The Role of Dysfunctional Subcortical Circuitry in Mouse Models of Developmental Disability

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Neurobiology in the Graduate School of Duke University

2015
ABSTRACT

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Abstract

Developmental disabilities, including intellectual disability (ID), attention-deficit hyperactivity disorder (ADHD), and autism spectrum disorders (ASD), affect approximately 1 in 6 children in the United States. Attempts to produce treatment for developmental disabilities have been hampered by our current lack of understanding of the molecular mechanisms underlying these disorders. Advancements in genome sequencing and animal modeling technologies have proven to be an invaluable resource in the elucidation of potential disease mechanisms, with recent studies reporting novel mutations of the Ptchd1 and Shank3 genes in patients with developmental disabilities. Though these two genes have been proposed to play important roles in neural development, their function in the normal brain and defective behavioral output are poorly understood.

In this dissertation, I characterize the circuit and behavioral dysfunction of the genetically-engineered Ptchd1 and Shank3 knockout mice. With respect to Ptchd1, I found that expression is developmentally enriched in the thalamic reticular nucleus (TRN), which is a group of GABAergic neurons serving as the major source of inhibition for thalamo-cortical neurons. Slice and in vivo electrophysiological experiments revealed that deletion of this gene in mice disrupts SK2 currents and burst firing mechanisms in the TRN, a region that has previously been shown to play an important role in sleep, attention, and cognition. Consistent with clinical findings, Ptchd1 knockout mice display behavioral phenotypes indicative of hyperactivity, attention deficits, motor dysfunction, hyperaggression, and cognitive impairment. Interestingly, attention-like deficits and hyperactivity
are rescued by SK2 pharmacological enhancement, suggesting a potential molecular target for developing treatment.

*Shank3* knockout mice display ASD-like phenotypes, including social interaction deficits and repetitive behaviors. In addition, biochemical, electrophysiological, and morphological abnormalities were discovered in the medium spiny neurons (MSNs) of these mice. However, the exact neural circuits and cell types responsible for the autistic-like behaviors have not been identified. To address this important question, I developed a new conditional Shank3 knockout mouse. Importantly, the behavioral abnormalities reported in the original *Shank3* knockout mice were recapitulated in this novel conditional *Shank3* knockout mouse, indicating that this mouse may be useful for future pathway-specific dissections of ASD-like behaviors. Together, these two sets of studies not only provide mouse models for dissecting the function of PTCHD1 and SHANK3 in normal and abnormal neural development, but also demonstrate a critical role for PTCHD1 in TRN neurons and SHANK3 in MSN cells and in the case of PTCHD1, identify potential cellular and circuit pathway targets for much-needed pharmacological intervention.
Dedication

To my grandmother, Mercedes L. Montalvo v. de Valencia.
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Normal functioning and behavioral output of the mammalian brain requires coordinated and carefully controlled neuronal communication. Disruption of these processes can lead to neuropsychiatric diseases, such as schizophrenia, bipolar disorder, and intellectual disability. Currently, the disease mechanisms underlying these disorders are poorly understood. In addition, few effective treatments are available for afflicted individuals, due largely in part to the lack of validated molecular targets for pharmacological intervention. Though human genetic studies have recently begun to identify possible genetic contributions, the general mechanisms and brain circuitry involved in the etiology of these disorders remain largely unknown.

Due to a plethora of societal and economic factors, the situation surrounding efforts to alleviate the burden of these diseases has become dire. This is especially true with respect to developmental disabilities, which include intellectual disability (ID), attention-deficit hyperactivity disorder (ADHD), and autism spectrum disorders (ASD). By definition, all developmental disabilities appear before/during adolescence and present as general impairment of intellectual functioning or
adaptive behavior (Crocker, 1988). Though sharing this common description, these disorders differ in core symptoms and criteria for diagnosis. One is diagnosed with ID if he or she tests with an IQ at or below 70 (Mefford, Batshaw, & Hoffman, 2012), while an ASD diagnosis requires the presence of verbal impairment, social interaction deficits, and repetitive or stereotyped behavior (Fakhoury, 2015; Kanner, 1946). The diagnostic checklist for ADHD, which is the most common and fortunately most treatable childhood developmental disability, includes hyperactivity with impulsive and/or inattentive behaviors (Willcutt, 2012). There is a wide variation of symptoms within each of these disorders, with the experiences of any given individual lying on a spectrum of severity. This fact, coupled with the relatively high rates of co-morbidity among these disorders (Antshel, Zhang-James, & Faraone, 2013; Bradley, Summers, Wood, & Bryson, 2004; Gautam, Bhatia, & Rathi, 2014), further complicates efforts to identify common disease mechanisms and drug targets.

Recent surveys estimate that developmental disabilities affect approximately 1 in 6 children in the United States (Boyle et al., 2011), making them some of the most commonly diagnosed mental impairments in this country. Unfortunately, the prevalence of these disorders has dramatically increased over the past two decades. In fact, the proportion of the US children diagnosed with ASD has doubled over that time span (Wingate et al., 2014). Though many factors ranging from paternal age (Hultman, Sandin, Levine, Lichtenstein, & Reichenberg, 2011; Kong et al., 2012) to exposure to various environmental factors (Newschaffer et al., 2007) have been proposed to explain this troubling increase, the major factor is probably the increased awareness and expanded diagnosis criteria.
Given the rising prevalence and the ballooning economic costs of developmental disabilities (Buescher, Cidav, Knapp, & Mandell, 2014), it is now more critical than ever for researchers to elucidate the cellular and molecular mechanisms underlying these disorders. Progress in this realm, much like past victories in the development of non-central nervous system drugs, will rely on the use of animal models. Recent advancements in animal modeling have proven to be a boon for neuropsychiatric disease research, with innovations in genome editing and circuit manipulation techniques leading the way in solving many impervious problems. Especially beneficial to the past and future success of psychiatric disease research is a species known as *mus musculus*, which is better known by its household name—the mouse.

### 1.1 Mouse models of psychiatric disease

The vast majority of cellular and molecular investigations of the mechanisms of developmental disability have relied on rodents as a model of the mammalian brain. The rise of the rodent model can be attributed to a few key factors. First of all, humans and rodents share a nearly identical set of genes. Recent improvements in genome sequencing techniques have led to the conclusion that a vast majority of genes found in humans are also found in rodents, with an average amino acid sequence homology of 85% (Batzoglou, Pachter, Mesirov, Berger, & Lander, 2000). These commonalities theoretically allow for discoveries in the rodent model to be extrapolated to the human condition. Secondly, rodents have a short incubation period (18-21 days) with large litter sizes (6-10 pups on average, depending on strain). This fact, combined with the relatively low cost of
housing and maintaining rodents compared to other mammals, allows for researchers to conduct quick and inexpensive experiments that can be easily replicated by other investigators. Finally and most importantly, rodent models have gained in popularity due to available advanced genetic manipulation techniques.

1.1.1 The use of mice versus rats

For most of the 20th century, rats were the standard animal of choice for pharmacological, biochemical, and behavioral assays. This began to change, however, in the early 1990s with the advent of knockout mouse technology (Robertson, Bradley, Kuehn, & Evans, 1986; Thomas & Capecchi, 1987). This method, which was pioneered by the 2007 winners of the Nobel Prize in Physiology or Medicine Mario Capecchi, Oliver Smithies, and Martin Evans, allowed researchers for the first time to alter specific segments of the genome of an animal. The targeted insertion or deletion of genetic material made it possible to study the role of individual genes on the behavioral output of an organism.

Targeted genome editing techniques involve the use of modified DNA constructs that are introduced into embryonic stem cells (Starkey & Elaswarapu, 2011). After homologous recombination between the exogenous and endogenous DNA takes place, a small percentage of stem cell colonies contain the desired gene mutation. These positively-targeted colonies are selected and injected into developing blastocysts, which are then introduced into pseudo-pregnant female mice. The end result is chimeric offspring that have a genome with both modified and endogenous DNA. Ideally, these chimeras will produce gametes containing the altered genome, which is necessary for successful transmission of the changes to subsequent generations. The original and still most commonly used techniques are
dependent on the successful culturing of embryonic stem cells. This process has been historically difficult in rats (Brenin et al., 1997; Buehr et al., 2003; Schulze, Ungefroren, Bader, & Fandrich, 2006), though it is worth noting that the recent advancements in rat ES cell culture and genome-editing technologies has led to a limited re-emergence of rat models for psychiatric research. On the contrary, mouse stem cell culturing methods are well-established and show high chimera viability (Hofker & Deursen, 2011). For these reasons, mice became the more often utilized animal in this system and have served as the foundation of decades of psychiatric disease research.

1.1.2 Commonly used mouse models of disease

One of the first mouse models of neuropsychiatric disease to be generated and characterized was the *Fmr1* knockout mouse, which mimicked the loss of FMR1 expression, but not the exact genetic aberration of humans with Fragile X Syndrome (FXS) (Pieretti et al., 1991). This mouse, which was produced by the Dutch-Belgian Fragile X Consortium in 1994, was originally found to have learning deficits and hyperactivity ("Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium," 1994). Subsequent work by Mark Bear and colleagues identified several other disease-relevant endophenotypes, including altered synaptic plasticity, increased dendritic spine density, and increased hippocampal protein synthesis (Bear, Huber, & Warren, 2004). These abnormalities, including deficits in learning, were rescued in mice through negative modulation of mGluR5 receptors (Dolen et al., 2007). In response to the positive results in mice, human trials involving mGluR5 antagonists have been conducted. These trials, however, have resulted in limited success in treating the hallmark behaviors of FXS (Scharf, Jaeschke, Wettstein, & Lindemann, 2015).
Drugs attacking other molecular targets, such as GABA (Lozano, Hare, & Hagerman, 2014) receptors, may still hold promise (Berry-Kravis et al., 2012; Schaefer, Davenport, & Erickson, 2015).

Another commonly used mouse model of psychiatric disease is the *Mecp2* knockout mouse, which models the lethal neurodevelopmental disorder known as Rett Syndrome (RS) (Amir et al., 1999; Couvert et al., 2001). This mouse displays abnormal social behaviors, increased anxiety, motor impairments, seizures, and premature death (Guy, Hendrich, Holmes, Martin, & Bird, 2001). Investigations on MECP2 have led to the conclusion that it acts as a transcriptional repressor and that aberrant gene expression could lead to the abnormal behaviors present in the *Mecp2* KO mouse (Moretti & Zoghbi, 2006; Nan, Campoy, & Bird, 1997). The growth factor BDNF has been found to alter MECP2 levels (Chang, Khare, Dani, Nelson, & Jaenisch, 2006) and in doing so corrects some of the defective behaviors of the KO mouse (Kline, Ogier, Kunze, & Katz, 2010). BDNF, however, is unable to cross the blood-brain barrier (BBB), which has limited its therapeutic potential. IGF-1 functions similarly to BDNF and has the added benefit of being able to cross the BBB. IGF-1 has been found to rescue many of the more severe symptoms displayed by the *Mecp2* KO mouse (Castro et al., 2014; Tropea et al., 2009) and is currently the focus of human clinical trials (Bray, 2014; Pini et al., 2014).

Together, the use of these mice and other candidate gene knockout models has resulted in a richer understanding of the mechanisms underlying these diseases as well as some of the basic principles of brain function. However, these gains in knowledge have yet to result in any effective and widely-available treatments, a problem common among neuropsychiatric diseases.
1.1.3 The role of mouse models in drug discovery

These aforementioned mouse models have been used as the foundation of extensive drug development by academic and industrial scientists. However, despite years of research and billions of dollars spent, these endeavors have generated few success stories in terms of producing FDA-approved treatments for complex neuropsychiatric diseases (Ivinson, 2014; Pankevich, Altevogt, Dunlop, Gage, & Hyman, 2014). For this reason, many major pharmaceutical companies have begun to contract, if not completely eliminate, neuroscience research divisions from their payrolls (Insel et al., 2013).

There are several reasons for this stagnation along the drug discovery pipeline. First of all, there is increased risk and cost associated with developing drugs targeting the central nervous system compared to other drug targets (Wegener & Rujescu, 2013). The FDA review time for CNS drugs, for example, is 35% longer than for non-CNS drugs (Choi et al., 2014), which is valuable time that reduces the patent shelf life. Secondly, the identification and validation process of drug targets for psychiatric diseases is significantly slower than other disorders (Pankevich et al., 2014). Finally, there is growing concern regarding the predictive value of animal models. Phase II clinical trial success rates are currently below 20% despite the efficacy of these drugs in mice and rats (Arrowsmith, 2011). When coupled with the lack of disease biomarkers for human psychiatric disease, it comes as no surprise that industrial pharmaceutical companies are fleeing the field of neuroscience. These failures to create life-improving treatments have long-lasting effects on society, both in terms of health-care costs and lost productivity.

One approach to overcome these issues is to use mouse models primarily to study the conserved molecular and circuitry mechanisms underlying disease while
de-emphasizing resemblances between mouse behavior and human symptoms. Rather than draw direct comparisons between abnormal behavior and disease, behavioral output could be used as a read-out of defective circuitry. This strategy could be used to identify behaviorally-relevant circuit-specific disruption in mouse models that could then be used as targets for drug development.

The shortcomings of the mouse model at the drug discovery level could also be improved by recent advancements in genome editing technology. Current methods can take as long as 12-18 months from the manufacture of DNA constructs to the analysis of behavior in backcrossed mice (Starkey & Elaswarapu, 2011). New methods, including the TALEN (Transcription Activator-Like Effector Nuclease) system and the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) approach, could save researchers both time and money by eliminating the need for cultured embryonic stem cells and allowing for the direct \textit{in vivo} gene targeting of fully-developed animals (Bedell et al., 2012; Dow et al., 2015; Gaj, Gersbach, & Barbas, 2013; Swiech et al., 2015). These editing methods can be used in cultured ES cells and induced pluripotent stem cell (iPSCs) to induce specific mutations with high efficiency (Li et al., 2015; H. Wang et al., 2013). Progress in the TALEN and CRISPR systems could also allow for the use of non-human primates (Niu et al., 2014), which share more genetic and behavioral similarities with humans, to be used as models of psychiatric disease. Successful targeted mutations of disease-relevant genes from these species have the potential to provide the breakthroughs necessary for drug development.

Though these improved technologies have generated much excitement about the future prospects of drug discovery, they are still in their early stages of development. As such, the employment of standard genome editing techniques in
mice to garner a better understanding of disease and non-disease states of the brain is a necessary step in the acceleration of the drug discovery process for neuropsychiatric diseases. These established methods allow for circuit-specific dissection of behavior. Initial inquiries into the etiology of neurodevelopmental disorders focused on the most recently evolved region of the brain, the neocortex (Goldman, 1953; Mesulam & Geschwind, 1978; Walker, 1957; Winkelman & Book, 1949). Recent studies, however, have begun to illuminate the contributions of more primitive subcortical circuits to the production of abnormal behavioral output in mice.

1.2 Thalamo-cortical circuitry and developmental disorders

1.2.1 Structure and function

One such subcortical structure linked to psychiatric disease is the thalamus. The thalamus serves as a relay and processing station between peripheral systems and the cortex. Most, if not all, afferents from sense organs (e.g. vision, hearing, pain, etc.) and other brain regions (e.g. cerebellum) pass through the thalamus on the way to cortical areas (Blum, Abraham, & Gilman, 1979; Krettek & Price, 1974; Shosaku, Kayama, & Sumitomo, 1984a; Shosaku & Sumitomo, 1983). Structurally, the dorsal thalamus is composed of several subdivisions that can be categorized as first order relay nuclei and higher order relay nuclei. First order relay nuclei, which include the lateral geniculate nucleus and medial geniculate nucleus, receive glutamatergic input from peripheral sense organs (the eyes and ears, respectively) and project onto cortical regions (Shepherd, 2004). Higher order relay nuclei, which include the pulvinar nucleus, receive glutamatergic afferents from the cortex itself and send this information to other regions of the cortex. Regulating these
interactions is a shell of GABAergic interneurons in the ventral thalamus known as the thalamic reticular nucleus (TRN) (Ohara & Lieberman, 1985). In rodents, the TRN is the predominant source of inhibition in the thalamus, while the primate brain contains inhibitory interneurons throughout the thalamic structure (Arcelli, Frassoni, Regondi, De Biasi, & Spreafico, 1997). TRN projections synapse primarily onto thalamic relay nuclei, thereby forming an inhibitory feedback loop that modifies thalamic relay cell firing onto the cortex (Pinault, 2004). Layer VI cortical cells also innervate the TRN as part of an inhibitory feedforward circuit that modulates thalamic nuclei activity. As such, the TRN serves an important role in regulating thalamo-cortical communication.

The TRN is also important for the development of thalamic circuitry. Cortico-thalamic circuits begin to develop as early as embryonic day 10 (E10) in mice (Grant, Hoerder-Suabedissen, & Molnar, 2012). Around this age, post-mitotic cortical cells start to migrate towards the preplate, which is the first stage of corticogenesis. At this point, the young cortical neurons form neurites that initiate their six-day journey towards the prethalamus. Once at the prethalamus, these cortical axons pause at the TRN until E18, after which point they begin to innervate dorsal thalamic nuclei (Jacobs et al., 2007; Miller, Chou, & Finlay, 1993; Molnar & Cordery, 1999). Directing the cortical axons to dorsal thalamic nuclei is a series of structural and molecular guidance cues. Early in the trek, cortical axons must first pass through regional boundaries (Lopez-Bendito & Molnar, 2003; Schuurmans & Guillemot, 2002), which is achieved through the use of molecular gradients (Harwell et al., 2012; Skaliora, Singer, Betz, & Puschel, 1998). Cortical axon innervation of thalamic relay nuclei takes place after TRN invasion of the thalamus (Mitrofanis & Baker, 1993; Molnar, Adams, & Blakemore, 1998),
suggesting that this connectivity plays a critical role in the maturation of cortico-thalamic circuitry.

Throughout the migration of cortical axons, thalamic nuclei are forming in the absence of cortical input. This process is mediated in part by the sonic hedgehog (SHH) pathway. Though not completely understood, it is believed that SHH gradients that are created from two sources (zli and basal plate) help to establish two distinct progenitor cell types (Jeong et al., 2011b). The caudal group of thalamic progenitors generates all sensory relay nuclei (Vue et al., 2007) while the rostral group gives rise to the ventrolateral geniculate nucleus and the intergeniculate leaflet (Horowitz, Blanchard, & Morin, 2004). In addition to producing major progenitor cells, SHH is known to play a critical role in thalamic interneuron identity (Flandin, Zhao, Vogt, Jeong, Long, Potter, Westphal, & Rubenstein, 2011; Sousa & Fishell, 2010). Through these TRN-dependent and TRN-independent mechanisms, thalamo-cortical circuits reach maturity as early as P9 in mice (Warren & Jones, 1997).

Due to its unique structure and connectivity with the cortex, the thalamus has been called the “gateway to the cortex” and the TRN acts as the “guardian of the gateway to the cortex” (Crick, 1984). This terminology, however, implies that the TRN acts like nothing more than a filter of cortex-bound information. In light of recent findings, the role of the TRN in the processing of information has expanded to include computations previously believed to be carried out exclusively by the cortex. The TRN has been hypothesized to function as a modulator of attention, with several reports detailing the link between the TRN and both visual and auditory attention (McAlonan, Cavanaugh, & Wurtz, 2008; Pinault, 2004; Yu, Xu, He, & He, 2009). Investigations in non-human primates revealed that a
mechanism involving the TRN and LGN functions in processing visual information prior to cortical involvement (McAlonan et al., 2008), a finding supported by human fMRI studies (O’Connor et al., 2002). Importantly, the TRN was found not only to function as a detector of attention-requiring stimuli (Zikopoulos & Barbas, 2007), but also as a suppressor of distracting information and a detector of deviant auditory stimuli (Yu et al., 2009).

The TRN is also known to regulate consciousness by modulating arousal during both wakefulness and sleep. During wakefulness, the TRN activity has been correlated with different levels of arousal and attention (McAlonan & Brown, 2002; Min, 2010). During sleep, mechanisms within the TRN generate sleep spindles (Cueni et al., 2008; Halassa et al., 2011; Steriade, Domich, & Oakson, 1986) that are recorded in the cortex by electroencephalography (EEG) measurements. Other known roles of the TRN, through modulation of thalamic nuclei output, include language processing (Klostermann, Krugel, & Ehlen, 2013), hippocampal-dependent memory consolidation (Mednick et al., 2013), and striatal-mediated behavioral flexibility (Floresco, Zhang, & Enomoto, 2009). As a whole, these results indicate that the TRN plays an active role in several cognitive processes and therefore should be considered more than a simple gateway.

1.2.2 Relevance to disease

Thalamic circuit dysfunction has been linked to several human brain disorders. Reductions in TRN-generated sleep spindles were found in patients with schizophrenia and ASD (Ferrarelli et al., 2007; Ferrarelli et al., 2010; Limoges, Mottron, Bolduc, Berthiaume, & Godbout, 2005; Wamsley et al., 2012). In addition, neuroimaging studies have revealed significant decreases in thalamic
volume in both adult and adolescent patients afflicted with schizophrenia or bipolar disorder (Csernansky et al., 2004; J. A. Frazier et al., 2005; Radenbach et al., 2010; Rao, Kalmady, Arasappa, & Venkatasubramanian, 2010). This finding was supported by post-mortem analysis of brains from adults with schizophrenia, who were found to have reduced volume and cell number in several association nuclei of the thalamus (Byne et al., 2002). Within the thalamus of bipolar and schizophrenic patients, abnormalities in dopamine content (Moghaddam, 2010) and NMDA receptors concentrations (Clinton & Meador-Woodruff, 2004) have been identified, though this work remains controversial and requires further investigation. Recent studies have also linked the thalamus to common neurodegenerative disorders, including Parkinson’s disease, Huntington’s disease, and Alzheimer’s disease (Braak & Braak, 1991; de Jong et al., 2008; Halliday, 2009).

Given the role of the TRN in attention, it comes as no surprise that youths diagnosed with ADHD were found to have thalamic morphological differences when compared to healthy controls. Neuroimaging methods revealed that children with ADHD had smaller pulvinar nuclei, which functions as an association region that receives input from multiple cortical sources. The size of the pulvinar nucleus was found to be influenced by whether or not the subject was undergoing pharmaceutical treatment, with treated patients showing an increase towards normalcy (Ivanov et al., 2010). Furthermore, other studies using diffusion tensor imaging identified decreases in white matter tracts connecting the thalamus to the prefrontal cortex (Xia et al., 2012), which could explain some of the deficits in attention and executive control exhibited by these individuals. While these findings point to a significant role of dysfunctional thalamo-cortical circuits in
neuropsychiatric disorders, few if any mouse models of developmental disability have directly probed TRN function in disease-relevant abnormal behavior. This issue will be addressed in Chapter 3 and Chapter 4 of this document.

1.3 Basal ganglia circuitry and developmental disorders

1.3.1 Structure and function

Another subcortical region—the basal ganglia—has been the subject of many investigations of circuit-specific causes of developmental disability. The basal ganglia is composed of a set of large nuclei that together play a critical role in goal-oriented behavior through the selection of specific movements and strategies among a plethora of options (Shepherd, 2004). In doing so, the basal ganglia functions in procedural learning and habit formation. This rather complicated circuitry is conserved across many species, suggesting that it is an essential component of normal central nervous system activity.

The major nuclei of the basal ganglia include the striatum (subdivided into the caudate and putamen in humans), globus pallidus (GP), substantia nigra (SN), nucleus accumbens (NAc), and subthalamic nucleus (STN). The basal ganglia receive input from both the cortex and thalamus, with excitatory afferents converging predominately in the striatum. These striatal cells then project to other basal ganglia, specifically the globus pallidus internal segment (GPi), globus pallidus external segment (GPe), and the substantia nigra pars reticulate (SNr). The GP and SNr then act as the main output structures of the basal ganglia, sending inhibitory connections to thalamic and, in the case of the GPe, cortical targets (Saunders et al., 2015). Together, these regions form the cortico-striato-thalamo-cortical loop that is critical for motor movements and action.
On the cellular level, the striatum consists almost entirely of medium spiny neurons (MSNs). MSNs are inhibitory projection neurons with highly branched dendritic arbors and large quantities of dendritic spines (DiFiglia, Pasik, & Pasik, 1976; Pasik, Pasik, Holstein, & Hamori, 1988). MSNs can be subdivided into two major subtypes—direct and indirect pathway (Smith, Bevan, Shink, & Bolam, 1998). This categorization is based on several discerning features of these cells. The direct pathway originates with inhibitory signals from the striatum that target the output nuclei SNr and GPi, which in turn send inhibitory information to the thalamus. Direct pathway MSNs express the D1 dopamine receptor and high concentrations of the neuropeptide substance P (Penny, Afsharpour, & Kitai, 1986). The indirect pathway consists of encephalin and D2 dopamine receptor expressing MSNs that innervate the GPe, which sends inhibitory projections to the STN. The STN then sends excitatory signals to the output nuclei of the basal ganglia (Steiner & Gerfen, 1999). Together, these pathways work in opposition of each other to influence thalamic output to cortical targets, with the direct pathway leading to an overall reduction of inhibitory tone (disinhibition) on the cortical-projecting thalamic neurons and the indirect pathway increasing said tone (Chevalier & Deniau, 1990).

The basal ganglia also include a limbic sector that consists of the NAc and ventral tegmental area (VTA). These structures work in concert to influence reward-seeking behavior and motivation by assigning a valence to motor decisions (Nicola, 2007; Stopper & Floresco, 2011). By assigning reward to different actions, the NAc and VTA play an important role in reinforcement learning. The NAc receives input from cortical, hippocampal, and amygdalar regions in addition to modulatory dopaminergic input from the VTA (Brady & O’Donnell, 2004;
Callaway, Hakan, & Henriksen, 1991; Jones, Mogenson, & Wu, 1981). The output of NAc computations is then sent to other basal ganglia nuclei where the information is integrated into the thalamic-bound circuits. Together, the integration of motor, sensory, and motivational circuits that takes place within the basal ganglia affords this structure a critical role in selection of behavior among competing goals and motivations (Dayan & Balleine, 2002).

1.3.2 Relevance to disease

Historically, the basal ganglia has been associated with movement disorders (Mettler, 1964). As early as the late 1970s, however, researchers hypothesized that striatal dysfunctions were a salient target for language deficits, repetitive behaviors, and motor problems displayed by autistic patients (Damasio & Maurer, 1978). Several MRI studies have identified an increase in caudate nucleus volume in both children and adults with ASDs (Estes et al., 2011; Hollander et al., 2005; Langen, Durston, Staal, Palmen, & van Engeland, 2007). Functional MRI approaches in ASD patients have also discovered abnormal striatal functional connectivity (Di Martino et al., 2011) and deficits in striatal activity during social reward processing tasks (Scott-Van Zeeland, Dapretto, Ghahremani, Poldrack, & Bookheimer, 2010). Interestingly, diffusion tensor imaging has also revealed fronto-striatal white matter defects in fragile X syndrome patients (Barnea-Goraly et al., 2003). At the same time, experiments conducted on healthy subjects have found strong evidence for striatal involvement during the anticipation of positive social feedback, as well as in response to social stimuli such as observing an attractive face (Aharon et al., 2001), viewing positive emotional expressions (Spreckelmeyer et al., 2009), and watching images of maternal or romantic love (Bartels & Zeki, 2004). As a whole, however, the limitations of these imaging techniques render
these data as merely correlative to the actual causation of ASD. As a result, the
direct relationship between the striatum and ASD remains poorly understood,
especially at the molecular, cellular, and circuit level. This issue will be addressed
in Chapter 5 of this document.

1.4 Discussion

In this chapter, I discussed the need for improved pharmacological
treatments for developmental disability and the benefits afforded by use of the
mouse model system in this journey. Despite the limitations of the mouse model,
its contribution to the field of psychiatric disease research cannot be understated.
This system has been, and in lieu of revolutionary technological advancements, will
continue to be the foundation of neuropsychiatric research for the foreseeable
future. Mouse-based investigations of two evolutionary subcortical brain regions—
the TRN and the basal ganglia—can greatly improve our understanding of these
diseases and could lead to the identification of molecular targets for future drug
treatments.

In the following chapters, I will detail my contributions to the
understanding of these regions in the diseased brain. More specifically, I will
discuss the results of my investigations of striatal dysfunction in the Shank3b
knockout mouse model of ASD and TRN deficits in the Ptchd1 knockout mouse
model of developmental disability. In addition to presenting the novel behavioral
and circuit abnormalities observed in these mice, I will argue the ways in which
these findings can help in the search for therapeutic intervention for humans
afflicted by mutations of these genes.
Materials and Methods

This chapter details the materials and methods used for experiments in subsequent chapters. All slice and electrophysiology experiments were conducted by Dr. Ralf Wimmer, Dr. Ian Schmitt, and Dr. Michael Halassa of New York University. The visual detection task was a joint effort between me and Dr. Wimmer. I performed all other experiments, with generous instruction and input from members of the Feng lab. The PCR primers and antibodies used in these experiments are summarized in Appendix A and B, respectively.

2.1 Production of genetically engineered mice

Generation of Ptchd1-YFP mouse

Ptchd1-YFP mice were generated by homologous recombination in R1 embryonic stem cells and implanted in C57Bl/6J blastocysts using standard procedures (Starkey & Elaswarapu, 2011). The targeting vector replaced Exon 1 of the Ptchd1 gene with ATG-eYFP-STOP cassette and a NEO cassette. Correctly targeted ES colonies were PCR screened using primers targeting the flanking the YFP insert and long-arm PCR (LA Taq) methods. Chimaeric mice were crossed to C57Bl/6J
females from Jackson Labs. Germline transmission was assessed through genotyping PCR of mouse tail DNA, using primers pFW (Pt1-YFPki) Gen 3a, pRV (Pt1-YFPki) Gen 3a, and pFW (Pt1-YFPki) Gen 3b for the wild-type allele (positive band = 190 base pairs) and the YFP positive allele (positive band = 316 base pairs). The F1 hybrids were backcrossed to C57Bl/6J mice for 2 generations. Female mice containing the YFP insertion (Ptchd1-YFP+/-) were used for all immunohistochemistry experiments while Ptchd1-YFP+/+ and Ptchd1-YFP+/− male mice were used for behavior experiments.

**Generation of Ptchd1 conditional knockout mouse**

The targeting vector flanked Exon 2 of the Ptchd1 gene with loxP sites and a NEO cassette. Chimaeric mice were crossed to C57Bl/6J females from Jackson Labs. Germline transmission was assessed through genotyping PCR of mouse tail DNA, using primers pFW (Pt1cKO) Gen 1a and pRV (Pt1cKO) Gen 3b for the wild-type allele (positive band = 187 base pairs) and the floxed allele (positive band = 227 base pairs). The F1 hybrids were crossed to C57Bl/6J β-Actin Flp mice to excise the NEO cassette. The floxed mice were then backcrossed to C57Bl/6J mice for 5 generations. After the 5th generation, speed congenic genotyping PCRs were conducted to determine the approximate purity of the background. Only mice showing >95% C57Bl/6J background were used for subsequent matings. Backcrossed Ptchd1 floxed mice were then bred with C57Bl/6J β-Actin Cre mice to produce germline knockouts of the floxed allele (termed Ptchd1 KO mice). Genotypes were determined by PCRs using the pFW (Pt1cKO) Gen 1a and pRV (Pt1cKO) Gen 4c for the knockout allele (positive band = 351 base pairs). For all behavioral experiments in the C57Bl/6J
background, \textit{Ptchd1}^{\pm} and \textit{Ptchd1}^{\pm\pm} males were bred with \textit{Ptchd1}^{\pm\pm} females. For the C57/129 mixed background behavioral experiments, 129^{\pm\pm} males were bred with \textit{Ptchd1}^{\pm\pm} C57Bl/6J females and the F1 offspring were used for experiments.

\textit{Generation of Shank3 conditional knockout mouse}

The targeting vector flanked Exons 13-16 (PDZ domain) of the \textit{Shank3} gene with loxP sites and a NEO cassette. Chimaeric mice were crossed to C57Bl/6J females from Jackson Labs. Germline transmission was assessed through genotyping PCR of mouse tail DNA, using primers \textit{pFW (Sh3cKO) Gen 1a} and \textit{pRV (Sh3cKO) Gen 3b} for the wild-type allele (positive band = 188 base pairs) and the floxed allele (positive band = 228 base pairs). The F1 hybrids were crossed to C57Bl/6J \(\beta\)-Actin Flp mice to excise the NEO cassette. The floxed mice were then backcrossed to C57Bl/6J mice for 5 generations. After the 5th generation, speed congenic genotyping PCRs were conducted to determine the approximate purity of the background. Only mice showing >95% C57Bl/6J background were used for subsequent matings. Backcrossed \textit{Shank3} floxed mice were then bred with C57Bl/6J \(\beta\)-Actin Cre mice to produce germline knockouts of the floxed allele (termed \textit{Shank3}b cKO mice). Genotypes were determined by PCRs using the \textit{pFW (Sh3cKO) Gen 1a} and \textit{pRV (Sh3cKO) Gen 5a} for the knockout allele (positive band = 290 base pairs). Pure C57Bl/6J heterozygous mice were bred to pure 129 mice to generate a mixed background. Mixed heterozygous mice were subsequently bred to each other to create \textit{Shank3}b^{\pm\pm\pm} and \textit{Shank3}b^{\pm\pm} male mice for behavioral tests.
2.2 Transcript and protein analysis

cDNA library preparation and qPCR

Whole brain tissue was dissected and flash frozen in liquid nitrogen prior to storage at -80°C. RNA was extracted using the TRIzol reagent (Invitrogen) protocol. In brief, tissue was dissociated using a glass-Teflon homogenizer followed by a phase separation step. RNA was precipitated with isopropyl alcohol and washed in 75% ethanol. The RNA pellet was resuspended in RNAase-free water and quantified using a NanoDrop 2000 device (Thermo Scientific). RNA samples then underwent a DNase inactivation step (Ambion) to remove contaminating DNA. Finally, DNA-free RNA samples were used as templates for reverse transcriptase PCR reactions using the iScript cDNA Synthesis kit (Bio-Rad).

The Ptchd1 quantity in the cDNA templates from different developmental ages was measured using an iQ5 Real-Time Thermal Cycler (BioRad). HPLC-purified primers were designed to produce an amplicon between 80-150bp from Ptchd1 and the control gene Tbp1. These primers were previously optimized by the Harvard PrimerBank and confirmed by sequencing of in-house PCR products. Initial qPCR reactions established an efficiency curve to confirm stability of the amplicon with successive cycles. Finally, samples were run in triplicate using SYBR Green qPCR Master Mix (Life Technologies) with a melting curve generated at the end of the reaction to confirm amplicon specificity. Relative Ptchd1 and Tbp1 amounts were calculated using the standard ΔΔCt method.

In situ hybridization

mRNA in situ hybridization was performed as described elsewhere (Peca et al., 2011). Reactions were performed with 20mm cryosections from freshly frozen
brain tissue from male mice using a mixture of two digoxigenin (DIG)-labelled probes against mouse *Ptchd1* cDNA (GenBank Accession NM’001093750.1; base pairs 372-1006 and base pairs 1290-2027). The hybridization signal was visualized using an alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche) and developed using 5-bromo-4-cloro-indolylphosphate/nitroblue tetrazolium (BCIP/NBT; Roche). Sections were mounted using 90% glycerol and imaged using an Olympus BX61 motorized fluorescent microscope.

**HEK293/COS-7 cell transfection**

Two days after plating onto glass coverslips, HEK293 or COS-7 cells were transfected overnight using Lipofectamine 2000 reagent (Invitrogen) with either the PTCH1-YFP (positive control), PTCHD1-YFP (modified from PTCH1-YFP construct; gift from Dr. Andrew McMahon), or EGFP-N1 (negative control) expression construct. After 24-48 hours (depending on when cells reached 50-70% confluency), the cells were exposed to recombinant SHH-N (10nM final concentration), media collected from SHH-FLAG overexpressed HEK293 cells, or protein lysate collected from SHH-FLAG overexpressed HEK293 cells. The presence of SHH-N in the media and lysate preparations was confirmed using immunocytochemical and Western blot techniques prior to exposure. The exposure ranged from 15 minutes to 2 hours at various temperatures (4°C, room temperature, or 37°C) which were chosen based on previous reports.

Post-incubation, cells were fixed with 4% paraformaldehyde and prepared for immunocytochemistry. Coverslips were washed 3 x 5 minutes in 1X PBS followed by a 1 hour incubation at room temperature in blocking solution (5% normal goat serum, 2% BSA, 0.1% Triton X-100 in 1X PBS). Sections were then
incubated overnight at 4°C in primary antibody. After a 3 x 10 minutes wash in 1X PBS, coverslips were incubated for 2 hours at room temperature in secondary antibody. Following a 3 x 10 minutes wash protocol, coverslips were mounted onto glass slides using Fluoro-Gel (Electron Microscopy Sciences) and imaged with an Olympus Fluoview 1000 confocal microscope.

**SDS-PAGE & Western blot**

Protein lysates were prepared from adult brains using standard procedures. In brief, tissue was placed in RIPA buffer containing protease inhibitors and dissociated on ice using a motorized homogenizer. Homogenate was then rotated on a nutator for 30 minutes at 4°C. Lysates were centrifuged at 900g at 4°C for 15 minutes to remove the nuclear pellet followed by an additional 15 minute centrifugation at 15,000g. The protein pellet was resuspended in RIPA buffer and quantified using the Pierce BSA method. Samples were then mixed with 2X Laemmli buffer and heated for 5 minutes at 95°C. Forty-microgram samples were then loaded into a 4%-12% SDS-PAGE gradient gel and run in 1X Protein Running Buffer for approximately 2 hours at 100V. Gels were then transferred to nitrocellulose membranes in 1X Protein Transfer Buffer for 100 minutes at 90V. After a 1 hour incubation step in blocking buffer (1X TBS + 5% Milk), nitrocellulose membranes were incubated in primary antibody overnight at 4°C. After a 3 x 20 minutes wash in 1X PBS, blots were incubated for 4 hours at room temperature in secondary antibody. After another 3 x 20 minutes wash in 1X PBS, blots were imaged using an Odyssey CLx Infrared Imaging system (LI-COR).
**Immunohistochemistry**

Mice were perfused with ice-cold 1X PBS and 4% paraformaldehyde. Brains were then fixed overnight at 4°C prior to vibratome sectioning. Fifty micron sections were washed with 1X PBS 3 x 5 minutes prior to a 1 hour room temperature incubation in blocking solution (5% normal goat serum, 2% BSA, 0.2% Triton X-100 in 1X PBS). Sections were then incubated overnight at 4°C in primary antibody solution. Following the 3 x 20 minutes wash in 1X PBS, tissue was incubated for 4 hours at room temperature in second antibody solution. Sections were once again washed 3 x 20 minutes in 1X PBS prior to mounting onto glass slides using Fluoro-Gel. Images were taken with an Olympus Fluoview 1000 confocal microscope and co-labeling was manually quantified.

**Production of PTCHD1 fusion antibody**

*Ptchd1* gene fragments were cloned into pET23b and pET28a expression constructs (Novagen) using standard PCR and restriction ligation methods. Constructs were heat shock transformed into either Rosetta or BL21 bacterial cells and incubated at 37°C for 2-4 hours. Induction of the T7 polymerase was initiated through the application of IPTG, which took place when the cultures reached an OD of 0.6-0.8 as read on a cuvette spectrophotometer. Several induction conditions were tested as follows: 30°C or 37°C incubation for 4 or 16 hours with 0.1μM or 0.4μM IPTG. Samples were quantified using the Pierce BCA method and loaded 30μg per well onto a 10% SDS-PAGE gel. After running the gel at 120V for 90-120 minutes, the SDS-PAGE gels were stained with Coomassie blue dye and washed before imaging with a digital camera.
2.3 TRN electrophysiology

Slice electrophysiology

Freshly prepared brain slices from P21-P28 male mice were superfused with oxygenated ACSF (in mM: 125 NaCl, 25 NaHCO$_3$, 25 glucose, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 1.2 MgCl$_2$, 2 CaCl$_2$, 1.7 ascorbic acid) and recorded at 30-34°C. Patch pipettes (2.5-4MΩ) contained the following intracellular solution in mM: 140 KMeSO$_4$, 10KCl, 10 HEPES, 0.1 EGTA, 4Mg-ATP, 0.2 Na-GTP, 10 phosphocreatine, (285 mOsm, pH 7.2). Calcium currents (T currents) were isolated through application of the SK channel blocker apamin (100 nM) while SK currents were estimated by digital subtraction of the isolated T current from control currents without apamin.

In vivo TRN recordings

Hyperdrives containing 12 individually adjustable microdrives loaded with 1-2 stereotrodes were built as previously described. Mice were anesthetized with 1% isofluorane and mounted on a stereotaxic frame. A 3 x 2.5mm craniotomy (center coordinate M/L 2.5mm, A/P −1.3mm) was drilled and the hyperdrive was implanted at a 15° angle relative to midline. At time of implantation, stereotrodes were lowered 500μm into the brain. Three stainless steel screws (one located prefrontal and two cerebellar) served as EEG electrodes and ground and anchored together with two additional fixation screws the hyperdrive to the skull. After recovery, mice were connected to a custom made 32-channel preamplifier headstage (Neuralynx, Bozeman, MT) and data was acquired using a Neuralynx Digilynx recording system. Stereotrode signals were amplified, filtered between 0.1 Hz and 9 kHz and digitized at approximately 30 kHz. Spikes were manually clustered using
the MClust toolbox for Matlab and bursts were identified as at least two spikes with inter-spike interval ≤ 10ms which were preceded by ≥ 70ms of silence.

**Fura-2AM Calcium imaging**

Freshly prepared brain slices from P21-P28 male mice were superfused with oxygenated artificial cerebral spinal fluid (ACSF, in mM: 125 NaCl, 25 NaHCO₃, 25 glucose, 2.5 KCl, 1.25 NAH₂PO₄, 1.2 MgCl₂, 2 CaCl₂, 1.7 ascorbic acid) and recorded at 30-34°C. Cells were filled with Fura-2AM dye (Molecular Probes) using a Picospritzer II apparatus that applied air pressure (10 psi) for 1 minute through a 1-2MΩ glass pipette. Slices were given 1 hour to recover in oxygenated ACSF prior to imaging. Individual cells were imaged using an Olympus BX61WI microscope with an attached CoolSnapF2 camera. Pixel intensity was measured using ImageJ software with whole-field background correction. Ratiometric (340nm/380nm) values were converted to [Ca²⁺]ᵢ using the following equation:

\[
[\text{Ca}^{2+}]_i = K_d \times \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \times \frac{S_{f2}}{S_{b2}}
\]

where \(K_d\) refers to the Ca²⁺ dissociation constant (140nM), \(R\) refers to the ratiometric measurement of the observed cell, \(R_{\text{max}}\) and \(R_{\text{min}}\) correspond to the ratio under conditions of saturated Ca²⁺ levels and in zero Ca²⁺, respectively. The values of \(S_{b2}\) (bound state) and \(S_{f2}\) (free Ca²⁺ state) are proportional to the fluorescence excited by 380nm under conditions of saturated Ca²⁺ levels and in zero Ca²⁺, respectively.

**2.4 Behavioral tests**

Animals were housed 3–5 by genotype per cage at a constant 23°C in a 12 hour light/dark cycle (lights on at 07:00) with ad libitum food and water (unless
otherwise noted). Age-matched males between 6-12 weeks were used for all behavioral experiments. All experimental procedures were reviewed and approved by the MIT Committee on Animal Cares.

**Visual detection task**

Mice were food restricted to 85-90% of their *ad libitum* body weight and training occurred in a custom built test chamber as previously described (Halassa et al., 2014). During testing, a white noise indicated that a new trial was available, and mice had to continuously break an infrared barrier for 500-700ms to initiate a trial. Upon successful initiation, a 50ms visual stimulus was presented either on the left or right side of the initiation nose-poke. Correct response at the corresponding nose-poke unit resulted in a milk reward (10μl evaporated milk, Nestle) that was available for 15 seconds. A new trial became available following an intertrial interval (ITI) of 5 seconds. Response at the incorrect location resulted in immediate blockage of poke access and a 30 second timeout before the next trial. After successful learning of the task, the mice were tested for performance changes that may result from the inclusion of distractors. Mice were subjected to 30-40 minute probe task for five continuous days. During these distractibility tasks, a 50ms visual distractor appeared at the opposite location of where the stimulus would be displayed during the 200ms time window prior to successful initiation in one-third of the trials. For drug treatment experiments, mice were injected with 1-EBIO (Tocris #1041; 25 mg/kg in 1% DMSO; subcutaneous injection) or vehicle 30 minutes prior to testing. Ranksum test was used for statistical analysis.
**Open Field**

Locomotor activity was evaluated over a 60 minute period in an automated Omnitech Digiscan apparatus (AccuScan Instruments) as previously described. Locomotor activity was measured as total distance travelled in centimeters. Anxiety-like behaviour was defined by number of rearings and time spent in the center as compared to time spent in the perimeter of the open field chamber. For drug treatment experiments, mice were placed in the open field arena for 30 minutes prior to amphetamine (3 mg/kg in saline; intraperitoneal injection), 1-EBIO (25 mg/kg in 1% DMSO; subcutaneous injection), or vehicle injections. Mice were then returned to open field arena for an additional 90 minutes. Two-way repeated measures ANOVA with Bonferroni post-hoc tests were used for statistical analysis.

**Elevated zero maze**

An elevated zero maze (Noldus) was set up with indirect illumination of the open (40 lux) and closed arms (10 lux). Testing began with an animal being introduced into a closed area of the maze. Behavior was assessed for 5 minutes and scored using an automated tracking system (Noldus Ethovision). Anxiety-like behavior was calculated using the percent time spent in the open areas. The animals used in the zero maze were previously tested in the open field test with a 2 day period in between tasks. For drug treatment experiments, mice were injected with 1-EBIO (25 mg/kg in 1% DMSO; subcutaneous injection) or vehicle 30 minutes prior to testing. Two-tailed t-tests were used for statistical analysis.
**Grooming**

Individually housed animals were habituated in the testing room for 1 hour prior to experimentation. Mice were video-taped for 2 hours under 2 lux (red light) illumination. Grooming behaviors were coded from 19:00–21:00 (2 hours beginning at the initiation of the dark cycle). This segment was analyzed using Noldus Observer software and the total amount of time in the 2 hour segment spent grooming was determined. A genotype-blind observer recorded all types of grooming, including incidences of face-wiping, scratching/rubbing of head and ears, and full-body grooming. Two-tailed t-tests were used for statistical analysis.

**Rotarod**

*Ptchd1* KO: Motor coordination was assessed in an accelerating rotarod test (Med Associates) over the course of two days. On the first day (training day) animals underwent three 5 minute trials at a constant speed (16rpm). On the second day (testing day), animals underwent three 5 minute trials at accelerating speeds (4-40rpm). For all trials, the latency to fall was determined. Animals were tested for three trials in a single day with an inter-trial interval of 30-60 minutes. Two-way repeated measures ANOVA with Bonferroni post-hoc tests were used for statistical analysis.

*Shank3* KO: No training day was employed. Animals underwent three 5 minute trials at accelerating speeds (4-40rpm) with an inter-trial interval of 30-60 minutes. For all trials, the latency to fall was determined. Two-way repeated measures ANOVA with Bonferroni post-hoc tests were used for statistical analysis.
**Grip strength**

Three different tests were used to assess grip strength. First, mice were tested on the upside-down wire rack test. For this test, mice were placed on top of a wire cage rack. The rack was then slowly inverted and the latency to fall was measured. Secondly, the mice were tested on the hanging wire task. Animals were suspended 40cm above the ground from a horizontal wire (2mm in diameter, 55cm in length). Finally, forepaw grip strength was directly measured using a force meter. For drug treatment experiments, mice were injected with 1-EBIO (25 mg/kg in 1% DMSO; subcutaneous injection) or vehicle 30 minutes prior to testing. For all tests, the average of three trials with an inter-trial interval of 5 minutes was recorded. Two-tailed t-tests were used for statistical analysis.

**Gait analysis**

The forepaws of animals were painted green and the hindpaws were painted pink. After a two minute habituation trial, the mice were allowed to walk down a homemade 50cm illuminated enclosed track attached to a dark chamber containing food. Fresh sheets of white paper were placed on the track for each trial. The sheets were then scanned and converted to digital files. The length and width of each stride were measured using Adobe Photoshop by an observer blind to genotype and the averages were recorded. Two-tailed t-tests were used for statistical analysis.

**Hot Plate**

Animals were placed onto a heating block set to 55°C surface temperature (Columbus Instruments). Latency to lick a forepaw or hindpaw was measured. The
average of three trials with an inter-trial interval of 5 minutes was recorded. Two-tailed t-tests were used for statistical analysis.

**Acoustic startle and pre-pulse inhibition**

Auditory abilities and sensory motor function was measured using Hamilton Kinder Scientific Pre-pulse Startle Monitor chambers with Startle Monitor software. On the first day, mice underwent a 5 minute acclimation trial in the acoustic startle boxes. On the second day, half of the mice were tested on the acoustic startle protocol while the other half was tested on the pre-pulse inhibition protocol. These protocol groups were switched on the third day. To test acoustic startle, mice are presented with pulses of various dB levels without pre-pulses for approximately 30 minutes. The testing session is preceded by a 5 minute exposure to 65dB background noise. Each mouse then receives a total of 92 stimuli (trials) with inter-trial intervals ranging from 7-23 seconds presented in pseudo-random order. The stimuli include a presentation of 8 pulse-alone trials (120dB, 40ms pulse, 4 at the beginning and 4 at the end of the session), 77 pulse trials (7 each of 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, and 120dB, 40 millisecond pulse), and 7 trials each without pulse or pre-pulse presentation. To test PPI, mice were once again exposed to 65 dB background for 5 minutes prior to testing. Each mouse received a total of 57 stimuli (trials) with inter-trial intervals ranging from 7-23 seconds presented in pseudorandom order. The stimuli include a presentation of 8 pulse-alone trials (120dB, 40 millisecond pulse, 4 at the beginning and 4 at the end of the session), 35 pre-pulse trials (7 each of 70, 75, 80, 85 and 90 dB, 20ms pre-pulse given 100 milliseconds prior to a 120dB, 40ms pulse), and 7 trials each without pulse or pre-pulse presentation. The PPI percentage within each test session was calculated as follows: $[100-(\text{mean Pre-pulse response/mean Pulse})$
response) x 100]. For all experiments, response to startle stimuli is measured in Newtons (N). Startle at each pulse level is averaged across trials and then across animals in a treatment group. Two-way repeated measures ANOVA with Bonferroni post-hoc tests were used for statistical analysis.

Three-chambered social arena

Ptchd1 KO: Three-chamber sociability test was performed as previously described. Briefly, littermate male animals were used for all tests. Age and size-matched 129 male target subjects (Stranger 1 and Stranger 2) were habituated to being placed inside wire cages for three days (20 minutes per day) before beginning of testing. Test mice were habituated to the behavior room for at least 1 hour prior to testing. The social test apparatus consisted of a transparent acrylic box with removable floor and partitions dividing the box into three chambers. The wire cages used to contain the stranger mice were cylindrical, 11cm in height, a bottom diameter of 10.5cm with the bars spaced 1cm apart (Galaxy Cup, Spectrum Diversified Designs). An inverted metal can was placed on the top of the cage to prevent the test mice from climbing on the top of the wire cage. For the sociability test, the test animal was introduced to the middle chamber and left to habituate for 10 minutes. Following this period, the middle chamber doors were opened and the test mouse was allowed to freely explore all three chambers for an additional 10 minutes. The test mouse was then returned to the middle chamber, after which an unfamiliar mouse (Stranger 1) was introduced into a wire cage on one of the side-chambers and an empty wire cage on the other side-chamber. The dividers were then raised and the test animal was allowed to freely explore all three chambers over a 10 minute session. Following the 10 minute session, the mouse was returned to the middle chamber while a novel stranger mouse (Stranger 2) was inserted in
the wire cage previously empty and again the test animal was left to explore for a 10 minute session. The time spent by the mouse (nose-point) in close proximity (5cm in diameter) to the wire cages was calculated using automated software (Noldus Ethovison 9). The release of the animals and relative position of social and inanimate targets was counterbalanced. However, for each individual test animal the location of Stranger 1 was maintained during Stranger 1 – E and Stranger 1 – Stranger 2 testing of the social behavior. One-way ANOVA with Bonferroni multiple comparison tests were used for statistical analysis.

Shank3 KO: Three-chamber sociability test was performed with modifications compared to the Ptchd1 protocol. For the sociability test, the test animal was introduced to the arena and left to habituate for 15 minutes. The test mouse was then returned to the middle chamber, after which an unfamiliar mouse (Stranger 1) was introduced into a wire cage on one of the side-chambers and an empty wire cage on the other side-chamber. The dividers were then raised and the test animal was allowed to freely explore all three chambers over a 15 minute session. The mouse was returned to the middle chamber while a novel stranger mouse (Stranger 2) was inserted in the wire cage previously empty and again the test animal was left to explore for a 15 minute session. Once again, the time spent by the mouse (center-point) in close proximity (5cm in diameter) to the wire cages was calculated using automated software (Noldus Ethovison 9). One-way ANOVA with Bonferroni multiple comparison tests were used for statistical analysis.

Resident intruder

Animals were individually housed for 2 weeks prior to testing with bedding left undisturbed for one week prior to testing. Animals were tested in a room with
10 lux lighting. On the test day, animals were habituated to the test room for 1 hour prior to testing. After acclimation, an age and weight-matched conspecific male stranger mouse was introduced to the home cage of the test animal. The subsequent interactions were videotaped for 5-10 minutes. All interactions were analyzed using Noldus Observer by a genotype-blind observer. Aggressive interactions were defined by instances of biting, fighting, and tail-rattling. Social interactions were defined by instances of following and sniffing. Two-tailed t-tests were used for statistical analysis.

Inhibitory avoidance

Animals underwent one training session followed by 24 hour and 48 hour post-training trials. On the training day, mice were placed in the light side of the IA box (Ugo Basile) and allowed to habituate for 30 seconds. The door to the dark side of the box was then opened and the latency to cross was measured. Upon entering the dark side of the box, the mice were given a 0.5mA shock for 2-4 seconds, followed by a 60 second post-shock habituation. On the probe trials, mice were placed in the light side of the box with the door to the dark side already opened. Latency to cross was once again measured with a maximum duration of 9 mins. For drug treatment experiments, mice were injected with 1-EBIO (25 mg/kg in 1% DMSO; subcutaneous injection) or vehicle 30 minutes prior to the shock training protocol. Two-way repeated measures ANOVA with Bonferroni post-hoc tests were used for statistical analysis.

Fear conditioning

Animals were tested for fear-induced freezing using Med Associates fear conditioning chambers encased in sound-attenuating cubicles optimized for near
infrared (NIR) video recording. The training protocol involved a 3 minute habituation period in the conditioning box, followed by three rounds of a 30 second tone + 2 second/0.75mA shock + 90 second rest. The final shock was followed by a 2 minute post-training habituation. The following day, mice were returned to the conditioning box and the time spent freezing was measured. Four hours after, the mice were returned to the conditioning boxes with modifications. A white triangular insert was added to change the dimensions of the box and 0.1% acetic acid was sprayed onto the base of the box in order to change the scent of the box. After a 3 minute interval, the sound cue was introduced for an additional 3 minutes. Time spent freezing pre-cue and during-cue was measured. All freezing was measured using Video Freeze software package analysis of NIR recordings. Two-tailed t-tests were used for statistical analysis.

**Morris water maze**

Spatial learning testing was conducted as describe elsewhere (Peca et al., 2011) with minor modifications. The testing pool was 120cm in diameter and the platform 8cm in diameter. The platform was submerged 1cm below the water surface in the northwest (NW) quadrant. Pool water was maintained at 23.0 ± 0.5 °C and made opaque by mixing-in white non-toxic white paint. During training, 90 second trials were used. If the animals did not find the platform within 90 seconds, the experimenter guided the animal to the platform. After reaching the platform the animals were left for 15 seconds on top of the platform before being removed. Trials were administered for 5-6 days with 4 trials per animal per day with the platform located in the NW quadrant. For two consecutive days after the training protocol, 60 second probe trials were performed (one per day). The reversal
training commenced with the platform in the southeast (SE) quadrant, and proceeded as described above. The experimenter followed the animals’ progress using tracking software outside of the testing room. Tracking and analysis were performed using the Noldus Ethovison software. Two-way repeated measures ANOVA with Bonferroni post-hoc tests and one-way ANOVA with Bonferroni multiple comparison tests were used for statistical analysis.

2.5 Analysis of striatal MSNs

Golgi stain and medium spiny neuron cell fill

Brains from 5-week old littermate male mice were sent for Golgi stain processing (FD Neurotechnologies, Inc.). MSN dendrites were traced using Neurolucida software to investigate cellular morphology and complexity. Sholl analysis revealed neuronal hypertrophy as measured by an increase in complexity of dendritic arbors, total dendritic length, and an increase in surface area in Shank3b/- MSNs.

To study dendritic morphology from WT and KO tissue, 4-6 week old male brains were perfused with ice-cold 1X PBS and fixed with 4% PFA, followed by a 2 hour post-fixation step at 4°C. Sections (200μm) were cut using a vibratome, followed by an additional 2 hour post-fixation step at 4°C. Sections were rinsed 3-4 times in ice-cold 1X PBS prior to luciferase yellow (LY) injections, which were carried out within 2 weeks of sectioning.

For intracellular LY injections, a micropipette (1-2μm in diameter) loaded with Lucifer Yellow dye (Sigma L-0259; 8% solution in 0.05M Tris buffer, pH 7.4) with 0.1M LiCl. Micromanipulators were used to bring LY dye into contact with a
cell body of interest, which were 2-3 focal planes into the surface of the slice. The cell body and proximal dendrites typically filled up within 10 seconds, though a 10-15nA current was applied for 5 minutes to completely fill dendritic spines. Following LY dye application, the sections were placed in Rabbit anti-Lucifer Yellow primary antibody diluted in stock solution (90 ml 1X PBS, 5g sucrose, 2g BSA, and 10 ml 10% Triton X-100) and incubated at 4°C for 2-4 days. Sections were washed 3 X 5 minutes with stock solution at room temperature, followed by a 2 hour room temperature incubation in biotinylated Goat anti-Rabbit antibody diluted in stock solution. Following an additional 3 X 5 minute wash in 1X PBS, sections were incubated for 2 hours at room temperature in streptavidin-conjugated Alexa 488 diluted in 1X PBS. Following a final 3 x 5 minutes wash in 1X PBS, sections were mounted using 22mm x 22mm coverslips and Fluorogel (Electron Microscopy Sciences). Z-stacked images of all sections were collected using an Olympus FV1000 confocal microscope. Stacked images were visualized using NeuronStudio software and differences in spine density were analyzed using two-tailed t-test.
Initial characterization of Ptchd1

It is a widely known fact that certain developmental disorders, including autism (ASD) and intellectual disability (ID), are more commonly diagnosed in males compared to females (T. W. Frazier, Georgiades, Bishop, & Hardan, 2014; Wing, 1981). Though many theories have been put forth by the scientific community, an increasing amount of data is supporting a genetic rather than environmental explanation for this phenomenon. One major genetic contributor to the male:female skew is the X chromosome. Given that males have only one copy of X-linked alleles, mutations in one allele are presumed to have more drastic effects compared to females who have two copies. In fact, while the X chromosome contributes approximately 4% of the genetic content of the entire human genome, it accounts for 10-15% of all genetically-linked cases of ID (Gecz, Shoubridge, & Corbett, 2009). Thus far, 90 of the 818 genes (~11%) found on the X chromosome have been associated with ID in human patients. Included in this list of genes are Fmr1 and Mecp2, the causative genes in Fragile X Syndrome and Rett Syndrome, respectively. Both have been the focus of many human and mouse model investigations of developmental disability over the past two decades.
In late 2010 and early 2011, several published papers reported on the link between deletions of the \textit{Ptchd1} (Patched-domain containing protein 1) gene and patients with ID and ASD. In this chapter, I present the basic characterization of this emerging yet poorly understood X-linked candidate gene for developmental disability. This rudimentary understanding is necessary to meet the future goals of identifying potential cellular and molecular targets of pharmacological intervention.

\section*{3.1 Introduction}

\subsection*{3.1.1 Ptchd1 and human disease}

With the recent breakthroughs in genome sequencing technology, more and more genetic underpinnings of human diseases are being discovered. These novel techniques, which range from analyzing the entire genome to sequencing point mutations in specific genes, have identified monogenetic and polygenetic risk genes of such diseases as schizophrenia, ASDs, and ID. Recently, these methods have been utilized to identify \textit{Ptchd1} as a candidate gene for ASDs and ID.

Initial reports found copy number variations (CNVs) of the \textit{Ddx53-Ptchd1} locus in seven patients with autism spectrum disorder using a SNP microarray system (Pinto et al., 2010). Given the absence of DDX53 protein from the brain, it was presumed that the lack of the \textit{Ptchd1} gene from these patients solely contributed to the presence of ASD symptoms. A subsequent report, which also used a SNP microarray to identify genome-wide CNVs, found maternally-inherited deletions of \textit{Ptchd1} in a pair of twin brothers with ASD (Marshall et al., 2008). This study was followed up by a survey of X chromosome CNVs in which \textit{Ptchd1} deletion was found in a family with ID (Whibley et al., 2010). The link between
Ptchd1 and human disease was further supported by a pair of papers which together identified Ptchd1 deletion in seven families with ASD and three families with ID (Noor et al., 2010). Importantly, in all of these cases, no deletion or mutation was found in samples from healthy controls.

Though this series of reports effectively described the relationship between disruption of Ptchd1 and human disease, little was known about the specific symptoms experienced by these patients and whether or not Ptchd1 deletion led to a syndromic condition. A detailed clinical report of twenty-two Ptchd1 deletion patients identified a highly variable, non-syndromic developmental disorder characterized by neurological, emotional, and cognitive abnormalities (Chaudhry et al., 2014). Patients presented such neurological deficits as hypotonia, mild motor incoordination, and irregularities in gait. Ptchd1 deletion patients also showed increased aggression, elevated anxiety levels, and impulsive behaviors. Finally, affected individuals displayed global developmental delay, ID, ADHD, and ASD (Table 3.1). Together, these reports suggest that mutations and deletions of Ptchd1 account for approximately 1% of all cases of ASD and ID (Noor et al., 2010), which puts Ptchd1 on par with such other neurodevelopmental disease candidate genes as Nrxn1 and Nlgn4 (Persico & Napolioni, 2013). The function of this gene and the ways in which it contributes to psychiatric disease, however, are poorly understood.

3.1.2 Structure of Ptchd1

Ptchd1 is predicted to encode a multi-pass transmembrane protein, a presumption supported by the results of our PTCHD1-YFP overexpression in HEK293 cells. The gene consists of three exons that together encode twelve transmembrane domains and a sterol-sensing domain in addition to several predicted glycosylation sites. The Ptchd1 gene appears to be evolutionarily
conserved, with the PTCHD1 protein sharing 99% and 98% sequence homology with marmoset and mouse homologs, respectively (Figure 3.1a-b).

Based on its domain structure, Ptchd1 has been classified as a late-evolving member of the Patched family of proteins. This family includes Ptch1 and Ptch2, which have been previously shown to be receptors for the sonic hedgehog (SHH) ligand. In addition, independent investigations in mice have identified Ptch1 as a

<table>
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<th>Category</th>
<th>Diagnosis</th>
<th>Prevalence</th>
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<tr>
<td>Growth</td>
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<td>Macroencephaly</td>
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<td>Broad-based gait</td>
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<td>Increased limb tone</td>
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<td>Intention tremor</td>
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<td>Hyperagression</td>
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<td></td>
<td>Impulsivity</td>
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cholesterol transporter (Bidet et al., 2011) located in primary cilia (Rohatgi, Milenkovic, & Scott, 2007) and in the synapse (Petralia, Wang, Mattson, & Yao, 2012). Deletions of Ptch1 in humans lead to the onset of early-life medulloblastomas (Goodrich, Milenkovic, Higgins, & Scott, 1997). Furthermore, the Patched family of proteins includes Npc1, deletions or mutations of which are responsible for 95% of all cases of Niemann-Pick’s Type C disease (Carstea et al., 1997). This lethal neurodegenerative disorder is believed to result from the accumulation of intracellular cholesterol as a result of improper cholesterol synthesis and transport (Carstea et al., 1997; Davies, Chen, & Ioannou, 2000; 

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**Figure 3.1: PTCHD1 amino acid sequence is highly conserved.**

*a*, PTCHD1 amino acid sequence alignment of five species: zebrafish, zebra finch, mouse, human, and marmoset.  
*  

*b*, Summary of alignment analysis shows 98% sequence homology between humans and mice.

<table>
<thead>
<tr>
<th>Species</th>
<th>Human</th>
<th>Marmoset</th>
<th>Mouse</th>
<th>Finch</th>
<th>Zebrafish</th>
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<td>Zebrafish</td>
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Lloyd-Evans et al., 2008). Given the known role of the sterol sensing domain in
mechanisms involving cholesterol, it comes as no surprise that some of the
members of the Patched family of proteins have been identified as playing a role in
cholesterol metabolism. The relationship between PTCHD1 and these similarly-
structured proteins might provide some insight into the function of this gene.

3.2 Results

3.2.1 Function of PTCHD1 protein

There are a plethora of in vitro and in vivo techniques commonly employed
to determine the cellular and molecular function of a given protein in a specific
pathway. Standard practice involves enhancing or inhibiting the action of the
protein under investigation, either through pharmacological intervention or
alterations in protein expression levels. The effects of these perturbations on the
system are then measured, typically through quantification of a specific metabolite
or downstream effector in the pathway. Critical to the success of these experiments
is the ability to visualize the target protein. Exogenously, this can be done through
the introduction of DNA constructs encoding fluorescently-tagged copies of the
target protein. To see the localization of endogenous protein, however, it is
necessary to use an antibody specifically directed against the protein. The ability
to visualize endogenous PTCHD1 in both intact and lysed brain tissue through
antibody staining, therefore, could greatly improve our ability to determine the
cellular and molecular role of PTCHD1. For this reason, I attempted to validate a
series of mouse PTCHD1-specific antibodies.
3.2.1.1 Attempts at generating PTCHD1 antibody

I attempted to procure and validate a series of commercially-available and custom-made anti-PTCHD1 antibodies (Figure 3.2a-b). I set three main criteria for the antibody validation procedure. First of all, the antibody had to be able to identify the correctly-sized band (~130kDa) in a Western blot using samples prepared from HEK cells overexpressing PTCHD1-YFP. Secondly, the antibody had to identify endogenous PTCHD1 protein (~101kDa) in a Western blot using samples prepared from P15 wild-type C57Bl/6J mouse cortex. Finally, the antibody had to identify the 101kDa band in the wild-type C57/Bl6 samples but not in the samples prepared from Ptchd1 knockout mouse cortex. Though some of the 15 tested antibodies showed potential, none of them met more than one of the criteria necessary for validation (Figure 3.2c). In fact, no antibody was able to correctly label the PTCHD1-YFP positive control in a Western blot.

To remedy this problem, I attempted to generate a PTCHD1 antibody using a fusion protein. This antibody required cloning either full-length PTCHD1 or PTCHD1 fragments into a pET expression construct. The pET system is a powerful method for expressing recombinant protein in *E. coli* that utilizes the T7 RNA polymerase from bacteriophage. Specifically, I used an inducible version of this system in which application of isopropyl-beta-D-thiogalactopyranoside (IPTG) turned on T7 polymerase activity after culture growth had reached a plateau. This strategy protects the bacterial cultures from potentially toxic proteins by activating target protein expression after the growth phase is complete. Successful induction of the recombinant protein in bacteria is the first step of many that concludes with purification of the antibody from a rabbit host. Using several variations of the pET expression system, however, I was unable to overexpress PTCHD1 in bacteria
Figure 3.2: Attempts at PTCHD1 antibody production.  

a. PTCHD1 domain structure and location of antibody epitopes.  

b. List of PTCHD1 antibodies tested for validation.  

c. Sample Western blot images from antibodies tested on PTCHD1-YFP expressing HEK293 cell lysates (anti-GFP positive control on left).  

d. Representative Comassie-stained SDS-PAGE gels from attempts at in-house fusion antibody production. The vendor supplied positive control (left) shows IPTG-induced protein expression (~120kDa) at all temperatures while the PTCHD1 gel (right) fails to show induction at the correct size (~90-100kDa).
without inducing cell death (Figure 3.2d). Importantly, several positive controls were successfully induced using this technique, suggesting that amplifying PTCHD1 protein beyond endogenous levels is toxic to this cell type.

The difficulties validating an antibody directed against PTCHD1 did not come as a complete surprise, as multi-pass transmembrane proteins commonly cause issues for antibody production. This stems from the fact that large portions of these proteins are typically located within the plasma membrane, which significantly reduces the size of potential epitopes. PTCHD1 has 12-transmembrane passes with only two small extracellular loops that could be used as epitope targets. Due to these restrictions, many of the antibodies generated against PTCHD1 are antibodies against short peptides (15 to 30 amino acids of the target protein). As a result of these small epitope regions, these antibodies are notorious for off-target binding. Until such obstacles are overcome, I am left without the ability to investigate the endogenous expression of the PTCHD1 protein.

3.2.1.2 PTCHD1 as a receptor for sonic hedgehog

Given the categorization of PTCHD1 as a member of the Patched family of proteins, mouse PTCHD1 is hypothesized to be a receptor for SHH (Noor et al., 2010). SHH is a secreted signaling protein that plays a critical role in nervous system development. SHH signaling is believed to take place primarily in the cilia of cells (Rohatgi et al., 2007), where extracellular SHH binds to PTCH1. By means of the SSD, it is believed that PTCH1 constitutively inhibits a seven-pass GPCR transmembrane protein called Smoothened (SMO) (Incardona, Gruenberg, & Roelink, 2002). SHH activation of PTCH1 results in the disinhibition of SMO, which subsequently triggers target gene transcription through the Gli family

SHH plays a significant role in prenatal development of the mammalian nervous system, primarily with dorsal-ventral patterning and neural cell fate (Fuccillo, Joyner, & Fishell, 2006). SHH has also been found to be necessary for the generation of dopaminergic and serotonergic neurons in the midbrain and hindbrain (Briscoe & Ericson, 1999; Patten & Placzek, 2000), which are critical for normal emotional and cognitive behavior. In adults, SHH released from cortical and basal forebrain neurons was found to regulate astrocyte and oligodendrocyte development (Garcia, Petrova, Eng, & Joyner, 2010; Orentas, Hayes, Dyer, & Miller, 1999; Pringle et al., 1996). In addition, several investigations have found that SHH plays a role in neural stem cells and adult neurogenesis (Ahn & Joyner, 2005; Balordi & Fishell, 2007; Hor & Tang, 2010; Ihrie et al., 2011) as well as interneuron patterning and identity (Flandin, Zhao, Vogt, Jeong, Long, Potter, Westphal, & Rubenstein, 2011; Gulacsi & Lillien, 2003; Jeong et al., 2011a). The absence of SHH in mammals can result in incomplete development of the neural tube, holoprosencephaly, and cyclopia (Chiang et al., 1996; Maity, Fuse, & Beachy, 2005; Roessler et al., 1996).

The link between PTCHD1 and the SHH pathway in mice is supported to a certain degree by early reports from collaborators at the University of Toronto. Dr. John Vincent and colleagues overexpressed PTCHD1-GFP in the hedgehog-responsive 10T1/2 cell line and measured Gli expression using a Gli-luciferase reporter. Similar to the PTCH1 and PTCH2 controls, PTCHD1 was found to inhibit the output of the SHH pathway in the presence of the Smoothened agonist purmorphamine (Noor et al., 2010; Sinha & Chen, 2006). This experiment,
however, did not address whether or not PTCHD1 functions as a receptor or as a downstream effector of SHH pathway activation.

To directly address the role of PTCHD1 in the SHH pathway, I assessed the ability of SHH protein to bind to PTCHD1-expressing cells using SHH-receptive cell lines. Previous reports using similar techniques found that epitope-tagged recombinant SHH-N protein bound specifically to HEK293 cells expressing mouse PTCH1 protein, but not to cells expressing the downstream effector Smoothened (Stone et al., 1996). This finding was supported by several subsequent studies using a variety of techniques, including immunoprecipitation experiments that confirmed the PTCH1:SHH-N interaction (Marigo, Davey, Zuo, Cunningham, & Tabin, 1996).

To probe for the interaction between PTCHD1 and SHH-N, I overexpressed a PTCHD1-YFP mammalian expression construct in HEK293 and COS-7 cells using standard transfection methods (Figure 3.3a). As a positive control, I used a PTCH1-YFP mammalian expression construct that had been previously used for similar studies and served as the backbone for the creation of the PTCHD1-YFP construct (Figure 3.3a). Overexpressed cells were then exposed to recombinant SHH protein, SHH-N containing media, or protein lysate from SHH-expressing cells. The conditions of this exposure ranged from a 15 minute to 2 hour incubation at 4°C, room temperature, or 37°C followed by cell fixation and antibody staining. After many attempts under these conditions, PTCHD1-YFP was never found to co-localize with SHH protein (Figure 3.3b, bottom). However, the PTCH1-YFP positive control was also found not to overlap with SHH protein (Figure 3.3b, top). Given the failure to replicate previously published results, no conclusions can be made from these experiments concerning the role of PTCHD1 in the SHH pathway.
3.2.2 Ptchd1 mRNA expression profile

One of the first steps in understanding the significance of a given gene in the mammalian brain is to study its expression profile. Despite the fact that the DNA substrate of every gene is located in every cell of the body, the presence of
transcription (mRNA) and translation (protein) products of that gene is cell-type dependent. To determine the cell-type specificity of the Ptchd1 gene in mice, I first attempted to understand the mRNA expression profile.
3.2.2.1 Quantification of Ptchd1 developmental expression

Previous work conducted by the St. Jude and Allen Brain Atlas identified Ptchd1 mRNA expression early in development (prenatal) and in adulthood (8 weeks). To further determine the developmental Ptchd1 expression patterns, I began by preparing cDNA libraries from whole brain tissue of C57Bl/6J mice at different developmental time points. For these initial experiments, I utilized the real-time quantitative PCR technique, which simultaneously amplifies and detects the number of copies of a specific amplicon. Preliminary results showed that Ptchd1 levels increased from E10 (embryonic day 12) to P36 (Figure 3.4d), indicating the Ptchd1 expression levels are developmentally regulated.

3.2.2.2 TRN enrichment of Ptchd1 expression

To characterize the region-specific localization of Ptchd1 transcript expression, I generated sense and anti-sense RNA probes directed for in situ hybridization experiments. Shortly after birth (i.e. P0-P1), Ptchd1 transcripts were exclusively found in the thalamic reticular nucleus (TRN) (Figure 3.4a). By P15, Ptchd1 mRNA expression was still enriched in the TRN (Figure 3.4b), but significant signal was detected in such other regions as the cortex, dorsal striatum, dentate gyrus of the hippocampus, and the granule cell layer of the cerebellum. This pattern was consistent into adulthood (P35) (Figure 3.4c) and matched the results provided by the online Allen Brain Atlas P56 mouse database. Though the in situ hybridization results provided critical insight into the expression dynamics of Ptchd1, they provided no information about the cell-type specificity and protein expression patterns of PTCHD1.
3.2.3 **PTCHD1 expression profile in a knock-in mouse**

The standard procedure for determining the cell-type specificity of a given protein involves the use of co-labeling immunohistochemistry protocols. In this method, the signal overlap (or lack thereof) between an antibody directed against the target protein and a cell-type specific antibody is quantified to assess co-expression. This approach was not feasible, however, given the absence of a validated PTCHD1 antibody. As a result, I generated a novel mouse in which the Yellow Fluorescent Protein (YFP) was used to label PTCHD1-expressing cells.

3.2.3.1 **Generation of Ptchd1-YFP mouse**

To visualize PTCHD1 expressing cells, I used targeted genome editing techniques to insert a YFP cassette in place of the first exon of the Ptchd1 gene (Ptchd1-YFP) (Figure 3.5a-b). The YFP expression cassette included a translational stop codon, meaning that no part of the Ptchd1 mRNA should be produced. GFP antibody-enhanced sections from Ptchd1-YFP mice revealed YFP expression patterns similar to Ptchd1 in situ hybridization at different ages (Figure 3.5c). YFP expression was enriched at P0 in the TRN, and once again showed expression in other brain regions throughout development. These results validate the use of the Ptchd1-YFP mouse for subsequent cell-type specific co-expression experiments.

3.2.3.2 **PTCHD1 is co-expressed with GAD67 in the TRN**

The TRN consists of entirely of GABAergic inhibitory neurons. Given that Ptchd1 is expressed in the TRN, as revealed by the in situ hybridization experiments, it can be inferred that PTCHD1 is expressed in inhibitory neurons, at least in the TRN. To determine if PTCHD1 is expressed in excitatory or inhibitory
neurons throughout the brain (Table 3.2), I stained brain sections from Ptchd1-YFP mice with an antibody directed against GAD67, a protein found in the cytosol of most subtypes of inhibitory neurons. Investigating brain slices from adolescent (P15) and young adult (P35) mice, I confirmed that YFP expression is found in GAD67-positive cells in the TRN (97%). Interestingly, YFP was only found in approximately 60% of TRN neurons (Figure 3.6a).

Outside of the TRN, however, PTCHD1 showed much more diversity in cell-type expression. In the cortex, PTCHD1 appears to be expressed predominantly in excitatory neurons, as indicated by the separation of the GAD67-positive and YFP-positive cell populations (Figure 3.6b; 4% overlap at P15, 3% overlap at P35). In the hippocampus, PTCHD1 cell-type specificity is region-
Table 3.2: Quantification of PTCHD1 co-expression.

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dependent (Figure 3.6c). In the dentate gyrus, YFP co-expresses with GAD67-negative cells approximately 97% of the time. This value drops to 60-80% in the CA1 and CA3 regions, indicating that PTCHD1 is expressed in both excitatory and inhibitory neurons in the hippocampus. YFP also showed layer-specific expression in the cerebellum (Figure 3.6d). YFP expression was enriched in the granule cell layer of the cerebellum, which consists predominantly of small excitatory neurons. YFP also showed minimal expression (20-25%) in GAD67-positive Purkinje cell layer. Finally, in the striatum, YFP did not co-label with GAD67-positive interneurons, indicating that its expression is confined to medium spiny neurons (Figure 3.6e).

3.2.3.3 PTCHD1 co-expresses with interneuron subtype-specific markers

The mammalian brain is composed of a wide range of different interneuron cell types, defined by different developmental, electrophysiological, and biochemical properties (Fishell & Rudy, 2011; Rancillac et al., 2010). Several recent studies have begun to decipher the role of different interneuron subtypes in various psychiatric diseases, including schizophrenia and autism spectrum disorders (Marin, 2012). Though the number of identified subtypes has increased over the past decade, a vast majority of interneurons fall into three categories: parvalbumin-positive (PV), somatostatin-positive (SOM), and calretinin-positive (CalR). To determine in which interneuron subtype PTCHD1 expresses, I co-stained brain sections from Ptchd1-YFP mice with antibodies directed against these subtype proteins.

Parvalbumin is a calcium binding protein found throughout the mammalian brain. PV-positive interneurons are typically fast-spiking and are categorized as Basket and Chandelier cells in the cortex (Connors & Gutnick, 1990; McCormick,
Figure 3.6: PTCHD1 is expressed in GAD67/PV-positive cells in the TRN. a-e, YFP co-labeling with GAD67 (left panels) and Parvalbumin (right panels) antibodies in (a) thalamic reticular nucleus, (b) cortex, (c) CA3 region of the hippocampus, (d) Purkinje layer of the cerebellum, and (e) striatum. Arrows denote overlap. Scale bar = 20μm.
Connors, Lighthall, & Prince, 1985). Given that the GAD67-positive inhibitory neurons of the TRN are mostly parvalbumin-positive, I quantified the overlap between YFP-positive cells and PV-positive cells in different brain regions at different ages. PV expression is typically not visible until P7-P10 (Taniguchi et al., 2011), so analysis was confined to samples from adolescent and young adult mice. Nearly 100% of YFP-positive cells were also positive for PV expression in the TRN at both ages. As found in the GAD67 experiments, only 60% of PV-positive cells were labeled with YFP. In the cortex, YFP was not found to co-label with PV, indicating that the small percentage of cortical inhibitory cells that express PTCHD1 are an interneuron subtype that differs from PV. In the hippocampus, YFP only co-expressed with PV in the CA3 region, labeling approximately 10% of all PV-positive cells in this area. In addition, YFP expression was found in a small subset (~20%) of PV-positive Purkinje cells in the cerebellum. As expected given the lack of co-expression with GAD67 in this region, YFP showed no overlap with PV in the striatum.

Somatostatin (SOM) interneurons compose nearly 30% of all neocortical inhibitory cells (Shepherd, 2004). Electrophysiologically, these interneurons are defined by their lower spike threshold and higher resting membrane potential, properties which result in some SOM-positive cells serving as pacemakers in the cortex. Calretinin, a calcium binding protein, labels a subtype of interneuron that is known to synapse onto non-CalR interneurons. This finding has led some to postulate that CalR-positive interneurons control the firing rates of other interneurons and may coordinate rhythmic inhibitory waves.

To test for PTCHD1 co-expression with these interneuron subtype markers, Ptchd1-YFP sections were co-stained for GFP enhancement and antibodies
directed against either SOM or CalR. I found limited to no co-localization between YFP and these interneuron subtypes throughout the brain, indicating that the small percentage of PTCHD1-expressing interneurons outside of the TRN do not fit into one of these three categories. These results are consistent with the known expression pattern of CalR protein, but are contrary to recent publications detailing the heavy expression of SOM in TRN neurons (Ahrens et al., 2015). This inconsistency could be the result of a less-than-perfectly optimized SOM antibody. As a result, current endeavors are underway to address for this issue. In addition, an expanded investigation involving other subtype markers, such as VIP and NPY, is necessary to identify other potential PTCHD1-expressing neuron subtypes.

3.3 Discussion

3.3.1 Unknown molecular function of PTCHD1

Given its domain structure and its similarity to the Patched family of proteins, PTCHD1 is presumed to be a receptor of the sonic hedgehog ligand and/or a component of cholesterol metabolism pathways. Unfortunately, many of the mechanistic studies described in this text failed to answer some of the basic questions regarding the cellular and molecular function of PTCHD1 in mice. These efforts were confounded by the lack of a validated antibody as well as an inability to replicate findings from investigations published two decades ago. Nevertheless, future studies can be conducted to help eliminate some of the uncertainties surrounding PTCHD1 function.
3.3.2 Ptchd1 developmental enrichment in TRN cells

Complete understanding of the in vivo role of a psychiatric disease-relevant protein begins with an investigation of the spatiotemporal dynamics of expression. Through the use of qPCR, in situ hybridization, and immunohistochemistry techniques, I have thoroughly described the expression profile of PTCHD1 and shown that this 12-pass transmembrane protein is developmentally enriched in the TRN. The TRN, which is a shell of GABAergic neurons that serves as the main source of inhibition for the thalamus, is known to play important roles in arousal, attention, and cognition. Given that Ptchd1 is expressed in the TRN early in development, the next issue that must be addressed concerns the role of this protein in the development and functioning of this structure as well as the behavioral ramifications of Ptchd1 disruption. These questions, and more, will be answered in the next chapter.
Mouse models of psychiatric disease have proven to be a valuable resource for advancing our knowledge of brain disorders. Despite the obvious clinical importance of the *Ptchd1* gene in humans, however, no such mouse model for deletion of this gene exists. To satisfy this need, I generated and characterized a novel *Ptchd1* conditional knockout mouse. In this chapter, I chronicle the disease-relevant abnormal behaviors displayed by these mice as well as the defective thalamo-cortical circuitry that may underlie some of these behaviors. In addition, I describe the preliminary results of pharmacological intervention experiments that may serve as a foundation for future drug development strategies.
4.1 Introduction

4.1.1 Role of the TRN in attention and cognition

The TRN is a group of GABAergic neurons that surround dorsal thalamic nuclei and provide the major source of their inhibition. Because of its unique attributes as an inhibitory structure that regulates interactions between thalamus and cortex, the TRN has been called “the guardian of the gateway” for the thalamus to cortex (Crick, 1984). Accordingly, the TRN has been shown to modulate cortical rhythms, sleep, and attention (Pinault, 2004).

By serving as the pacemaker and generator of cortical sleep spindles (Halassa et al., 2011; Steriade et al., 1986), the TRN plays a critical role in sleep and learning processes (Astori, Wimmer, & Luthi, 2013). Sleep spindles, which are 7-15Hz oscillations observed in the electroencephalogram (EEG) during NREM sleep, are believed to be important for sleep stability and facilitation from NREM to REM sleep (Dang-Vu, McKinney, Buxton, Solet, & Ellenbogen, 2010; Vyazovskiy, Achermann, Borbely, & Tobler, 2004). In addition, several studies in humans have correlated sleep spindle activity to general cognitive ability, learning efficiency, and hippocampal memory formation (Fogel, Nader, Cote, & Smith, 2007; Kaestner, Wixted, & Mednick, 2013; Lustenberger, Maric, Durr, Achermann, & Huber, 2012; Mednick et al., 2013; Tamminen, Lambon Ralph, & Lewis, 2013). Importantly, both medicated and un-medicated patients afflicted with schizophrenia consistently present with decreases in sleep spindle activity (Ferrarelli et al., 2007; Ferrarelli et al., 2010; Ferrarelli & Tononi, 2011). Together, these reports suggest that the TRN plays an integral role in cognition.

The TRN also serves an important function in awake, behaving animals. Countless publications have chronicled the significance of the TRN in attention,
alertness, and consciousness (McAlonan & Brown, 2002; Min, 2010; Zikopoulos & Barbas, 2006). In rodent and non-human primate subjects, the TRN has been shown to play a role in both visual and auditory change detection (McAlonan et al., 2008; Yu et al., 2009), both of which are processes necessary for diverting attention towards relevant stimuli. Furthermore, the TRN is known to receive inputs from both the amygdala and the posterior orbitofrontal cortex (Zikopoulos & Barbas, 2012), thereby serving as a convergence point of emotional and attentional pathways. As such, dysfunction of TRN circuitry could have drastic negative effects on behavioral output of an organism.

4.1.2 TRN burst firing mechanisms

The TRN is known to be a vital component in the generation of thalamo-cortical oscillations through burst firing. The mechanisms governing TRN burst firing are not completely understood, though some of the major players involved have been identified. In order for a burst to initiate, the TRN cell must be hyperpolarized, which typically takes place when the animal is asleep. The source of the hyperpolarizing inputs is both intrinsic and extrinsic. Intrinsically, the TRN cells contain active GABA_A and NMDA receptors, which generate a continuous outward Cl⁻ current and facilitate GABA release, respectively (Crabtree, Lodge, Bashir, & Isaac, 2013). Extrinsically, mGluR2 receptors in cortical glutamatergic terminals decrease the resting membrane potential of TRN neurons. Together, these mechanisms are sufficient to hyperpolarize the TRN cell to the membrane potential necessary to activate burst firing (-70mV).

Under hyperpolarizing conditions, low-voltage T-type Ca²⁺ (CaV₃.3) channels are de-inactivated. This allows for Ca²⁺ to enter the TRN cell, which both
63
depolarizes the cell enough to initiate a Na⁺/K⁺ action potential and activates the functionally coupled small-conductance K⁺ (SK2) channels (Cueni et al., 2008). The activation of SK2 channels results in an outward flow of K⁺, which creates the afterhyperpolarization (AHP) necessary to return the TRN cell to a hyperpolarized membrane potential. This activity re-establishes conditions for CaV₃.3 channel de-
inactivation, which were inactivated by the depolarization that resulted from Ca\(^{2+}\) influx. The AHPs, therefore, allow for subsequent Ca\(^{2+}\) spikes to occur until the process is hindered by dampening of the Ca\(_{V_{3.3}}\) channels or a reduction in intracellular Ca\(^{2+}\) levels by sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pumps, which compete with SK2 channels for free intracellular Ca\(^{2+}\). Disruption of this mechanism could interfere with thalamo-cortical communication, while elongation of bursts could lead to hypersynchrony and seizures (Astori et al., 2013; McCormick & Contreras, 2001).

4.2 Results

4.2.1 Generation of Ptchd1 conditional knockout mouse

As previously discussed, Ptchd1 expression is enriched in the TRN early in development and remains present throughout adulthood. To study the in vivo effects of Ptchd1 deletion on behavior and TRN circuit function, I generated a Ptchd1 conditional knockout mouse. This mouse line was produced using standard targeted homologous recombination in mouse embryonic stem cells. The targeting construct consisted of loxP sites flanking Exon 2 of the Ptchd1 gene. Chimaeric mice containing the floxed allele were backcrossed 5-6 generations to produce a pure C57Bl/6J genetic background (Figure 4.1a). The Ptchd1 conditional knockout mice were then bred with β-Actin Cre mice to create a germline knockout line. PCR genotyping confirmed the successful removal of Ptchd1 Exon 2 from the genome (Figure 4.1b).

Exon 2 was chosen based on early predictions by the NIA gene index that indicated the presence of two Ptchd1 isoforms in mice—one full-length isoform
containing exons 1-3 and a smaller isoform lacking Exon 1. Subsequent updates to the NIA gene index and the Ensembl databases, which took place after the generation of the Ptchd1 conditional knockout mouse, made alterations to this preliminary prediction. These updates identified a novel small isoform that does not lack Exon 1, but instead lacks Exon 2. PCRs using cDNA libraries from Ptchd1 WT mice as a template confirmed the presence of the full-length and Exon 1+3 isoforms (Figure 4.1c). As expected, the full-length isoform was absent from Ptchd1 KO samples while the Exon 1+3 isoform remained. Sequencing of the smaller isoform revealed a transcript with a missense mutation that resulted in the removal of nine of twelve transmembrane domains and the sterol-sensing domain (Figure 4.1d). Therefore, it is presumed that this remaining transcript in the Ptchd1 KO mice does not produce a functional protein, though this claim cannot be adequately tested without the employment of a verified Ptchd1 antibody.

4.2.2 In vitro TRN dysfunction in Ptchd1 knockout mice

4.2.2.1 Reduced TRN burst firing in Ptchd1 KO slices

Given the enriched expression of Ptchd1 in the TRN, we investigated the electrophysiological properties of TRN cells in the Ptchd1 KO mice. To assess the direct effects of Ptchd1 disruption on TRN function, my collaborators Dr. Ralf Wimmer and Dr. Michael Halassa conducted slice recording experiments from the TRN in young mice. Previous studies have shown that TRN neurons display two discharge modes depending on their membrane potential. At depolarized levels they discharge tonic Na⁺ spikes. When hyperpolarized below -65mV, an interplay between CaV_{3.3} channels (Astori et al., 2011; Huguenard & Prince, 1992) and Ca^{2+}-activated SK2 channels enables them to generate repetitive Ca^{2+} spikes crowned by
Figure 4.2: Reduced repetitive bursting and SK2 currents in *Ptchd1* KO TRN neurons *in vitro*. 

a. Representative TRN rebound burst traces from WT and KO slices. 

b. Repetitive burst firing in *Ptchd1* KO TRN neurons is reduced independent of passive cellular properties as resting membrane potential and input resistance are comparable (inset). 

c. Representative T-current and SK2 current traces from WT and KO slices. 

d-g. Pharmacological (100nM apamin) isolation of T and SK-currents during burst discharge shows normal T- (d-e) and decreased SK2-current (d, g) in KO. 

h. Free [Ca$^{2+}$]$_i$ is reduced in KO TRN cells at P21. Heat maps show pixel intensity of fluorescence images taken at 340 and 380nm (top) as well as the 340/380nm ratio (bottom) for a WT and KO example cell. All data are presented as mean ± s.e.m. from 12 cells (6 mice) per genotype (d-g) or 35-40 cells (4-5 mice) per genotype (h). *P<0.05; ***P<0.001; N.S. = not significant.
high-frequency Na$^+$ spikes known as bursts (Cueni et al., 2008). Whole-cell patch-clamp recordings from TRN neurons revealed a significant decrease in rebound bursting in brain slices from Ptchd1 KO mice compared to WT controls (Figure 4.2a-b). Of note, the resting membrane potential and input resistance did not differ between the two genotypes, indicating that gross cellular morphology and gross electrophysiological properties are intact.

4.2.2.2 Weakened SK2 currents in Ptchd1 KO slices

In order to determine the contribution of T channels and SK channels to the observed burst firing deficits in the Ptchd1 KO mice, Dr. Wimmer isolated individual currents using the SK channel blocker apamin (Hallworth, Wilson, & Bevan, 2003). Using a digital subtraction method in which overall current is compared under two different conditions (pre-apamin and post-apamin treatment), he found that while T currents remained unaltered, peak SK2 currents were reduced by 50% in the Ptchd1 KO TRN slices (Figure 4.2c, f-g). Typically, increases in T current are met with comparable increases in SK2 current. While this relationship holds in the Ptchd1 KO slices, varying degrees of SK2 current reduction were found at many different levels of T current (Figure 4.2d).

4.2.2.3 Altered baseline intracellular calcium levels in Ptchd1 KO slices

T-type Ca$^{2+}$ channel to SK2 channel coupling has been shown to depend on intracellular Ca$^{2+}$ ([Ca$^{2+}]_i$) homeostasis, and disrupted [Ca$^{2+}]_i$ has been previously reported in neurodevelopmental disorders. To determine whether there is a defect in Ca$^{2+}$ homeostasis in TRN neurons of Ptchd1 KO mice, I measured free [Ca$^{2+}]_i$ levels in TRN neurons in acutely prepared brain slices with the ratiometric Fura-2AM Ca$^{2+}$ indicator. I found an approximately two-fold reduction of free [Ca$^{2+}]_i$ in
Ptchd1 KO TRN neurons compared to controls, suggesting that altered Ca\(^{2+}\) homeostasis could underlie reduced SK2 (Figure 4.2h).

4.2.3 In vivo TRN dysfunction in Ptchd1 knockout mice

4.2.3.1 Reduced TRN bursts in Ptchd1 KO mice

To assess the direct effects of reduced SK2 currents and rebound bursts in the Ptchd1 KO mice, Dr. Wimmer and Dr. Halassa performed in vivo recording experiments on the TRN using implanted hyperdrives containing an array of independently adjustable microdrives loaded with 1-2 stereotrodes (Figure 4.3a). They found that extracellularly detected TRN bursts were significantly reduced in sleeping Ptchd1 KO mice compared to wild-type littermate controls (Figure 4.3b-c). Interestingly, the total number of bursts is unchanged in the Ptchd1 KO mice, indicating that the mechanisms the govern the production of Na\(^+/\)K\(^+\) action potentials are intact and that the deficit is restricted to burst firing.

4.2.3.2 Cortical sleep spindle deficit in Ptchd1 KO mice

One hypothesized consequence of TRN burst firing in sleep is the generation of cortical sleep spindles. Sleep spindles have been linked to sleep stability and memory consolidation, two processes that may be disrupted in neurodevelopmental disorders. EEG analysis of chronically implanted mice revealed normal length of cortical sleep spindles but a reduction in the total number of spindles in Ptchd1 KO mice compared to WT littermates (Figure 4.3d-e). This finding is consistent with the observed deficit in TRN bursting in both slice and in vivo preparations. In addition, wild-type TRN bursts are synchronized with cortical spindles, while this synchrony is absent in the Ptchd1 KO mice. Overall, the results of the slice and in
vivo recordings reveal the presence of dysfunctional thalamo-cortical circuitry in the Ptchd1 KO mouse, which could have a significant impact on the behavioral output of these mice.
4.2.4 Behavioral characterization of Ptchd1 knockout mice

The rodent model has been used for decades to study a plethora of human-like behaviors, including aggression, anxiety, and general cognitive ability. This model is useful due to its genetic similarity to humans, in addition to a relatively short gestation period (~18-21 days). Historically, the rat model has been used to study complex emotional and cognitive behaviors. However, within the past 20 years, advancements in mouse genome manipulations have resulted in the mouse models becoming more commonly used to study behavior. For these reasons, the behavioral effects of Ptchd1 deletion were assessed using the mouse model.

4.2.4.1 Assessment of sensory skills

I first assessed the sensory and motor skills of the Ptchd1 KO mice using a battery of behavioral tasks. The completion of these tests was necessary for several reasons. First of all, given the presence of such neurological abnormalities as gait dysfunction, grip weakness, and mild motor incoordination in humans with Ptchd1 deletion (Chaudhry et al., 2014), these tests would sufficiently probe for defects that mirror the human condition. Secondly, global deficits in sensorimotor function in the Ptchd1 KO mice could confound the results of cognitive or emotional tasks and therefore need to be properly screened prior to analysis of these complex behaviors. Finally, given the known role of the TRN in sensorimotor function (Hartings, Temereanca, & Simons, 2000; Shosaku, Kayama, & Sumitomo, 1984b), these tests could provide insight into the in vivo role of PTCHD1 in the TRN.

Initial experiments focused on the sensory abilities of the Ptchd1 KO mice. The Ptchd1 KO mice showed normal responses on the acoustic startle test. This test involves measuring the force of bodily startle responses to pure auditory tones of increasing intensity (65dB-120dB). These results indicate that the deletion of
Figure 4.4: *Ptchd1* KO mice have intact sensory responses but defective motor skills. a-c, Normal acoustic startle (a), pre-pulse inhibition (b) and hot plate response in *Ptchd1* KO mice (c). d-e, Normal motor coordination on the accelerating rotarod test (d), but reductions in grip strength as measured by the hanging wire test (e). f, Representative images of WT (black) and KO (red) strides. Forepaw represented by green paint and hindpaw represented by pink paint (scale bar = 2cm). g-h, Elongated stride length and slightly increased width. Two-tailed t-tests (c, e, g-h) and two-way repeated measures ANOVA with Bonferroni post-hoc tests (a-b, d) were used for statistical analysis. All data are presented as mean ± s.e.m. from 10-20 mice per genotype. *P<0.05; **P<0.01; ***P<0.001; N.S. = not significant.
Ptchd1 in mice does not significantly impact the auditory system (Figure 4.4). To further explore sensory perception in the Ptchd1 KO mice, I subjected them to the pre-pulse inhibition (PPI) task. This task is based on the observed phenomenon that an organism's response to a stimulus (typically auditory) is reduced if said stimulus is preceded by the presentation of the same stimulus at a weaker intensity. PPI is commonly used to measure sensorimotor gating and the system's ability to modulate its sensitivity to incoming sensory information (Geyer, McIlwain, & Paylor, 2002). Humans suffering from schizophrenia and bipolar disorder commonly show deficits in the PPI task (Gogos, van den Buuse, & Rossell, 2009; Kumari, Soni, Mathew, & Sharma, 2000). In the PPI task, Ptchd1 KO mice showed no response differences when compared to WT littermates (Figure 4.4b). Finally, to assess pain perception in the Ptchd1 KO mice, I performed the hot plate task. This task involves measuring the amount of time it takes for a test mouse to show pain, usually in the form of forepaw or hindpaw licking, in response to being placed on a hot surface. Once again, the Ptchd1 KO mice showed normal motor responses to this noxious stimulus (Figure 4.4c). As a whole, these results indicate the sensorimotor function is intact in these knockout mice.

4.2.4.2 Assessment of motor abilities

Motor dysfunction is common in mouse models and patients with developmental disabilities (Jongmans, Smits-Engelsman, & Schoemaker, 2003; Leehey et al., 2008; Ming, Brimacombe, & Wagner, 2007). To probe for potential motor defects that result from Ptchd1 deletion, I first subjected the mice to the rotarod task. In this test, mice are forced to walk along a rotating rod that accelerates from 4 RPM to 40 RPM over the course of 5 minutes. Typically, mice
are subjected to three training trials followed by three probe trials the following
day. As such, it can be used not only to measure basic coordination, but also motor
learning (Shiotsuki et al., 2010). Ptchd1 KO mice showed intact motor
coordination and learning in the rotarod assay (Figure 4.4d), indicating that gross
motor abilities are intact.

A subset of humans with Ptchd1 deletion have presented with hypotonia
(Chaudhry et al., 2014). To measure general muscle strength, I subjected the
Ptchd1 KO mice to three separate grip tests. First, the inverted wire cage test was
conducted and Ptchd1 KO mice showed drastic deficits in their ability to hang
from the wire cage. Given that the inverted wire cage test involves turning the
mice upside-down, there exists the potential confound that decreased hanging times
could be the result of undiagnosed differences in vestibular function. To eliminate
this potential confound, the mice were tested on the hanging wire test. This test
involved hanging the mice from a thin metal wire (2mm in diameter) by their
forepaws. The mice were then assessed for latency to fall. Consistent with the
inverted wire cage results, the Ptchd1 KO mice showed a reduction in fall time
compared to WT littermates (Figure 4.4e). Finally, forepaw grip strength was
directly measured using a force meter. Using the average of three trials, the Ptchd1
KO mice showed a decrease in total force. Together, these data support the use of
the Ptchd1 KO mouse as a model for the hypotonia phenotype found in human
deletion patients.

Gait analysis is a common metric for general motor function in several
mouse models of psychiatric and neurodegenerative diseases (Gadalla, Ross,
Riddell, Bailey, & Cobb, 2014; Tsai et al., 2012). In addition, Ptchd1 deletion
patients have been found to show abnormalities in gait. I assessed gait in the
Ptchd1 KO mice using the painted paw system. Using this setup, I was able to document and measure the length and width of strides from the test mice. I found mild gait dysfunction in the Ptchd1 KO mice, as indicated by increases in gait length and width (Figure 4.4f-h). These results point to specific motor deficits in the Ptchd1 KO mice and are consistent with the human condition.

4.2.4.3 Description of locomotor activity and anxiety-like behaviors

Some Ptchd1 patients have presented with hyperactivity and anxiety disorders (Chaudhry et al., 2014). For this reason, I measured locomotor and anxiety-like behaviors in the Ptchd1 KO mice using the open field arena and the elevated zero maze. In the open field arena, which consists of a large well-lit chamber with motion detectors, the Ptchd1 KO mice showed an increase in distanced traveled over the course of the 60 minute test (Figure 4.5a-b), indicating both that the Ptchd1 KO mice have a hyperactive phenotype and that the previously identified gait abnormalities do not significantly affect overall locomotion. The significance of this finding is further elevated by the fact that several patients with Ptchd1 deletion have been diagnosed with ADHD.

By comparing the number of rearing bouts (i.e. vertical behavior) and the amount of time spent in the center of the open field arena, one can extract information about anxiety-like phenotypes in mouse models (Prut & Belzung, 2003). Using these metrics, no significant differences were found between WT and KO mice during any of the 5 minute time bins (Figure 4.5c-d). There did, however, appear to be a trend towards an increased amount of time spent in the arena center in the Ptchd1 KO mice, which would indicate a reduction in anxiety levels. In fact, when the amount of time spent in the center is summated over the 60
Figure 4.5: *Ptchd1* KO mice are hyperactive and display normal to reduced anxiety-like behaviors. **a-b**, KO mice show increased locomotion at several time bins (a) and over the 60-minute experiment (b) in open field assay. Representative WT (black border) and KO (red border) traces are shown. **c-f**, KO mice display more time in center (c, e) but normal rearing behavior. **g-h**, KO mice spend more time in the open arm (g) and increased locomotor behavior (h) during elevated zero maze task. Two-tailed t-tests (b, e-h) and two-way repeated measures ANOVA with Bonferroni post-hoc tests (a, c-d) were used for statistical analysis. All data are presented as mean ± s.e.m. from 20-35 mice per genotype. *P*<0.05; **P*<0.01; ***P*<0.001; N.S. = not significant.
minute test, rather than analyzed at each individual time bin, statistical significance is reached (Figure 4.5e).

The mildly hypoanxious phenotype in the *Ptchd1* KO mice is supported by the results of the elevated zero maze. In this assay, mice have the choice between spending time in a dark closed arm (~4 lux) or a brighter open arm (~36 lux) of a circular platform elevated off the ground. Typical wild-type mice will spend a vast majority of the time in the closed arm as the open arm is perceived as more dangerous and therefore more anxiety-inducing (Walf & Frye, 2007). As such, *Ptchd1* WT and KO mice can be compared by using the percentage of time in the open arm as a metric for general levels of anxiety. In this test, the *Ptchd1* KO mice showed a significant increase in time spent in the open arm (Figure 4.5g-h), consistent with the results of the open field assay.

4.2.4.4 *Ptchd1* KO mice are hyperaggressive

Some humans with *Ptchd1* deletion have been found to display increased aggressive behaviors (Chaudhry et al., 2014). To probe for aggression in the *Ptchd1* KO mice, the resident-intruder test was conducted. The validity of the resident-intruder test hinges on the fact that male mice tend to defend against perceived threats to their home cages (Koolhaas et al., 2013; Miczek, Maxson, Fish, & Faccidomo, 2001). To simulate this natural behavior, test mice are socially isolated for two weeks. For one week prior to the test date, the home cage bedding is left undisturbed in order to maintain olfactory cues. When an age-matched and size-matched stranger mouse was introduced to the home cage of the test mouse, the interactions between resident and intruder mice showed a similar pattern between *Ptchd1* WT and KO mice early in the test. Initially, mice from both genotypes spent a majority of their time following and sniffing the intruder, both of which are
standard social behaviors (Figure 4.6a). However, after this initial investigative phase, Ptchd1 KO mice initiated fights, as measured by bouts of pouncing, wrestling, pining, and biting, while few Ptchd1 WT mice exhibited this behavior. These low levels of wild-type attack are consistent with other published reports using the C57Bl/6J mouse strain (Olivier & Young, 2002). Overall, the Ptchd1 KO mice were quicker to initiate an attack and showed an increase in attack duration compared to wild-type controls (Figure 4.6b-c).

4.2.5 Cognitive deficits in Ptchd1 knockout mice

Several studies have linked dysfunction of the TRN to cognitive impediments in humans. One such TRN-dependent deficit pertains to the ability to filter out distractors. Distraction filtering is necessary in order to attend to relevant stimuli, whether that is visual, auditory, or emotional. To assess this behavior, Dr.
Wimmer and I designed a novel visual detection task to test distractibility in the *Ptchd1* KO mice. The potential outcome of this task was particularly important given the significant subset of *Ptchd1* human deletion patients that has been diagnosed with ADHD. As a result, we hypothesized that attention-like deficits may be present in the *Ptchd1* KO mice.

### 4.2.5.1 Visual detection task

The visual distractibility task was modified from a similar behavioral assay pioneered by Dr. Wimmer and Dr. Halassa (Halassa et al., 2014). In brief, test mice were trained to initiate a trial by breaking an infrared beam located in a nose poke. Upon trial initiation, a visual stimulus directed the test mouse to approach the left or right nose poke. If the animal selected the correct side, it was rewarded with a small amount of milk. If the incorrect choice was made, the milk reward was withheld and a 30 second lockout period was initiated (Figure 4.7a). We found that after three weeks of training, the *Ptchd1* KO mice performed equally as well as the WT controls in this learning phase of the task (Figure 4.7b). For the probe test, a distractor stimulus was added to one-third of all trials. The visual distractor was displayed early in the initiation phase of the task at the opposite side of the correctly cued side. We found that the inclusion of the distractor stimuli did not significantly affect the performance of the WT mice. However, while the baseline/non-distractor performance was normal, the *Ptchd1* KO mice performed significantly worse on the trials that included the distractor stimulus (Figure 4.7c). These results suggest that the deletion of *Ptchd1* influences the ability to filter non-relevant information.
Figure 4.7: *Ptchd1* KO mice are more susceptible to distractors in a visual detection task. **a**, Design of the visual detection task. White noise signals availability of a trial. Trials are self-initiated by breaking an infrared barrier for 0.5-0.7 sec which ensures proper head orientation when the 50ms stimulus is displayed. In a third of the trials, a 50ms visual distractor appeared at the opposite location of where the stimulus would be displayed. **b**, Under baseline conditions, *Ptchd1* KO mice showed comparable performance to WT littermates. **c**, However, relative to baseline performance, KO mice displayed decreased accuracy in the presence of distractors. Ranksum test was used for statistical analysis from 8-10 mice per group. *P*<0.05; **P**<0.01; N.S. = not significant.
4.2.5.2 Fear learning

I conducted the most commonly used method for studying fear learning in mouse models—the conditioned fear conditioning task (Fanselow, 1980). This associative learning assay involves placing the mice into a fear conditioning chamber and subjecting them to a series of mild foot shocks that were paired with a high intensity auditory tone (Wehner & Radcliffe, 2004). Well-optimized training protocols typically result in the test subject forming an association between the visual, olfactory, and auditory cues of the chamber with the painful shock. After training, two different types of associative fear learning can be assessed (Figure 4.8a). Contextual fear memory can be measured by placing the mice back into the same chamber and measuring the time spent freezing. If fear learning circuits are intact, the test mouse will freeze in response to the visual and olfactory cues of the conditioning box. In fact, typical WT mice can spend anywhere from 20%-80% of the time freezing depending on the severity of the shock protocol. Deficits in contextual fear learning are classically attributed to dysfunctional hippocampal circuitry (McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998; Phillips & LeDoux, 1992).

Cued fear conditioning can also be measured after the aforementioned training protocol. This type of fear learning is tested by altering the visual and olfactory dimensions of the conditioning chamber. Mice are then placed in the altered conditioning box and the auditory tone is played. If cued fear learning circuits are intact, test mice will freeze in response to the tone, which was previously paired to the foot shocks during training. Deficits in cued fear learning are classically attributed to defects in both hippocampal and amygdala function (Phillips & LeDoux, 1992).
In both the contextual and cued fear conditioning paradigms measured 24 hours post-training, Ptchd1 KO mice showed a significant decrease in the percentage of time spent freezing when compared to WT littermates (Figure 4.8b-c). Together, these results suggest that fear learning circuits are disrupted as a result of Ptchd1 deletion.

To assess operant fear learning in the Ptchd1 KO mice, I ran the hippocampal and amygdala-modulated inhibitory avoidance task (Detrait, Hanon, Dardenne, & Lamberty, 2009; Wilensky, Schafe, & LeDoux, 2000). In this test, mice are first placed into the bright side of a narrow light-dark box (Figure 4.8d). The mice are blocked from entering the darker, less anxiety-inducing portion of the box by a small magnetic door. After a brief acclimation period (door delay), the doors open and the latency to cross into the dark chamber is measured. Upon entering the dark portion of the box, the magnetic doors close and the mice are subjected to a mild foot shock. Probe trials, which consist of measuring the latency to cross from light to dark without the door delay, are conducted 24 and 48 hours post-training. Typically, mice with intact operant fear learning circuits will avoid entering the dark box, despite the inherent desire to seek safety in the darkened chambers. Many WT mice will not enter the dark box at all, which is why many inhibitory avoidance protocols involve a set maximum time point (9 minutes). In multiple cohorts of mice, Ptchd1 KO mice showed a similar latency to cross in the training session after the door delay when compared to WT controls (Figure 4.8e), indicating once again that the hyperactive phenotype is not influencing this behavior. When latency to cross was measured in the probe trials, however, the
Ptchd1 KO mice were found to cross into the dark chamber much more quickly than WT controls. This phenomenon is consistent with the fear conditioning results, supporting the claim that mechanisms governing fear learning and memory are impaired in the Ptchd1 KO mice. In addition, these behavioral results support the use of the Ptchd1 KO mouse as a model for human Ptchd1 deletion patients. 

Figure 4.8: Ptchd1 KO mice display impaired fear learning behaviors. a, Schematic of experimental protocol used for contextual and cued fear conditioning task. b-c, Ptchd1 KO mice showed significant decreases in fear-induced freezing during the (b) contextual and (c) cued fear conditioning probe trials when compared to WT controls. d, Schematic of experimental protocol used for inhibitory avoidance task. e, Ptchd1 showed normal pre-shock latency to cross into the dark chamber values, but significantly reduced latencies during the 24 hr and 48 hr probe trials compared to WTs. Two-tailed t-tests (b, c) and two-way repeated measures ANOVA with Bonferroni post-hoc tests (e) were used for statistical analysis. All data are presented as mean ± s.e.m. from 12-24 mice per genotype. *P<0.05; **P<0.01; ***P<0.001; N.S. = not significant.
majority of which have been diagnosed with intellectual disability (Chaudhry et al., 2014).

4.2.5.3 Spatial learning

Fear learning and spatial memory have different neural substrates. To probe for deficits in spatial memory, I conducted the Morris water maze experiment (Bromley-Brits, Deng, & Song, 2011). This task involves placing the mice in an opaque water tank containing a submerged platform in one quadrant. Over the course of several trials over several days, mice are trained to locate the platform in order to end the task and escape the tank. After training, a probe trial is conducted in which the platform is removed and the time spent in the quadrant that previously contained the platform is measured. The process can be repeated to measure reversal learning in the mice by changing the location of the platform. Both the learning curve and the probe trial generate valuable information concerning the spatial learning skills of the test mice. When spatial and reversal learning were tested using the Morris water maze, Ptchd1 KO mice showed no differences between WT controls in either learning efficiency or the 24-hour and 48-hour probe trials (Figure 4.9a-d). These results indicate that cognitive dysfunction is not global, but rather is restricted to specific circuits.

4.2.6 Contribution of genetic background to Ptchd1 knockout mouse behaviors

Much like the global human population, the mouse (Mus musculus) species encompasses populations with a highly diverse genetic background. These differences in genetic composition, which are typically classified by strain (e.g. C57Bl6/J, S129, etc.), can be the source of variability in controlled behavioral tests
For example, it is known that mice in the C57Bl/6J background show more locomotive activity than BALB/c mice, while mice in the BALB/c background outperform C57Bl/6J mice in avoidance learning tasks (Crawley et al., 1997). For this reason, many laboratories have adopted the “pure” background strategy. This

(Heiman-Patterson et al., 2011; Montagutelli, 2000; Wolfer, Crusio, & Lipp, 2002).

**Figure 4.9: Ptchd1 KO mice show normal spatial memory.** a, Comparable learning curves between *Ptchd1* WT and KO mice during cued training protocol. b, Intact spatial learning demonstrated in 24 hour probe trial. c, *Ptchd1* mice show normal reversal learning curve. d, No significant difference between WT and KO mice in 24 hour probe trial after reversal learning protocol. Two-way repeated measures ANOVA with Bonferroni post-hoc tests (a, c) and one-way ANOVA with Bonferroni multiple comparison tests (b, d), were used for statistical analysis. Data are presented as mean ± s.e.m. from 10 mice per genotype. N.S. = not significant.
method employs experimentation on strain-specific mice that are generated after several rounds of breeding to mice established on said strain (termed “backcrossing”). The logic behind this strategy states that wild-type mice with identical genetic backgrounds will behave in similar ways, and that the behavioral effects of targeted mutations of individual genes within this background will therefore be more obvious. Furthermore, use of this strategy allows other laboratories to more easily replicate the genetic background in their own studies, which increases the chances of data replication.

Other laboratories, however, employ a different experimental strategy. The “mixed” background method uses mice with a genetic composition from two or more different strains. This method has grown in popularity due to the elimination of expensive and time-consuming backcross breedings, which can take anywhere from 6-12 months depending on the desired purity of the strain. In addition, laboratories using this breeding strategy have argued that the mixed background more closely resembles the genetic heterogeneity of the human population and therefore is more relevant for disease modeling. The major theoretical drawbacks with use of this strategy in disease modeling, however, include higher intra-genotype behavioral variability and a decreased probability in inter-laboratory data replication.

4.2.6.1 Behavioral characterization of mixed background Ptchd1 KO mice

To address this issue, I produced mixed background (C57Bl/6J/S129) Ptchd1 KO mice and assessed their behaviors. These mice were generated by crossing pure C57Bl/6J Ptchd1+/- females with pure S129 males to produce
littermates that are on average an even mixture of C57Bl/6J and S129 (i.e. 50% C57/50%129) in terms of genetic background.

As with the pure C57Bl/6J Ptchd1 mouse, I conducted a battery of behavioral tests to probe for neurological, emotional, and cognitive abnormalities. The mixed background Ptchd1 KO mice performed similarly to the pure C57Bl/6J. In the open field assay, the mixed background KO mice showed increased locomotor activity (Figure 4.10a), as previously shown in the pure C57Bl/6J KO mice. The mixed background KO mice showed an increase in time spent in the center of the arena (Figure 4.10b) as well as increased number of rearing bouts at several time points (Figure 4.10c), suggesting a more salient decreased anxiety-like phenotype compared to the pure C57Bl/6J background. This finding was supported by the results of the elevated zero maze, in which mixed background KO mice spent more time in the open arm when compared to WT controls (Figure 4.10d).

When tested for sensory and motor deficits, the mixed background Ptchd1 KO mice displayed behaviors identical to the pure background mice. Ptchd1 KO
mice performed normally on the rotarod (Figure 4.10f) and the acoustic startle/pre-pulse inhibition tasks (Figure 4.10g-h), but showed a marked deficit in the hanging wire test (Figure 4.10e), suggesting a weakened grip phenotype. In addition, the mixed background *Ptchd1* KO mice showed normal responses to noxious stimuli in the hot plate test (Figure 4.10i), indicating that pain and tactile modalities are intact. The mixed background *Ptchd1* KO mice displayed normal social abilities in the three-chambered social interaction test (Figure 4.10j-k). Finally, these mice performed poorly in the inhibitory avoidance task (Figure 4.10l), much like their pure C57Bl/6J counterparts. Overall, these results support the claim that the abnormal behaviors prevalent in the *Ptchd1* KO mouse are strain-independent and targeted mutation-dependent.

4.2.7 Contribution of *Ptchd1* isoforms to abnormal behaviors of *Ptchd1* knockout mouse

As previously mentioned, when the *Ptchd1* conditional knockout mouse was initially designed, it was predicted to produce a full deletion of the mouse *Ptchd1* gene. This was based on preliminary results from the NIA Gene Index, which stated that two major *Ptchd1* isoforms existed: (1) the full-length Exons 1-3 isoform and (2) the shortened Exons 2-3 isoform. In an attempt to eliminate both isoforms, Exon 2 was targeted in our initial construct design. Later, it was shown that the mouse *Ptchd1* gene transcribes the full length isoform in addition to a Exon 1+3 isoform. In other words, a novel isoform lacking Exon 2 was identified and predicted to generate a protein product. Though our assessment of this Exon 1+3 isoform indicates that it does not produce a functional protein, it is still possible that this remaining isoform in some way influences behavior, perhaps by creating a dominant-negative conformation of the PTCHD1 protein. Given that the
targeting strategy involved the complete removal of Exon 1 in favor of a “YFP + translational stop” cassette, it is predicted that all isoforms of Ptchd1 will be missing from this mouse. For this reason, I tested the Ptchd1-YFP mice, which were previously used for immunohistochemical co-expression experiments, in a series of behavioral tests.

4.2.7.1 Behavioral characterization of Ptchd1-YFP mice

The Ptchd1-YFP mice (mixed C57/129 background) underwent many of the same neurological, emotional, and cognitive behavioral tests conducted on the Ptchd1 KO mice. Mice lacking the YFP insertion (Ptchd1-YFP<sup>y/+</sup>) served as the wild-type controls and were compared to littermate mice containing the YFP insertion (Ptchd1-YFP<sup>y/-</sup>). As expected, the Ptchd1-YFP mice, in which Exon 1 is deleted, phenocopied the Ptchd1 KO mice, in which Exon 2 is absent from the genome.

In the open field assay, the Ptchd1-YFP<sup>y/-</sup> mice showed increased locomotor activity (Figure 4.11a), as previously shown in the Ptchd1 KO mice. Like the mixed background Ptchd1 KO mice, the Ptchd1-YFP<sup>y/-</sup> mice showed an increase in time spent in the center of the arena (Figure 4.11b) as well as increased number of rearing bouts at several time points (Figure 4.11c), once again suggesting a reduction in anxiety-like behaviors. This finding was supported by the results of the elevated zero maze, in which the Ptchd1-YFP<sup>y/-</sup> mice spent more time in the open arm when compared to Ptchd1-YFP<sup>y/+</sup> controls (Figure 4.11d).

Furthermore, the Ptchd1-YFP<sup>y/-</sup> mice displayed increased aggression-like behaviors in the resident intruder task (Figure 4.11e-f). These mice performed normally on the rotarod motor coordination task (Figure 4.11h) and showed a
Figure 4.11: Hyperactivity, reduced anxiety, motor, and learning deficits in Exon 1 deletion *Ptchdl-YFP* mice. a, Representative traces from open field assay for WT (black border) and YFP Exon 1 deletion (green border) mice. YFP mice show increased locomotor activity. b-c, Increased time spent in center (b) and greater number of rearings (c) in the open field assay suggest reduced anxiety in the YFP mice. d, YFP mice spend more time in open arm of elevated zero maze. e-f, YFP mice display hyperaggressive behaviors in the resident intruder task. g-h, YFPs show decreased mean holding time in the hanging wire test (g) but normal motor coordination in the rotarod task (h). i, YFP mice show impaired associative learning and memory in the inhibitory avoidance task. Two-tailed t-tests (d-g) and two-way repeated measures ANOVA with Bonferroni post-hoc tests (a-c, h-i) were used for statistical analysis. All data are presented as mean ± s.e.m. from 12-15 mice per genotype. *P<0.05; **P<0.01; ***P<0.001; N.S. = not significant.
significant deficit in the hanging wire test (Figure 4.11g). Finally, the \textit{Ptchd1-YFP}^+/− mice appear to have impaired fear learning, as shown by the results of the inhibitory avoidance task (Figure 4.11i). Overall, these findings support the claim that the behaviors displayed by the \textit{Ptchd1-YFP}^+/− mice are consistent with those found in the \textit{Ptchd1} KO mice, suggesting that the protein product of the Exon 1+3 isoform in the \textit{Ptchd1} KO mice is not the source of these abnormalities.

4.2.8 Contribution of dysfunctional TRN circuits to abnormal behaviors of \textit{Ptchd1} knockout mice

Thus far, I have described a series of abnormal behaviors that may or may not be linked to the identified deficits in TRN burst firing properties. Though the connection between TRN dysfunction and attentional and cognitive deficits has been shown in several studies, the relationship is purely correlative in this \textit{Ptchd1} KO mouse model. One way to show causality between the behavioral and the circuit dysfunctions in the \textit{Ptchd1} KO mouse is to specifically disrupt TRN circuitry and assess the resulting behaviors.

4.2.8.1 Generation of TRN-specific knockout of \textit{Ptchd1}

One of the most powerful uses of targeted knockout mouse technology is the ability to alter the genome in a time-specific and cell-specific manner. This is accomplished through the insertion of region of interest-flanking loxP sites, which when in the presence of the Cre recombinase enzyme, allow for the removal of the flanked genetic segment (Nagy, 2000). Given the fact that the \textit{Ptchd1} knockout mouse was generated with loxP sites flanking Exon 2, I decided to utilize the conditional nature of this mouse line in order to remove \textit{Ptchd1} from TRN interneurons.
Currently, there are no mouse lines that express Cre exclusively in the TRN. However, the results of the *Ptchd1-YFP* mouse co-expression data suggest that the overlap of PTCHD1 and parvalbumin is limited to the TRN. The PV-Cre mouse line, which was developed by Dr. Sylvia Arber (Hippenmeyer et al., 2005), expresses Cre in PV-positive cells throughout the brain, including the TRN. This expression was found to initiate around two weeks of postnatal age and plateau by eight weeks of age (Carlen et al., 2012). Importantly, this mouse line has been found to be 90% efficient in Cre-dependent excision. Based on these observations, I bred Ptchd1 conditional knockout mice to PV-Cre mice in a pure C57Bl/6J background (*PV-Cre:Ptchd1*) in order to generate a knockout line that, except for approximately 10% of CA1 hippocampal neurons and roughly 25% of the cerebellar Purkinje cells, confines Ptchd1 deletion to the TRN inhibitory neurons.

4.2.8.2 Behavioral characterization of PV-Cre:Ptchd1 mice

I ran a battery of behavioral tests on the *PV-Cre:Ptchd1* mice to more directly test the role of TRN dysfunction in the behavioral abnormalities present in the *Ptchd1* KO. Given the known role of the TRN in attentional processes, Dr. Wimmer first tested the *PV-Cre:Ptchd1* mice on the previously described visual detection task. As expected, the TRN-specific *Ptchd1* KO mice (*PV-Cre*: *Ptchd1*\(^{-/-}\)) mice showed normal baseline performance in the absence of the distractor cue when compared to the WT littermate controls (*PV-Cre*: *Ptchd1*\(^{+/+}\) and *PV-Cre*: *Ptchd1*\(^{+/+}\)). Like the germline *Ptchd1* KO mice, the *PV-Cre*: *Ptchd1*\(^{-/-}\) mice displayed an increased susceptibility to the distractor cue (Figure 4.12a), suggesting that dysfunctional TRN circuits underlie this behavioral abnormality.
As previously stated, one of the more salient phenotypes displayed by the Ptchd1 KO mice is hyperactivity in the open field arena. When PV-Cre:Ptchd1 mice were tested in this paradigm, I found no genotype-based differences in distance traveled among the PV-Cre⁺:Ptchd1⁺, PV-Cre⁺:Ptchd1⁻⁻, and PV-Cre⁺:Ptchd1⁻⁻⁻ littermates (Figure 4.12b-c). When analyzing the anxiety-like rearing behavior, however, I found a significant decrease in the PV-Cre⁺:Ptchd1⁻⁻⁻ mice when compared to PV-Cre⁺:Ptchd1⁺⁺ (Figure 4.12e-f), suggesting that the conditional deletion of Ptchd1 from the TRN could lead to increased anxiety. This finding was supported by the results of the elevated zero maze, in which PV-Cre⁺:Ptchd1⁻⁻⁻ showed a decrease in time spent in the open arm (Figure 4.12g). This data is confounded, however, by the normal levels of time spent in the center of the open field arena and the lack of statistical significance when compared to all control groups (Figure 4.12d). Therefore, due to these inconsistencies, the connection between TRN-specific deletion of Ptchd1 and anxiety-like behaviors is not conclusive.

Though the TRN has been implicated in several cognitive processes in several species, there is little to no published data directly linking manipulation of the TRN to cognitive deficits in rodent models of psychiatric disease. Given the known TRN dysfunction and impaired fear learning in the Ptchd1 KO mice, I tested the PV-Cre:Ptchd1 mice in the inhibitory avoidance task. I found that all three genotype groups displayed a similar latency to cross into the darkened chamber prior to shock training. Unlike the Ptchd1 KO mice, however, the PV-Cre⁺:Ptchd1⁻⁻⁻ mice showed latencies to cross that were similar to controls at both 24 hours and 48 hours post-training, implying that the circuitry governing fear learning is intact in the TRN-specific knockout of Ptchd1 (Figure 4.12i). Finally,
the PV-Cre:Ptchd1 mice were tested for motor deficits, as assessed by the hanging wire test. Using this approach, I found no genotype-based differences among the littermates (Figure 4.12h). This is consistent with the established literature identifying the spinal cord and cerebellum as the main systems controlling grip strength. Overall, this behavioral characterization supports the claim that the TRN is critical for normal attention-like behaviors, but shows little influence of this brain region on the presence of other defective behaviors in the Ptchd1 KO mice.

4.2.9 Pharmacological rescue of abnormal behaviors in Ptchd1 knockout mice

4.2.9.1 The effects of acute amphetamine treatment on Ptchd1 KO mouse behaviors

Many individuals coping with ADHD are often prescribed with amphetamines (e.g. Adderall) to alleviate hyperactivity (Kolar et al., 2008). Based on this observation, one could argue that the increased locomotor behaviors displayed by the Ptchd1 KO mice could be rescued by acute amphetamine treatment. To test this claim, I measured activity before and after drug exposure in
the open field assay. Prior to drug treatment, the \textit{Ptchd1} KO mice once again showed elevated activity levels compared to \textit{Ptchd1} WT littermates. Exposure to amphetamine at 3 mg/kg has previously been shown to drastically increase locomotor activity in wild-type C57Bl/6J mice. As expected, this phenomenon was observed in the \textit{Ptchd1} WT mice (Figure 4.13a). However, contrary to the hypothesis that amphetamine treatment would rescue \textit{Ptchd1} KO mice to untreated wild-type levels, this group also showed the dramatic increase in behavior (Figure 4.13b). Importantly, \textit{Ptchd1} WT and KO mice treated with saline showed no such post-injection increase in activity, indicating that the effect was caused directly by drug exposure and not a generalized response to intraperitoneal injections. Overall, this experiment suggests that the \textit{Ptchd1} KO mouse hyperactivity phenotype does not favorably respond to standard ADHD treatments.

4.2.9.2 The effects of acute 1-EBIO treatment on \textit{Ptchd1} KO mouse behaviors

Through his thorough investigation of the disease mechanism underlying \textit{Ptchd1} KO mouse TRN burst deficits, Dr. Wimmer identified the SK2 channel as a potential target for therapeutic intervention. As a follow-up experiment, we decided to test the effects of SK2 current modification on the \textit{Ptchd1} KO mice using the well-studied SK2 modulator 1-EBIO for behavioral rescue experiments. 1-EBIO, which is known to enhance SK2 channel activity, shows no signs of toxicity or disruption of basic neurological functions when given at 25 mg/kg (J. Lam, Coleman, Garing, & Wulff, 2013; Vick, Guidi, & Stackman, 2010). When subcutaneously injected with 1-EBIO 30 minutes prior to testing, \textit{Ptchd1} WT mice were unaffected (Figure 4.14a) while \textit{Ptchd1} KO mice showed a marked improvement in error rates in the presence of distractor cues during the visual
Figure 4.13: Acute amphetamine exposure does not rescue hyperactivity in Ptchd1 KO mice. 

**a**, Ptchd1 KO mice show normal responses at every time bin in response to acute amphetamine (3 mg/kg) treatment in the open field arena. **b**, Summary of results in which time before injection (Pre) and time after saline injection (Sal) confirm hyperactive phenotype of Ptchd1 KOs. Total distance traveled after amphetamine treatment (Amph) are comparable between WT and KOs. Two-way repeated measures ANOVA with Bonferroni post-hoc tests were used for statistical analysis. All data are presented as mean ± s.e.m. from 6 mice per genotype per treatment. *P<0.05; ***P<0.001; N.S. = not significant.
detection task (Figure 4.14b). Subsequent tests in the open field arena revealed that acute 1-EBIO exposure was sufficient to rescue the Ptchd1 KO hyperactivity phenotype (Figure 4.14c). There also appears to be a trend towards the return of Ptchd1 KO anxiety levels to baseline (Figure 4.14d), though this experiment requires replication. 1-EBIO was also found to have no effect on hanging wire performance (Figure 4.14e). Finally, when exposed to 1-EBIO 30 minutes prior to shock training, the treated Ptchd1 KO mice still displayed impaired fear learning (Figure 4.14f), indicating that 1-EBIO has no beneficial effects on this cognitive process.

4.3 Discussion

In this chapter, I presented data supporting the use of the Ptchd1 KO mouse as a mouse model of developmental disability. The Ptchd1 KO mice display many abnormal behaviors consistent with those found in human patients suffering from deletion of the Ptchd1 gene. These behavioral aberrations, which include hyperactivity, hyperaggression, and cognitive deficit, were found in multiple genetic backgrounds and targeted disruptions of the mouse Ptchd1 gene. Electrophysiological analysis by my colleagues Dr. Michael Halassa and Dr. Ralf Wimmer identified in vitro and in vivo thalamo-cortical pathway dysfunction that we believe to underlie the attention-like deficits present in our mouse model. Finally, by isolating the specific channel underlying the circuit perturbation, we have identified a potential target for therapeutic intervention and successfully shown that modulation of this target is sufficient to rescue certain behavioral abnormalities.
4.3.1 Implications of TRN circuit dysfunction

Dr. Halassa and Dr. Wimmer identified defective TRN circuitry in both the slice and in vivo preparations. In TRN slices from P21 mice, Dr. Wimmer found significant decreases in TRN rebound bursts. These findings were corroborated by the burst deficits found in vivo, which were followed by decreases in TRN-generated cortical sleep spindles. These experiments were followed up by more nuanced pharmacological experiments that revealed a drastic reduction in Ca$^{2+}$-activated SK2 currents, which serve as the main generator of the afterhyperpolarizations required for burst firing. Finally, Fura-2AM Ca$^{2+}$ imaging experiments on P21 TRN slices found decreases in resting Ca$^{2+}$ levels, suggesting that SK2 current deficiencies could be caused by aberrant Ca$^{2+}$ homeostasis.

The ability to decipher the mechanisms underlying dysfunctional thalamo-cortical circuitry is of utmost clinical importance. Elucidating the cellular and molecular etiology of the observed reduction in TRN-generated sleep spindles could lead to potential treatments of neuropsychiatric diseases, such as schizophrenia and autism, as well as to a better understanding of the role of sleep spindles in learning and memory processes. By discovering the reduction in SK2 channel-mediated
current, we have not only identified a potential therapeutic target for the \textit{Ptchd1} KO mouse TRN dysfunction, but also established the framework for using SK2 modulators to correct both circuit and behavioral perturbations.

\textbf{4.3.2 Implications of \textit{Ptchd1} KO mouse behavioral profile}

The overarching goal of this project was to generate a mouse model of developmental disorder in order to better understand disease mechanism and potentially identify targets for drug development. For this goal to be met, the genetically-modified mouse would first have to display abnormal behaviors found in humans harboring the same genetic mutation, which would establish face validity for the model and could indicate the presence of conserved circuit dysfunction. Fortunately, the \textit{Ptchd1} KO mouse presented with many of the behavioral abnormalities found in their human counterparts. The \textit{Ptchd1} KO mice showed increased locomotor activity and aggression with decreased anxiety-like behaviors. They displayed intact sensory skills but struggled during the hanging wire and gait analysis tests. The \textit{Ptchd1} KO mice performed poorly in a distractibility task and displayed behaviors consistent with impaired fear learning. Contrary to some \textit{Ptchd1} deletion patients, the \textit{Ptchd1} KO mice did not show some of the hallmark behaviors of ASD, such as repetitive behaviors and social interaction deficits. Importantly, these behaviors were found in two differently targeted \textit{Ptchd1} KO mice in two different genetic backgrounds. The combination of the neurological, emotional, and cognitive phenotypes indicates that the \textit{Ptchd1} KO mouse meets the face validity requirements as a model of developmental disorder (J. N. Crawley, 2007).

Though there is a strong connection between the behaviors exhibited by mice and humans with \textit{Ptchd1} deletion, there are still some unanswered questions
concerning the use of this mouse for future investigations of therapeutic intervention. First of all, our knowledge of the human condition is still underwhelming. Currently, there are only twenty-two documented clinical cases of \textit{Ptchd1} deletion. There is tremendous phenotypic heterogeneity within this group, and many of the patients are still too young to test for certain motor and cognitive abilities. Larger sample sizes are needed to more accurately describe the effects of \textit{Ptchd1} deletion in humans and to make correlations between the type of \textit{Ptchd1} deletion (e.g. full deletion vs Exon 2-only deletion) and the presence of specific symptoms.

Secondly, the fact that the \textit{Ptchd1} KO mice displayed normal social behaviors indicates that this mouse should not be used for the development of pharmacological treatments of hallmark ASD-like behaviors. The term “autism” encompasses a large population of individuals experiencing a set of symptoms of varying severity and underlying cause. Autistic patients may suffer from social interaction deficits that may be the manifestation of defects in theory of mind or the effects of high levels of social anxiety. It is the variability of the etiology of these abnormal behaviors that makes it difficult to establish animal models. Furthermore, mice are solitary animals while primates are highly social. As such, from an evolutionary view, the circuits governing certain aspects of social behavior diverged millions of years ago. These nuances, and other possible explanations, could account for the lack of ASD-like social interaction behaviors in the \textit{Ptchd1} KO mouse.

\textit{4.3.3 Relationship between TRN circuit dysfunction and Ptchd1 KO behaviors}
The TRN is a strip of inhibitory neurons critical for effective communication between the thalamus and cortex. As such, it plays an important role in attention and cognition. We found specific thalamo-cortical pathway deficits in the *Ptchd1* KO mouse model of developmental disability. In addition, we found several abnormal behaviors that could or could not have been linked to the observed TRN dysfunction. To help answer this question, we generated a TRN-specific knockout of the *Ptchd1* gene and assessed the behavioral ramifications. We found that attention-like mechanisms were disrupted in the TRN-specific *Ptchd1* KO mouse, but that all other tested behaviors appeared to be normal. These results confirm what was previously known about the role of the TRN in awake, behaving animals.

Though this strategy was successful in identifying one TRN-mediated behavioral abnormality in the *Ptchd1* KO mouse, it was not without its significant drawbacks. First of all, the Cre-driver line used to generate the TRN-specific *Ptchd1* KO was based on the promoter for parvalbumin (PV-Cre). This mouse begins to express Cre in a PV-dependent manner around P14, which is two weeks after Ptchd1 expression appears in the TRN. The TRN is known to play a role in early pre- and post-natal development of the thalamo-cortical circuitry and it is therefore possible to speculate that PTCHD1 also plays a role in said development. If such is the case and the genetic ablation of *Ptchd1* does not begin until the 3rd week of life, this strategy would not be sufficient to elucidate the importance of the TRN in *Ptchd1* KO behavioral abnormalities. To conclusively answer this question, one would have to breed the Ptchd1 conditional knockout mouse to a germline TRN-specific Cre driver mouse line. Currently, no such mouse exists.
4.3.4 Pharmacological rescue of Ptchd1 KO behaviors

As previously discussed, the Ptchd1 KO mice showed deficits in SK2 current, which presumably led to the TRN burst firing dysfunction. The SK2 modulator, 1-EBIO, is known to enhance SK2 activity \textit{in vivo} with little to no toxicity and limited off-target effects. To determine if global SK2 current deficits governed the Ptchd1 KO behaviors, we conducted pharmacological rescue experiments in adult males. Dr. Wimmer first tested the Ptchd1 KO mice in the visual detection task and found that systemic 1-EBIO treatment (25 mg/kg) administered 20-30 minutes prior to testing was sufficient to rescue the Ptchd1 KO mouse error rates in the presence of the distractor cue to baseline levels. Using the same dosage parameters, I also found that 1-EBIO was sufficient to rescue the hyperactivity phenotype in these mice. This finding suggests that the increased locomotor behavior is at least partially governed by a SK2-modulated mechanism. This is particularly interesting given the inefficacy of standard ADHD treatments (i.e. Amphetamine) on Ptchd1 KO mice and identifies a novel target for intervention of this behavior in humans.
Autism spectrum disorders (ASD) comprise a group of neurodevelopmental disorders that affect approximately 1 in 68 children (Wingate et al., 2014). Although heterogeneous, ASDs are characterized by the presence of two core symptoms: deficits in social interaction and communication, and restricted repetitive patterns of behaviors or interests (Fakhoury, 2015; Kanner, 1946). Currently, the most effective treatment for ASDs hinges on cognitive and behavioral therapies (Koning, Magill-Evans, Volden, & Dick, 2013; White et al., 2010), which are not very effective. The inability to develop effective drug treatments for ASDs stems from the lack of understanding of the exact cause and pathophysiology of the disease. Attempts at deciphering the disease mechanisms underlying these disorders have proven difficult, though recent human genetics studies have identified several ASD candidate genes (Geschwind & State, 2015).
One of the most promising ASD candidate gene is *Shank3*. *Shank3* is one of the genes deleted in the Phelan-McDermid Syndrome (originally known as 22q13.3 deletion syndrome), a disease characterized by neurodevelopmental defects and ASD-like behaviors (Delahaye et al., 2009; Phelan, 2008; Wilson et al., 2003). Mutations of *Shank3*, including microdeletions and a recurrent breakpoint within the *Shank3* gene, have also been identified in patients with idiopathic autism (Durand et al., 2007; Gauthier et al., 2010). The *Shank3* gene encodes a postsynaptic density (PSD) protein that interacts with several key scaffolding proteins, including SAPAP3, HOMER1, and Cortactin (Lim et al., 1999; Naisbitt et al., 1999). These proteins in turn associate with NMDA receptors, mGluR5 receptors, and actin filaments, respectively. The product of these interactions is the linkage of cytoskeletal structures to metabotropic and ionotropic glutamate receptor complexes. Due to the abundance of protein-protein interactions and its localization deep in the dendritic spine (Valtschanoff & Weinberg, 2001), *Shank3* has been labeled the “master” scaffolding protein in the PSD (Kreienkamp, 2008; Sheng & Kim, 2000).

### 5.1 Introduction

#### 5.1.1 The Shank3b knockout mouse as a model of ASD

Motivated by the relationship between mutations of the *Shank3* gene and the presence of ASD symptoms in humans, members of our lab generated and characterized a *Shank3b* knockout mouse (Peca et al., 2011). The *Shank3b* mutant mouse was created with a deletion of Exons 13-16, which encode the PDZ-binding domain that mediates many protein-protein interactions including NMDA receptor interactions. This deletion resulted in the elimination of the two major isoforms of...
Shank3 and lead to the onset of ASD-like behaviors. Shank3b mutant mice displayed social interaction deficits, as measured by the three-chamber social interaction task and the reciprocal interaction task. These mice also displayed increased and injurious repetitive behaviors as identified through measurements of time spent grooming and the appearance of self-induced skin lesions. Interestingly, in situ hybridization experiments conducted in our lab revealed an enrichment of Shank3 mRNA in the mouse striatum. Though the Shank family of proteins showed significant overlap of expression throughout the brain, only Shank3 mRNA was predominant in the striatum. Based on this finding, I decided to probe for striatal dysfunction in the Shank3b KO mice.

5.2 Results

5.2.1 Striatal defects in Shank3b knockout mice

Proper development of synapses is necessary for normal brain functioning, as evidenced by the plethora of neurological diseases associated with improper synapse formation. In particular, human studies of ASD patients have found defects in both spine number and morphology (Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011). Importantly, several in vitro reports have detailed the importance of Shank3 in synaptic development (Durand et al., 2012; Roussignol et al., 2005; Sala et al., 2001; Sala, Roussignol, Meldolesi, & Fagni, 2005). To test the in vivo role of Shank3 on synaptic development, I quantified the spine densities of medium spiny neurons (MSNs) from Shank3 WT and Shank3 KO mouse striatal tissue using the Lucifer Yellow patch-assisted cell filling technique. Experiments were conducted in MSNs because of the striatal enrichment of Shank3 and the
Figure 5.1: Defective MSN morphology and spine density in Shank3b KO mice. **a**, Sholl analysis reveals an increased neuronal complexity of Shank3b KO MSNs (red) when compared to WT mice (black). Representative WT (top) and KO (bottom) neurons are shown in inset. **b-c**, MSNs from Shank3b KO mice show an increase in total dendritic length (b) and surface area (c) compared to WTs controls. **d**, Confocal stacks from representative WT (top) and KO (bottom) dye-filled MSNs. Scale bar = 1 μm. **e**, Spine density in MSNs from Shank3b KO mice are lower than WT controls. Two-way repeated measures ANOVA (a) and two-tailed t-tests (b-c, e) were used for statistical analysis. Data from b, c, and e are presented as mean ± s.e.m. from 3 mice per genotype. For Sholl analysis (b-c), 36 cells were analyzed per genotype while 41 cells per genotype were measured for spine counts (e). *P < 0.05; **P < 0.01.
involvement of this brain region in repetitive behaviors. Confocal stack images of filled cells revealed a significant reduction in spine density in Shank3b KO mice MSNs (Figure 5.1d-e). In addition, Scholl analysis of Golgi-stained tissue found an increase in dendritic length and arborization in the Shank3b KO mice MSNs (Figure 5.1a-c), which may serve as a compensatory response to the reduced spine density.

To determine the effects of these defects on cortico-striatal circuitry, members of the Feng lab conducted electrophysiological experiments in the striatum of WT and KO mice (Peca et al., 2011). Extracellular and whole-cell slice electrophysiological recordings identified a reduction in cortico-striatal synaptic strength, suggesting that the decrease in spine number influences overall striatal function. Though this investigation strongly suggests the importance of the striatum in the development of normal behavior, it did not address the hypothesis that striatal dysfunction is necessary and sufficient for the onset of ASD-like behaviors in the Shank3b KO mice. For this reason, I decided to take the initial steps necessary to directly test whether the observed striatal circuitry dysfunction plays a causative role in ASD-like behaviors by generating a conditional Shank3 knockout mouse.

5.2.2 Generation of Shank3b conditional knockout mouse

To investigate the role of the specific brain regions, including the striatum, in the abnormal behaviors of this animal model of ASD, I generated a Shank3b conditional knockout mouse using standard targeting of mouse ES cells techniques. This mouse was designed with loxP sites that flank Exons 13-16, the same genetic segment altered in the original Shank3b KO mouse (Figure 5.2a). Chimeric mice containing the floxed allele were backcrossed 5-6 generations to produce a pure
C57Bl/6J background prior to being crossed to 129 mice to create a C57/129 mixed strain for all behavioral testing. When the Shank3 floxed mice were bred with β-Actin Cre mice to generate a germline knockout line (Shank3b cKO), PCR genotyping confirmed the successful removal of Exons 13-16 from the genome (Figure 5.2b) while Western blot analysis of brain lysates confirmed the ablation of Shank3 isoforms.
the large SHANK3B protein isoform (Figure 5.2c). In order to validate the future use of the Shank3b cKO mice in cell-type specific investigations, I conducted a series of behavioral tests to confirm that this mouse line exhibited the same ASD-like behaviors found in the previously-studied Shank3b KO mouse.

5.2.3 Behavioral characterization of germline Shank3b cKO mouse

5.2.3.1 Assessment of motor and sensory skills

The initial investigation of the Shank3b KO mouse found no motor abnormalities and did not probe for sensory deficits. However, subsequent unpublished studies conducted by our lab have since found deficits in motor coordination and normal perception of pain. To test for motor abilities in the germline Shank3b cKO mice, I conducted the rotarod task. Using a one-day rotarod task in which animals did not undergo a training day, I found significant deficits in latency to fall in the Shank3b cKO mice compared to littermate controls (Figure 5.3e). This result suggests dysfunctional motor coordination in the germline Shank3b cKO mice, which may have not been present in the initial Shank3b KO mouse study due to the exclusion of the training day in the past procedure.

5.2.3.2 Description of locomotor and anxiety-like behavior

Initial reports suggested that Shank3b KO mice showed no differences in total distance traveled in the open field arena (Peca et al., 2011), but did show decreases in rearing behavior and time spent in center, both measures of anxiety-like behaviors. While the anxiety-like behaviors have been replicated within the lab, it has been shown that the Shank3b KO mice also show considerable decreases in total distance traveled (Karen Mei, personal correspondence), which is indicative
of a hypoactive phenotype. In addition to the results of the open field arena, the Shank3b KO mice showed increases in self grooming and associated self-induced skin lesions, suggesting the presence of an anxiety-based repetitive behavior phenotype.

Figure 5.3: Normal locomotion, motor deficits, and increased anxiety-like behaviors in Shank3b cKO mice. a, Shank3b cKO mice display locomotor behavior comparable to WT littermates. b-c, Germline KOs showed increased total time spent in center (c) and but no difference in number of rearings in open field (d). d, Shank3b cKO mice show increased anxiety-like behaviors in the elevated zero maze paradigm. e-f, cKOs show motor deficits in the rotarod (e) and hanging wire (f) tasks. Two-way repeated measures ANOVA (a, e) and two-tailed t-tests (b-d, f) were used for statistical analysis. All data presented as mean ± s.e.m. from 15-20 mice per genotype. *P<0.05; **P<0.01; N.S. = not significant.
To assess locomotor and anxiety-like behaviors in the germline Shank3b cKO mice, I conducted the open field assay and grooming analysis. I found no differences in total distance traveled between the WT and KO mice (Figure 5.3a), consistent with original reports. In addition, the Shank3b cKO mice were found to spend less time in the center of the arena (Figure 5.3b), consistent with previous anxiety-like behaviors. However, grooming analysis revealed no differences in grooming time at 4 months of age and no signs of self-induced lesions (Figure 5.3c).

5.2.3.3 Reduced social interaction in Shank3b cKO mouse

Shank3b KO mice showed significant deficits in both social interaction and recognition of social novelty in the three-chambered social interaction assay. To test for similar defects in the germline Shank3b cKO mice, I conducted the three-chambered social interaction assay with slight protocol adjustments. This study replicated the deficits in social interaction and social novelty previously observed identified in the original Shank3b KO mice (Figure 5.4a). However, it should be noted that the wild-type controls also failed to show recognition of social novelty (Figure 5.4b), which limits the conclusions that can be made regarding that aspect of the assay.

5.3 Discussion

5.3.1 Implications of Shank3b cKO mouse behavioral profile

Prior to using the Shank3b cKO mouse as a tool for the dissection of circuit contributions to ASD-like behaviors, it is critical to show that germline disruption of the floxed Shank3b produces behavioral abnormalities consistent with those found in the original Shank3b knockout mouse. While many of the aberrant
behaviors were replicated in the Shank3b cKO mouse, there were notable exceptions. I found genotype-based differences in rotarod performance not previously observed in the Shank3b KO mice. In addition, the original assessment of the normal locomotor activity of the Shank3b KO mouse is not in alignment with subsequent research conducted by other members of the Feng lab that identified a hypoactive phenotype (Karen Mei, personal correspondence). Finally, while lesions and repetitive grooming were previously shown to be a salient feature of the Shank3b KO mice, no such qualities were observed in the Shank3b cKO mice.

The source of the behavioral inconsistencies between the Shank3b KO and the germline Shank3b cKO could lie in the choice of genetic background. The
original *Shank3b* KO mice were tested in a hybrid C57/129 background consisting of unknown contributions from each strain. This strategy is contrary to that employed for the *Shank3b* cKO mouse, in which the mixed genetic background was more carefully controlled. As discussed in the previous chapter, it is known that different strains of mice have different baseline behaviors. Such differences could explain how the original assessment found social interaction deficits and increased repetitive grooming as young as six weeks of age in the *Shank3b* KO mice, while experiments conducted by members of the Feng lab on the *Shank3b* KO mouse in a more controlled mixed C57/129 background found that these behaviors are not persistent until much later in life. In fact, excess grooming is not typically observed until 6-8 months of age in these mice (Karen Mei, personal correspondence), which is two months older than test mice used for my assessment of the *Shank3b* cKO mice. Analyzing the mice at a more advanced age could remedy this issue and those experiments are underway.
Future directions and conclusions

Our understanding of the molecular and cellular mechanisms underlying developmental disorders is scarce and in great need of improvement. The increasing prevalence of these disorders, compounded by the lack of effective treatments, has placed a tremendous burden on society that can be alleviated by disease-relevant research and eventual development of effective treatment. Here, I have presented data supporting the use of two mice—Ptchd1 knockout and the Shank3b knockout—to elucidate the roles of subcortical circuits in complex neurodevelopmental disorders.

Through my investigation, I identified a unique expression pattern of Ptchd1 that favored early enrichment in the TRN. Subsequent work conducted by my colleagues identified deficits in TRN burst firing that appear to be the result of reduced Ca\(^{2+}\)-activated SK2 current. My thorough characterization of the Ptchd1 KO mouse behaviors identified several behavioral abnormalities consistent with human Ptchd1 deletion patients. The Ptchd1 KO mice displayed hyperactivity,
decreased anxiety, hyperaggression, attention-like deficits, abnormal gait, limb weakness, and fear learning impairments.

Importantly, the attention-like deficits were found to be TRN-dependent, as revealed by our analysis of the TRN-specific Ptchd1 KO mice. The TRN-specific knockout, however, did not show behavioral deficits in other realms, suggesting that postnatal deletion of Ptchd1 from the TRN is not sufficient to recapitulate all of the behavioral abnormalities. Nevertheless, the increased susceptibility to distraction and hyperactive phenotypes were found to be responsive to SK2 current enhancement, suggesting that SK2 aberrations found in the TRN may be present in other circuits in these mice. As such, SK2 channels serve as a potential target for future attempts at pharmacological intervention.

My contribution to the initial characterization of the Shank3b mouse model of autism focused on deformities in striatal neuron morphology. These findings were supplemented by work conducted in the Feng lab that identified electrophysiological and biochemical defects in these cells, which were suggested to underlie the ASD-like behaviors displayed by these mice. In order to better understand the role of the striatum in these behaviors, I generated a conditional replica of the Shank3b knockout mouse. My initial biochemical and behavior characterization of this mouse indicates that it is a suitable tool for future investigations of circuit-specific Shank3 deletion.

Though the work conducted by myself and collaborators has provided considerable insight into the disease mechanisms underlying developmental disorders, many unanswered questions remain. Future experiments aimed at minimizing the gap between the known and unknown are the focus of this chapter.
6.1 Cellular and molecular mechanisms of PTCHD1

Due to technical issues, I was unable to adequately decipher the basic roles of PTCHD1 in the cell. Advancements in antibody production could be a first step in making progress on this endeavor. In addition, SHH-ligand binding assays could be performed to determine whether or not PTCHD1 acts as a receptor for SHH (Marigo et al., 1996). These experiments can determine the temporal dynamics of potential PTCHD1-SHH interactions while also providing insight into the binding capacity of the receptor. In addition, experiments elucidating the role of PTCHD1 in cholesterol metabolism could be conducted in vivo and in PTCHD1-expressing HEK cells. Staining techniques could identify aberrant cholesterol depletion or accumulation, similar to investigations conducted in mouse models of Niemann-Pick type C disease (Liao et al., 2007; Reid et al., 2004). Success in these undertakings could pave the way for future drug target screens.

6.2 TRN circuit dysfunction in Ptchd1 KO mice

6.2.1 Relationship between Ptchd1 and SK2

Our initial assessment of the Ptchd1 KO mouse found dysfunction in disease-relevant SK2-mediated TRN firing mechanisms. One of the most pressing issues surrounding these findings is the fact that the relationship between PTCHD1 and SK2 proteins has not yet been investigated. While it is known that both PTCHD1 and SK2 are transmembrane proteins expressed in the TRN, it is unclear if these two proteins interact. If these two proteins interact, it would be critical to determine the spatiotemporal dynamics of this interaction. For example, it is possible that direct interaction with PTCHD1 is necessary for the localization and/or stabilization of SK2 channels in close apposition to CaV\(_{3.3}\) channels. In this
scenario, the loss of PTCHD1 protein could lead to the subsequent loss of properly positioned SK2 channels, which could explain the reduction in Ca\textsuperscript{2+}-mediated SK2 current. Future experiments involving co-immunoprecipitation Western blots and co-labeling immunohistochemistry could be conducted to begin to answer some of these questions. At present, these experiments are unfeasible due the lack of dependable SK2 and PTCHD1 antibodies.

It is also possible that the SK2 current deficiencies are not directly caused by the absence of PTCHD1 protein interactions and instead are the result of an unidentified indirect and/or compensatory mechanism in this knockout mouse. As previously stated, the Ptchd1 KO TRN slices showed decreased resting levels of free intracellular Ca\textsuperscript{2+} as shown by Fura-2AM imaging methods. This reduction in baseline Ca\textsuperscript{2+} levels could be the result of defective global Ca\textsuperscript{2+} input mechanisms or disrupted intracellular Ca\textsuperscript{2+} buffering mechanisms. The former possibility seems unlikely, given that the TRN electrophysiological defects appear to be restricted to mechanisms governing burst firing, and not tonic firing. The later possibility, in which increased buffering or storage of free intracellular Ca\textsuperscript{2+} leads to the decrease in activation of SK2 channels, is plausible but would require further investigation. Advanced Ca\textsuperscript{2+} imaging experiments could be performed to identify potential culprits in the observed increase in Ca\textsuperscript{2+} buffering, such as the SERCA pumps (Cueni et al., 2008). It is also equally as plausible that the deletion of Ptchd1 leads to the upregulation of specific Ca\textsuperscript{2+}-binding proteins (e.g. Calb2, Calmodulin, etc.) in the TRN. RNA sequencing or proteomic experiments comparing Ptchd1 WT to Ptchd1 KO samples could be conducted to identify such proteins. However, initial attempts at conducting RNA sequencing experiments on WT vs KO TRN samples failed to identify credible candidates (data not shown).
6.2.2 TRN influence on Ptchd1 KO behaviors

The results of the PV-Cre:Ptchd1 KO study suggest that dysfunctional TRN circuits affect distractibility but not hyperactivity or fear learning in adult mice. As previously discussed, it is possible that the limited behavioral defects in this particular mouse line are the result of the temporal expression dynamics of this Cre recombinase driver line lying outside of a critically important developmental window. To remedy this potential confound, attempts are underway to identify a Cre mouse line that shows early TRN expression. Currently, the front runner in this race is the Som-Cre mouse (Ahrens et al., 2015), which uses the somatostatin promoter to elicit Cre-dependent excision before birth. Prior to establishing the Som-Cre:Ptchd1 line, immunohistochemical co-labeling experiments will need to be performed to confirm PTCHD1/SOM co-expression in the TRN. In addition, long-term plans are ongoing to develop a TRN-specific Cre recombinase driver line.

Though suffering from the same temporal shortcoming as the PV-Cre strategy, adult rescue of PTCHD1 function could answer some important questions concerning the use of this mouse for future drug development. Recent advancements in viral vector technology have opened the door for detailed in vivo circuit manipulations in mouse models of disease (Bourdenx, Dutheil, Bezard, & Dehay, 2014; Deyle & Russell, 2009; Tenenbaum et al., 2004). Future experiments on the Ptchd1 KO mouse could focus on the altering PTCHD1 expression specifically in the TRN followed by an assessments of both behavior and circuit dysfunction. Currently, I am in the process of generating a series of AAV viral vectors for the study of PTCHD1 function in the TRN. In order to rescue expression in Ptchd1 KO mice, I have produced an AAV-hSyn-FLEX-HA-Ptchd1 virus that can be bilaterally injected into the TRN as early as P21. To restrict
expression to TRN cells, and not neighboring striatal or thalamic tissue, this virus will be injected into *Ptchd1* KO mice expressing VGAT-Cre (*VGAT-Cre*⁺:*Ptchd1*⁺⁻⁻). Though this strategy will be unsuccessful if the main role of PTCHD1 is perinatal, it will nonetheless reveal whether or not adult rescue of TRN function in the *Ptchd1* KO mice is possible.

### 6.2.3 The role of *Ptchd1* in TRN functional sub-networks

Quantification of the *Ptchd1-YFP* mouse expression profile showed that the PTCHD1 is expressed in only 60-70% of all TRN cells. Recent work published by Dr. Halassa and Dr. Wimmer has described the existence of different functional subnetworks within the TRN (Halassa et al., 2014). Limbic-projecting TRN neurons function mainly in arousal, while sensory-projecting TRN neurons generate sleep spindles and modulate attentional states. Interpreting these findings in conjunction with the known spindle and attentional dysfunction in the *Ptchd1* KO mice, it is plausible to speculate that PTCHD1 is expressed in the sensory-projecting subset of TRN neurons. Future experiments could attempt to probe for electrophysiological, morphological, or biochemical differences between PTCHD1-expressing and non-expressing TRN cells. Anterograde viral tracers could be injected into YFP-positive cells of *Ptchd1-YFP* heterozygous females to determine if PTCHD1-expressing TRN cells project to specific sensory thalamic nuclei. Such characterization of these cells could answer questions regarding the role of PTCHD1-expressing cells on the TRN-dependent behavior.
6.3 Advanced behavioral assessments of *Ptchd1* KO mice behavior

6.3.1 Tests for cognition and attention skills

While the learning and memory tasks presented in this document are some of the most commonly utilized for mouse models of disease, there are several other tests that could be used in the future to probe for additional cognitive deficits in the *Ptchd1* KO mice. The T-Maze task could be performed to test for deficits in working spatial memory (Jacqueline N. Crawley, 2007). In addition, the *Ptchd1* KO mice could be subjected to the novel object recognition task to identify problems with mechanisms governing both short-term and long-term memory.

Further steps can be taken to better understand the influence of TRN defects on attention-like behaviors. It is well-documented that the TRN receives information from all sensory modalities (Y. W. Lam & Sherman, 2011). Recent studies have begun to elucidate the significance of these multiple inputs and more importantly, the ways in which the TRN filters signals from different senses in order to attend to the most relevant stimuli. To probe for deficits in sensory selection, *Ptchd1* KO mouse will undergo a cross-modal distractibility test. The tenets of this behavioral assay are similar to the previously described visual detection task with the important addition of a reward information-containing sound cue. Mice will be trained to respond to a light cue that signals reward location and in separate training sessions, will be trained to respond to a sound cue that signals reward location. Once animals are trained under both paradigms, they will be trained to respond to the light cue when the sound cue is simultaneously presented. In probe trials, the mice will have to choose the target nose poke correctly when both the light and sound cue signal the same reward location (congruent/enhancer signal) and when the two cues provide contrasting
information (incongruent/distractor signal), in which case the sound will serve as the distractor. In a separate cohort of mice, this experiment can be repeated, with the main difference being that the visual cue will serve as the distractor. The results of these experiments can provide insight into whether or not the increased distractibility present in the Ptchd1 KO mice is specific to the vision perception or if the effect applies to multiple sensory modalities.

6.3.2 Tests for sensorimotor skills

The Ptchd1 KO mice displayed normal sensory perception but impaired motor function. While three separate tests were conducted to describe Ptchd1 KO mouse grip weaknesses, only one test was performed to analyze gait. To get a more thorough and accurate measure of the observed gait abnormalities, the Ptchd1 KO could be tested on an automated track (e.g., Noldus CatWalk™ XT). These devices digitally capture each footprint and automatically analyze stride length and width. Use of the automated track would allow for more high-throughput gait analysis.

6.3.3 Tests for ASD-like behaviors

My initial characterization of the Ptchd1 KO mouse revealed no hallmark ASD-like behaviors despite the strong link between Ptchd1 deletion and ASD in humans. These experiments, however, were performed on adult mice. As such, future studies could focus on ASD-like behaviors in juvenile Ptchd1 KO mice. The ultrasonic vocalizations of neonatal Ptchd1 KO pups could be analyzed and assessed for abnormalities (Scattoni, Crawley, & Ricceri, 2009). This experiment, which is conducted on newborns that have been temporarily separated from their mothers, is frequently used as a metric for communication in mice. Another
commonly used measure of ASD-like behaviors in mice is the juvenile play paradigm. In this task, the social approach levels of 3-week old mice are quantified and compared between WT and KOs. These tests have the possibility of unearthing ASD-like behaviors not observed in our behavioral assessment.

6.4 The SK2 channel as a target for rescue of TRN dysfunction and behavioral abnormalities

SK2 modulation via 1-EBIO treatment was found to improve performance on the visual detection task in *Ptchd1* KO mice without affecting WT behavior. At the same time, this drug was found to rescue *Ptchd1* KO hyperactivity to WT levels. Together, these results introduce the exciting possibility that pharmacological rescue of these behaviors in humans with *Ptchd1* deletion is possible.

Given that SK2 is expressed throughout the brain, however, the effects of the 1-EBIO treatment cannot be directly correlated to changes in TRN circuitry. Therefore, to determine the TRN-specific effects of SK2 modulation, I have generated an AAV-hSynP-FLEX-SK2-P2A-YFP virus to conduct viral-mediated rather than pharmacological-mediated rescue of the *Ptchd1* KO mice. Once again, this virus will be bilaterally injected at P21 into *VGAT-Cre*:*Ptchd1*+/− and *VGAT-Cre*:*Ptchd1*+/+ mice in order to confine SK2 overexpression to the TRN. These mice will be tested for slice and *in vivo* differences in TRN burst generation, in addition to such behaviors as sensitivity to distractors. Such findings could further strengthen the link between SK2-mediated TRN defects and disease-relevant behaviors as well as provide a more thorough understanding of the *Ptchd1* KO mouse disease mechanisms.
6.5 Future investigations of cell-type specific influence on Shank3b KO behaviors

While further testing is required to verify some of the Shank3b cKO mouse behaviors, my initial study suggests that this mouse can be useful for future mechanistic studies. To determine the role of the striatum in the ASD-like behaviors exhibited by Shank3b KO mice, the conditional Shank3b mice could be crossed to the Gng7-Cre mouse line and the behaviors of the resulting progeny could be assessed. To further dissect the influence of striatal circuits on Shank3b KO mouse behavior, the conditional line could be bred with direct and indirect pathway Cre recombinase driver lines (Drd1a-Cre and A2AR-Cre, respectively). The behavioral characterization of these different Shank3b deletion mice could be reinforced by slice and in vivo electrophysiology experiments to identify circuit-specific defects. If successful, these studies could provide the first direct evidence for striatal dysfunction as a mechanism for repetitive behaviors and abnormal social interaction behaviors in an animal model of ASD. In addition, use of the conditional Shank3b deletion with other cell-type specific Cre driver lines could help elucidate the roles of other brain regions in the observed ASD-like behaviors. Furthermore, crossing the conditional Shank3b mouse with cell-type specific Cre-ER mice could produce valuable information concerning circuit-specific developmental aspects of these behaviors. Such insight could guide future inquiries into the treatment of neurodevelopmental diseases.

6.6 Conclusion

The results of the work presented in this document underscore the utility of genetically-modified mice in modeling neurodevelopmental disorders. The initial
characterization of the *Ptchd1* knockout mouse model of developmental disability identified cellular and circuit defects in the thalamic reticular nucleus, a key regulator of thalamo-cortical communication. Moreover, the production and validation of a conditional version of the *Shank3b* mouse model of autism spectrum disorder has set the stage for future investigations of specific circuits involved in social and anxiety-like behaviors. In detailing the circuit and behavioral abnormalities of these two novel mouse models, I have established the foundation of future inquiries into the defective cellular and molecular mechanisms governing psychiatric disease. Progress in this field could lead to the development of much-needed pharmacological interventions.
### Appendix A

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Function</th>
<th>Description</th>
<th>5′-3′ Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPW (Pt1cKO) Gen 1a</td>
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<td>Forward short homology arm</td>
<td>GGATGTTACCCACCTACAATATGCG</td>
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<td>Pchd1 cKO Targeting (SHA)</td>
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<td>pPV (Pt1cKO) PCR B1b</td>
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127
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CTCGCGCGCGCGCTCGCGCG
CAGCGCTGCGCTTGAAG +
GGGGCGCGCTTA
CTCGAGCGTT
|
| pRV (Pt1-YFPki) PCR 8a | Ptcld1-YFP Targeting (LHA) | 5' Ptcld1 LHA + AscI | AACTTCAAAATAACAC
TGAATTAATTTTGCTTG
GCCATTTACTGCTG +
GTAACCGGCGGCGCGG
ATCCAG
|
| pFW (Pt1-YFPki) ES Scr 5b | Ptcld1-YFP ES screen | Forward ES SHA screen | TGCCAGGATCCGAGATT
CC
|
| pRV (Pt1-YFPki) ES Scr 5b | Ptcld1-YFP ES screen | Reverse ES SHA screen | CACTTGAATGGATTTTC
TGGAC
|
| pFW (Sh3cKO) Gen 1a | Shank3cKO Genotyping | Forward short homology arm | CAGCAATATACATGAC
CTGAGAC
|
| pRV (Sh3cKO) Gen 3b | Shank3cKO Genotyping | Reverse Fmr/Wt | GGAGTATAGACGTGAAT
AACC
|
| pRV (Sh3cKO) Gen 5a | Shank3cKO Genotyping | Reverse long homology arm | CGGGGCGAAACGGTCTC
AGG
|
| pFW (Sh3cKO) PCR H1b | Shank3cKO Targeting (SHA) | KpnI + 5' Shank3 SHA | GCC + GGTACC +
CTGGCTGTGTCGCGTG
CTC
|
| pRV (Sh3cKO) PCR H1b | Shank3cKO Targeting (SHA) | 3' Shank3 SHA + Apal | GCC + GGGCCC +
AGCAATTGCGCGCTCG
|
| pFW (Sh3cKO) PCR H1a | Shank3cKO Targeting (Ex1-16) | Apal + LoxP + 5' Exon 13 | GCC + GGGCCC +
ATACGTCCGATATACGATA
CATTATAAGAAGTTAT +
GCCCTGTGA
TGCTGTGCTGAAT
|
| pRV (Sh3cKO) PCR H1a | Shank3cKO Targeting (Ex1-16) | 3' Exon 16 + Sall | GCC + GTGAC +
CTCCAGGTGCGCATGAC
|
| pFW (Sh3cKO) PCR J3 | Shank3cKO Targeting (LHA) | 3' Shank3 LHA + NotI | GCACCCATGAAACAAC
AGTGAAATATACAGTCTG
AAGGGCAAGAAAGAGCTTA +
ACGTAAAGGGCC
GCACACGGGGTTGG
|
| pRV (Sh3cKO) PCR J3 | Shank3cKO Targeting (LHA) | 5' Shank3 LHA + PacI | GCACCCATGAAACAAC
AGTGAAATATACAGTCTG
AAGGGCAAGAAAGAGCTTA +
ACGTAAAGGGCC
GCACACGGGGTTGG
|
| pFW (Sh3cKO) ES Scr1b | Shank3cKO ES screen | Forward ES SHA screen | CTCTATAGACTCCCACTCC
AGG
|
| pRV (Pt1cKO) ES Scr2 | Shank3cKO ES screen | Reverse ES SHA screen | CGTATAATGATGCTGTAA
AGGAAAATATAGGG
|
| pFW (Pt1 ISH) PCR A2 | In situ probe #1 | NotI + 5'Pctdh1 | GCCGCCGCGCGC+CATA
CTCGAGTGACCCAAGATC
|
| pRV (Pt1 ISH) PCR A2 | In situ probe #1 | 3'Pctdh1 + XhoI | GCCGCCGCGCGC+CATA
CTCGAGTGACCCAAGATC
|
| pFW (Pt1 ISH)PCR A1a | In situ probe #2 | NotI + 5'Pctdh1 | GCCGCCGCGCGC+CATA
CTCGAGTGACCCAAGATC
|
| pEV (Pt1 ISH) PCR A1 | In situ probe #2 | 3’Fchd1 + XhoI | GCC + CTGGAG + CCTCCCTCAGAGTCTCCAA GAG |
## Appendix B

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<th>Type</th>
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<td>Santa Cruz sc-30193</td>
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<td>Primary</td>
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<td>Chicken anti-GFP</td>
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<td>Mouse anti-FLAG</td>
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<td>LT A-10521</td>
<td>1:1000</td>
</tr>
<tr>
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<td>Secondary</td>
<td>LT A-21422</td>
<td>1:1000</td>
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<td>Secondary</td>
<td>LT A-11039</td>
<td>1:1000</td>
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Biography

Michael Frederick Wells was born in Columbus, OH on January 9, 1986. He attended Saint Charles Preparatory in Bexley, OH before earning a Bachelor's of Science degree from the University of Notre Dame. He joined the Department of Neurobiology program at Duke University in 2008 and conducted research at the Massachusetts Institute of Technology as a visiting scholar from 2010 until 2015. He was awarded a Ruth L. Kirschstein National Research Service Award Individual Predoctoral Fellowship in 2012. He was an author on a Nature publication titled “Shank3 mutant mice display autistic-like behaviors and postsynaptic dysfunction” in 2011 and is the co-first author on a recently submitted manuscript titled “Ptchd1, a neurodevelopmental disorder gene, impacts thalamic reticular function in attention and cognition.” He recently accepted a post-doctoral position in the laboratory of Dr. Kevin Eggan of the Harvard University Stem Cell Institute and will begin his work there in the Fall of 2015.