunction to TD might lead to further speculation about the varied etiology of TD.

Mitochondrial Ribosomal Protein L3

Whole exome sequencing of a multiplex TD family showed three rare novel non-synonymous mutations in the *MRPL3*, *DNAJC13*, and the *OFCC1* genes (150). The three variants were not found in controls or dbSNPs/1000 Genomes databases. However, a targeted-sequencing study of the same three variants in Han Chinese TD patients did not replicate these findings (151).

Mitochondrial ribosomal protein L3 (*MRPL3*) is a mitochondrial ribosome protein involved in mitochondrial protein translation (152). Compound heterozygous mutations in the *MRPL3* gene were associated with mitochondrial respiratory

chain deficiency in a pedigree of French origin (153), but no psychiatric diseases were reported.

Genes Associated with Other Diseases DnaJ (Hsp40) Homolog, Subfamily C, Member 13 (DNAJC13)

A missense variant (A2057S) in the *DNAJC13* gene was found to segregate with TD or chronic tic disorder (CTD) in a multiplex pedigree (150). Subsequently, a novel missense mutation Asn855Ser in the *DNAJC13* gene was found in a multi-generation family with PD and in an additional four other PD patients (154). Human *DNAJC13* is a membrane-associated protein involved in endocytosis, specifically in the process of early endosome-mediated membrane trafficking (155, 156). Knocking down the *DNAJC13* gene in mammalian cells did not cause obvious dysfunction of endocytosis. However, introducing a C-terminus truncated mutant *DNAJC13* protein into the cells did affect the normal distribution and formation of early endosomes (156). No *DNAJC13* knockout animal model is available.

Orofacial Cleft 1 Candidate 1

After an initial study suggesting that the *OFCC1* gene led to orofacial clefts (157), it was later linked to schizophrenia (158). Recently, a genome-wide association study (GWAS) of OCD found a significant association with *OFCC1* (159). Sequence variants in *OFCC1* have been found in patients with neurodevelopmental or neuropsychiatric disorders: a missense mutation (R129G) segregating with TD and CTD in a multiplex family (150) and a non-sense and a missense mutation were found in a single autism family (160).

The function of the orofacial cleft 1 candidate 1 (*OFCC1*) protein is unclear but one study suggested that the *OFCC1* protein was an interacting partner and methylation substrate of protein arginine methyltransferase 1 (161). However, *Ofcc1* knockout mice did not show any behavioral abnormalities (158).

Hypocretin (Orexin) Receptor 2 (HCRTR2/OX2R)

The coding regions of the orexin-1/hypocretin-1 (*OX1R*) receptor gene, the orexin-2/hypocretin-2 (*OX2R*) gene, and the prepro-orexin gene were examined in patients with Excessive Daytime Sleepiness, TD, or ADHD and healthy controls (162). A C29T nucleotide change in the *OX2R* gene producing a Pro10Ser amino acid substitution was detected in only one TD patient with comorbid ADHD. The Pro10Ser variant reduces responsiveness of the Orexin2 receptor to its ligands, Orexin-A, and Orexin-B. The Orexin receptor 2 is a G protein-coupled receptor that participates the regulation of feeding and sleep-wakefulness in mammalian brains (163). *Ox2r* knockout mice do not show any tic-like behaviors (164).

FUNCTIONAL ANALYSES OF GENES DISRUPTED IN TD

As indicated in the previous section, evidence for TD susceptibility genes exists. The mutations discussed were found in only a small number of individuals with TD, and replication remains elusive. This lack of replication may be due to the extreme locus

heterogeneity, similar to what has been found in ASD (165). What evidence beyond stronger statistical association might help establish their potential role in TD pathogenesis? Some of these genes are found within neurobiological pathways that are presumed to be disrupted in TD (e.g., neural signal transmission/modulation) while others are found in novel pathways. Hence, the evidence that these genes are true susceptibility genes remains insufficient. Consequently, more convincing functional studies are needed to determine how variants in these genes could contribute to TD. In this next section, we review the *in vitro* and *in vivo* functional studies and techniques (beyond the knock out experiments referred to above) that will likely be useful to evaluate the consequences of mutations found in these presumptive TD susceptibility genes and for discovery of cellular and molecular phenotypes in disease.

Transgenic Mammalian Cell Lines

Functional studies using neuronal cells from individuals with TD can provide insights into the molecular basis of TD and potentially help to clarify the biological pathways altered in TD. However, one of the difficulties is the inability to obtain relevant biomaterials (e.g., neurons or neural stem cells) from affected individuals. Since TD is not a lethal disorder, there is very limited access to neural tissue from individuals with TD, particularly from individuals with a known causal variant.

Transgenic human non-neuronal cell lines have been used to characterize the cellular and molecular phenotypes resulting from specific mutations. Typically, plasmids carrying a wild type or a mutant gene of interest are delivered into the cell lines. Once the protein products of the transgene are expressed in the cells, assays are developed to evaluate the functional consequences of the mutant proteins. For example, in the *CDH2* gene, both the wild type and mutant *CDH2* genes were cloned into expression plasmids and subsequently delivered into human embryonic kidney (HEK293) cells and reduced expression of mutant CDH2 proteins was reported (106). While easily done, functional studies in non-neuronal cell lines are suboptimal for a variety of reasons. During transgenesis, the gene of interest is often transiently overexpressed or is controlled by a conditional and/or inducible gene expression system (166). Therefore, the level of transgene expression might not faithfully represent the endogenous gene expression level. Also, the expression levels of many genes are tissue-specific (167). This is particularly relevant for genes with multiple transcript isoforms where the isoform expression pattern in transgenic cell lines may not be comparable to patterns in neurons. Furthermore, because transgenic cells would not be expected to have the same gene expression profile as neurons, they may not provide relevant cellular environment for the transgene to execute its genuine neuronal function(s). Neuronal samples from TD patients are therefore preferred for *in vitro* functional studies but these are very difficult if not impossible to obtain. A recent technological advance, induced pluripotent stem cells (iPSCs), now makes functional studies of neuronal samples with a known causal variant possible.

Induced Pluripotent Stem Cells Use of iPSC-Derived Neuronal Cells to Model Neuropsychiatric and Neurodevelopmental Disorders

The relatively new reprogramming technique that converts human somatic cells into iPSCs allows researchers to model diseases *in vitro* using patient-derived cells. Since the first generation of iPSCs from mouse fibroblasts (168), the ability to produce differentiated cells from iPSCs has been intensively studied and improved. Recently, iPSC neuronal differentiation has become routine (169). Generating patient-specific neurons carrying mutations in disease candidate genes is invaluable for researchers who wish to study the cell-autonomous effects of mutations and to understand the cellular basis of neurological and neuropsychiatric disorders. To date, no study using iPSC-derived neurons to model TD has been published. However, mutations associated with other neuropsychiatric disorders have been studied in iPSC-derived neurons, and cellular abnormalities have been demonstrated (170–174). For example, Rett syndrome is a neurodevelopmental disorder occurring mainly in females characterized by mental retardation. Loss-of-function mutations in the methyl-CpG binding protein 2 (*MeCP2*) gene were reported in the majority of Rett Syndrome cases (170). Therefore, neurons with functional null mutation in the *MeCP2* gene were generated from the iPSCs of an individual with Rett Syndrome. In culture, the neurons showed smaller soma size (171). Similarly, iPSC-derived neurons have been used to understand ASD. In an individual with ASD, a balanced translocation spanning the transient receptor potential 6 channel (*TRPC6*) gene was identified. Neurons derived from the iPSCs exhibited decreased *TRPC6* expression, altered morphology and reduced Ca^{2+} influx (172). In schizophrenia, the *DISC1* gene has been considered an important risk factor (175) and in iPSC-derived *DISC1* mutant neurons, pre-synaptic vesicle release was impaired (173).

Unfortunately, *in vitro* neural differentiation from iPSCs yields mixed populations of neurons rather than a homogenous population. For diseases with clear neuropathology, pure cultures of the specific neuron types involved in the disorder are preferred in order to recapitulate disease-related cellular phenotypes. For instance, PD is characterized by loss of substantia nigra dopamine neurons (176). Cultures containing a high percentage of dopaminergic neurons were generated from PD patients who carry monogenic mutations (177–180), and defects in cellular functions such as autophagy, mitophagy, oxidative stress response, and dopamine release were found in these neurons. In patients with amyotrophic lateral sclerosis (ALS), motor neurons degenerate. Therefore, motor neurons were derived from iPSCs of ALS patients carrying known casual mutations (174, 181). As hypothesized, the mutant motor neurons exhibited neurite degeneration (181).

Use of Single-Cell Analysis to Overcome Culture Heterogeneity

One method to overcome cell culture heterogeneity is to analyze single cells, all of the same type. Looking at the transcriptome

of single cells using microarray or RNA-seq analysis holds promise of detecting gene dysregulation in particular populations of neurons, which might not be identified by analyzing heterogeneous mixtures of cells. For instance, single-cell gene expression analysis of iPSC-derived dopamine neurons from PD patients with a LRKK2 mutation unveiled dysregulation of oxidative stress genes in mutant dopamine neurons (182). In another study, neurons were generated from the iPSCs of Timothy Syndrome patients with a mutation in the *CACNA1C* gene. Single cells were taken from the culture containing mixed neuron populations, and gene expression was analyzed by microarray. As a result, the distribution of neuron subtypes was altered in the Timothy Syndrome neuronal culture compared to the control cells (183). Compared to microarray, RNA-seq is gaining greater popularity for analyzing the transcriptome of single cells due to its ability to unbiasedly detect any transcript in cells within a broader dynamic range of expression. Generally, there are four important steps to achieve single-cell RNA-seq: (1) single-cell isolation, (2) RNA capture, (3) cDNA synthesis and, (4) next generation sequencing. The microfluidic system is becoming popular for single-cell RNA-seq because it can isolate single cells, lyse the cells, purify RNA, synthesize cDNA or even conduct gene expression analysis all in one run (184, 185).

Use of iPSC-Derived Cerebral Organoids to Model Neuropsychiatric and Neurodevelopmental Disorders

In contrast to PD or ALS, the neuropathology of many neurodevelopmental and neuropsychiatric disorders, such as TD or ASD, is unclear or is heterogeneous (18). As described above, mutations associated with TD indicate dysregulations of various neurotransmitter pathways or of neural circuits involving multiple brain regions. Hence, studying specific type of neurons may not help to explain the pathogenesis of TD. The recently developed “cerebral organoid” cellular system enables the differentiation of iPSCs into a three-dimensional miniature organ in a bioreactor, with minimum external interferences (186). Such self-organized spherical structures resemble the human brain at very early stages of development. In comparison to monolayer neuronal cultures, the cerebral organoids contain more diverse neuronal populations that define distinct brain regions. Also, within the cerebral organoid, neuronal migration and human-specific brain structures (e.g., the outer subventricular zone) were observed (187). Therefore, the cerebral organoid has been used to model neurodevelopmental diseases. For microcephaly, premature neural differentiation was recapitulated in organoids derived from microcephaly patients' iPSCs (187). In cases of idiopathic ASD, overproduction of GABAergic inhibitory neurons in patient-derived cerebral organoids was reported (188). At the molecular level, the cellular phenotype was explained by overexpression of the transcription factor *FOXP1*. However, use of cerebral organoids to model neurodevelopmental diseases has limitations. The various “brain regions” in the organoids are fairly disorganized. Therefore, the cerebral organoid would not be suitable to study neural circuits. Furthermore, neuronal cells within the organoids are mostly neural progenitor cells, and their differentiation is restricted by limited growth of the organoids, which in turn is

probably due to the lack of internal nutrient and oxygen supply. More importantly, each organoid is “unique” because the self-organization process is random and is not controlled by external factors. This “uniqueness” will generate variation among organoids, which may mask phenotypic differences between normal and patient-derived cells.

Use of Genome Editing to Generate Isogenic Control iPSC Lines

One challenge in identifying the phenotypic effects of a given mutation in iPSC-derived neurons is finding an appropriate control sample. While age, gender, and ethnicity-matched control samples with the wild-type allele are typically available, they are not matched for all of the other common genomic variants. Failure to control for such variability in genetic background can lead to spurious results. The recent technological advance of highly specific genome editing now allows the production of more comparable isogenic controls for functional studies. Several genome editing systems, such as zinc finger nuclease (189, 190), TALEN, and CRISPR-Cas9 (191, 192), are able to reverse the mutation to wild type at one genomic locus at a time in iPSCs. Comparing neuronal cells generated from mutant iPSCs and their edited, isogenic control neuronal cells with the mutation removed allows identification of molecular and cellular changes that are due only to the mutation (180). However, “off target” mutations at unrelated loci inadvertently introduced by editing remain a potentially important technological hurdle (193).

Gene Expression and Gene Network Analyses

The major goal of genomic sequencing of patients with neurodevelopmental and neuropsychiatric disorders is to identify disease-associated mutations. Once such genes are found, systematic approaches including genome-wide gene expression analysis and gene network analyses can be used to implicate common biological pathways altered in patients with different mutant genes.

Gene Expression Analysis

Gene expression analysis, primarily through the RNA-seq approach, aims to quantify transcript level of target genes or of the whole transcriptome in biological samples from patients and healthy controls to identify genes dysregulated in human diseases (194). For neurodevelopmental disorders, post-mortem brain samples are often used for the transcriptomic analyses. The first transcriptomic analysis of TD patients' post-mortem striatum samples revealed that interneuron disruption might be involved in the pathophysiology of TD (34). However, to evaluate particular mutations, post-mortem samples meeting specific research criteria are usually difficult or virtually impossible to obtain. With the emergence of somatic cell reprogramming techniques, iPSC-derived neurons with and without a putative disease-causing mutation can be produced *in vitro* (195). The transcriptomes of these iPSC-derived neurons can be compared by microarray or RNA sequencing (RNA-seq) (172, 173). To further dissect the cellular phenotype at single neuron level and to detect abnormalities only shown in particular populations of

neurons, single-cell transcriptome analysis can also be performed (182, 183). Multiple bioinformatic tools have been developed for RNA-seq data to detect differentially expressed genes (DEGs) from distinct cell types or under different experimental conditions (196–198). Among these RNA-seq analysis methods, none outperforms the others in all aspects. Selecting an optimal method for a study requires an understanding of the benefits and limitations of each method as well as the parameters of the study (196). Once the DEGs between experimental conditions are determined, gene and pathway annotation tools, gene and protein expression and interaction databases can help to explore the gene pathways underlying the disorder. Gene and pathway annotations tools such as IPA¹, KEGG (199, 200), DAVID (201, 202), ConsensusPathDB (203) report biological pathways in which DEGs are enriched or reduced and take these into account to predict how these pathways might be affected. However, the data from which these tools were constructed come from non-neuronal samples which could lead to associations not found in neuronal tissues or failure to detect neuronal associations (204). In order to annotate neuronal gene expression in a temporal and spatial manner, human brain gene expression databases, for example, the Allen Human Brain Atlas (205), BrainSpan (206), GTEx (207) were built using microarray and RNA-seq data from post-mortem brain samples. Mapping DEGs identified in neuronal samples of patients with neurodevelopmental disorders to human brain gene expression databases revealed specific brain regions and neural developmental stages that were affected (34, 208). A more detailed human brain gene expression atlas that annotates gene expression at single cell level has been initiated by a group in Stanford University (209). Single-cell RNA-seq was used to analyze neurons from human adult and fetal cerebral cortex and it identified more diverse populations of neurons within the cortical region (209). Constructing a comprehensive human brain gene expression database at single neuron resolution is quite challenging due to limited access to healthy human brain samples and the high cost of single-cell RNA-seq. Therefore, collaborative work with standardized experimental protocols is required.

Gene Network Analysis

Differential gene expression from transcriptome analysis is sample-dependent and tissue-specific. In order to explore the etiology of complex neurodevelopmental disorders such as TD, disease-associated genes can be mapped to gene networks to visualize relationships between disease candidate genes and, further, to pinpoint annotated or novel pathways. The gene networks can be gene co-expression networks (205, 206), gene regulatory networks², protein–protein interaction networks (210) or networks constructed with combined criteria (203, 211). For example, in ASD, disease-associated genes have been evaluated using spatiotemporal gene co-expression networks constructed from BrainSpan (206) and were found to be enriched in sub-networks that represent specific brain regions and time periods during human brain development (212). Furthermore, ASD-associated

mutations identified by previous genetic studies were mapped to a “background network” which scores each pair of human genes based on very comprehensive information about every known human gene (211). Cell types and brain areas affected in ASD were implicated (213). With a growing number of mutations associated with TD, the same approaches could be utilized in TD.

Animal Models

Another approach to the study of the functional effects of a specific mutation in a gene is to use animal models. Most often, a putative disease gene is knocked out or modified in the animal model. Then, the mutant and wild type animals from the same genetic background are compared. (Some such animal knock-out studies of putative TD genes have been described above). Conventional animal models are designed to study a small number of genes, usually one or two genes at a time. If, however, TD is caused by the combined effect of multiple variant genes, multi-transgenic animals, whose genomes are modified at multiple loci, would be required. Although more challenging, generation of such multi-transgenic animals can be achieved by genome editing as well. (214, 215).

In order to model TD in animals, the following criteria should be met: (1) the gene to be studied is strongly associated with the disease; (2) the gene and the neurological component phenotypes involved in TD are relatively well conserved between humans and the animal; (3) the gene is thought to have similar functions in both humans and the model animals; and (4) the disease phenotype can be experimentally characterized in the model animals by biochemical and/or behavioral approaches (216). One mouse model in TD that meets these criteria is *HDC*. As indicated previously, a rare dominant non-sense mutation W317X in *HDC* cosegregated with all TD individuals in a two generation pedigree. (25). Subsequently, a study using *HDC* knockout mice demonstrated behavioral and molecular abnormalities caused by the loss of the HDC activity in the brain (28). The knockout and heterozygous mice showed tic-like stereotypic movements after psychostimulant administration. Also, the striatal dopaminergic pathway was dysregulated due to the HDC deficiency. Specifically, the dysregulation of dopamine receptors in the basal ganglia region of the *HDC* knockout and heterozygous mice recapitulated the dysregulation of the same types of dopamine receptors in TD patients carrying the W317X mutation.

SUMMARY AND FUTURE DIRECTIONS

Tourette's disorder is likely caused by a complex multigenic inheritance pattern that includes locus and allelic heterogeneity of both common and rare variants that interact with environmental factors (14, 217, 218). Despite long-standing interest in the genetic contribution to TD, the overall genetic architecture of TD remains elusive. Some of the genes identified as causal of TD are involved in neurotransmitter pathways presumed to be altered in TD, while others are novel. In this respect, the genetics of TD may resemble that of other complex neuropsychiatric disorders. Indeed, there is evidence of some overlap with subsets of similar genes involved in multiple disorders. Furthermore, there also appears to be an increased rate of comorbidity between some such

¹www.qiagen.com/ingenuity

²http://www.braineac.org/

disorders, such as TD and ASD (27, 219). It may also be that current DSM-based psychiatric nosology does not sufficiently “carve nature at its joints,” and that other classification schemes, such as Research Domain Criteria (RDoC)(220–224), might reveal etiologically more coherent groupings of disorders or patients.

International consortiums including the Tourette International Collaborative Genetics (TIC Genetics), the Tourette Syndrome Association International Consortium for Genetics (TSAICG), the European Multicenter Tics in Children Studies (EMTICS), the European Society for the Study of Tourette Syndrome (ESSTS), the Tourette Syndrome Genetics The Southern and Eastern Europe initiative (TSGeneSEE), and sharing repositories (New Jersey Center for Tourette Syndrome Repository) (225, 226) have initiated large collaborations to collect many patient and family samples in an effort to understand the genetics of TD. Continued efforts in gene discovery from large open-access repositories are needed to find additional risk variants.

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All authors wrote, reviewed, and critiqued the manuscript.

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