ESSENTIAL PATHWAYS AND CIRCUITS OF AUTISM PATHOGENESIS

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ESSENTIAL PATHWAYS AND CIRCUITS OF AUTISM PATHOGENESIS

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Photograph of the Third Bosphorus Bridge being built in 2015 (Istanbul, Turkey). The image is representative of the gap between basic science advances in autism and the development of successful therapeutics for these disabling disorders. The papers in this e-book focus on convergent cellular pathways and brain circuits that may underlie large number of genetic conditions leading to autism. Further understanding these essential pathways and circuit can enable to bridge this important gap. (Photo by M. Sahin)

The Centers for Disease Control and Prevention estimate that 1 in 68 children in the United states is afflicted with autism spectrum disorders (ASD), yet at this time, there is no cure for the disease. Autism is characterized by delays in the development of many basic skills, most notably the ability to socialize and adapt to novelty. The condition is typically identified in children around 3 years of age, however the high heritability of autism suggests that the disease process begins at conception. The identification of over 500 ASD risk genes, has enabled the molecular genetic dissection of the pathogenesis of the disease in model organisms such as mice. Despite the genetic heterogeneity of ASD etiology, converging evidence suggests that these disparate genetic lesions may result in the disruption of a limited number of key biochemical pathways or circuits. Classification of patients into groups by pathogenic rather than etiological categories, will likely aid future therapeutic development and clinical trials. In this set of papers, we explore the existing evidence supporting this view. Specifically, we focus on biochemical cascades such as mTOR and ERK signaling, the mRNA network bound by FMRP and UBE3A, dorsal and ventral striatal circuits, cerebellar circuits, hypothalamic projections, as well as prefrontal and anterior cingulate cortical circuits. Special attention will be given to studies that demonstrate the necessity and/or sufficiency of genetic disruptions (e.g. by molecular deletion and/or replacement) in these pathways and circuits for producing characteristic behavioral features of autism. Necessarily these papers will be heavily weighted towards basic mechanisms elucidated in animal models, but may also include investigations in patients.

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Table of Contents

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EDITORIAL
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Editorial: Essential Pathways and Circuits of Autism Pathogenesis

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The Editorial on the research topic

Essential Pathways and Circuits of Autism Pathogenesis

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social communication skills, as well as stereotyped movements and restricted interests (DSM-5; American Psychiatric Association, 2013). Appreciation of the genetic etiology of ASD began with epidemiological studies in the 1970s, revealing the extremely high heritability of the disorder. Since then over 700 genes have been implicated in the etiology of ASD. A handful of these are highly potent rare variant mutations (e.g., Mendelian disorders like Fragile X; structural, or copy number variants, CNVs, like 16p11.2 deletion/duplication; and *de novo,* rare variant exonic mutations like chromodomain helicase DNA-binding protein 8, *CHD8,* gene mutations). Nevertheless, ASD risk mutations are also incompletely penetrant (only a subset of patients who have the mutation also have ASD), pleiotropic (all known mutations are also causes of intellectual disability, schizophrenia, and/or epilepsy), and likely highly polygenic (i.e., one characteristic is controlled by two or more genes; estimates for ASD range from 400 to 1000 genes). Accumulating evidence suggests that, in the face of this etiological complexity, we may be able to understand the emergence of key core clinical symptoms by examining a limited number of convergent biochemical pathways or brain circuits. This Frontiers Research Topics brings together a set of review articles, which explore the existing evidence supporting this view.

Although, deficits in social interactions and restrictive, repetitive patterns of behavioral output are seemingly unrelated symptom domains, growing appreciation of striatal function suggests that this brain region regulates behavioral flexibility, motivational state, goal-directed learning, and attention. The review articles by Fuccillo and Rothwell consider whether alterations in striatal physiology might be a central node mediating a range of autism-associated behaviors, including social and cognitive deficits that are hallmarks of the disorder. Similarly, the cerebellum is classically thought to control fine motor function, but more recent evidence implicates this brain region in higher cognitive functions as well. Three manuscripts critically review the hypothesis that the cerebellum is essential for many, if not most of the processes that are perturbed in ASD, including language and communication, social interactions, stereotyped behavior, motor activity and motor coordination, and higher cognitive functions (Hampson and Blatt; D'Mello and Stoodley; Mosconi et al.).

Circuit-level explanations of ASD pathogenesis are appealing because they most directly account for the emergence of clinical symptoms; however, because ASD genes are expressed across the whole brain, it is at this time unclear how specific circuits, cell types and brain regions are more likely to be involved in producing symptoms. One compelling possibility is that this pattern emerges through pathoclisis (i.e., the whole brain is exposed to the insult, in this case genetic

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lesion, but certain subtypes of cells, localized to distinct brain regions or circuits, are more susceptible to injury). Alternatively, the selective involvement of certain brain regions or circuits may reflect the case that these circuits are recruited as a compensatory shunting mechanism for symptoms that arise in a distributed fashion throughout multiple brain regions and circuits (this alternative is perhaps analogous to "abdominal guarding" whereby the muscles of the abdominal wall contract when there is injury to any of the organs found within the abdominal cavity). In the case of pathoclisis, future studies aimed at understanding how certain cell types become selectively susceptible may yield important pathogenic mechanisms and therapeutic targets. In the case of compensatory shunting, examining underlying, and unifying biochemical or molecular mechanisms will be critical for understanding the pathogenesis.

The first suggestion that ASD might be thought of as a "synaptopathy" was driven by the observation that the dozen or so ASD risk genes known at the time, all encode synaptic proteins, and could be linked together by biochemical signaling pathways that regulate synaptic pruning and plasticity during early post-natal development. Later, the Fragile X mental retardation protein (FMRP), which is absent in patients with Fragile X (the first identified and most common cause of ASD) binds to nearly one quarter of identified ASD candidate genes, suggesting that this protein might serve as a central node for ASD pathogenesis. Interestingly, even as we discover novel unifying mechanisms, these early speculations are also being borne out by genetic pathway analysis. Several reviews in this volume address these unifying mechanisms revealed by molecular, biochemical, and genetic pathway analysis.

Baribeau and Anagnostou summarize the known associations between the oxytocin and vasopressin neuropeptide systems and social neurocircuits in the brain. Meffert and colleagues review evidence that genetically diverse forms of ASD may be usefully parsed into entities resulting from converse patterns of growth regulation at the molecular level, which lead to the correlates of general synaptic and neural overgrowth or undergrowth (Subramanian et al.). Sell and Margolis examine the hypothesis that changes in UBE3A protein levels alter the levels of a collection of protein substrates giving rise to the unique phenotypic aspects of *UBE3A* associated ASDs. Huang and Hsueh focus *T-brain-1* (*TBR1*) and argue that this gene serves as a node for ASD pathogenesis, as well as reviews their recent evidence that $Tbr1^{+/-}$ ASD model mice show amygdalar wiring and NMDAR hypoactivity phenotypes. O'Roak and colleagues focus on genes involved in transcriptional regulation, such as chromatin modifiers and summarizes evidence that *CHD8*, a chromatin remodeling factor, may serve as a "master regulator" of a common ASD etiology (Barnard et al.). Dougherty and colleagues consider evidence from genetic pathway analysis that reveal clusters of ASD associated genes, which are involved in a handful of cellular functions, as well as the developmental time course, brain region and cell-type specificity of those functions (Kopp et al.).

We are currently at a critical juncture in ASD research. As we discover more and more pathogenic mechanisms, it is important to step back and synthesize so that we may generate novel testable hypothesis about whether and how these mechanisms may intersect to produce the common symptoms of ASD. We hope that the papers brought together in this Frontiers Research Topic will serve to stimulate that conversation and provide the readers with new ideas and perspectives toward such convergent mechanisms and circuits.

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All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Autism Spectrum Disorders and Drug Addiction: Common Pathways, Common Molecules, Distinct Disorders?

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Autism spectrum disorders (ASDs) and drug addiction do not share substantial comorbidity or obvious similarities in etiology or symptomatology. It is thus surprising that a number of recent studies implicate overlapping neural circuits and molecular signaling pathways in both disorders. The purpose of this review is to highlight this emerging intersection and consider implications for understanding the pathophysiology of these seemingly distinct disorders. One area of overlap involves neural circuits and neuromodulatory systems in the striatum and basal ganglia, which play an established role in addiction and reward but are increasingly implicated in clinical and preclinical studies of ASDs. A second area of overlap relates to molecules like Fragile X mental retardation protein (FMRP) and methyl CpG-binding protein-2 (MECP2), which are best known for their contribution to the pathogenesis of syndromic ASDs, but have recently been shown to regulate behavioral and neurobiological responses to addictive drug exposure. These shared pathways and molecules point to common dimensions of behavioral dysfunction, including the repetition of behavioral patterns and aberrant reward processing. The synthesis of knowledge gained through parallel investigations of ASDs and addiction may inspire the design of new therapeutic interventions to correct common elements of striatal dysfunction.

Keywords: autism, addiction, striatum, accumbens, synapse, dopamine, medium spiny neuron

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INTRODUCTION

Autism spectrum disorders (ASDs) are prevalent and devastating neuropsychiatric conditions with a pathophysiology that remains poorly understood. The high heritability of ASDs has motivated the widespread application of advanced sequencing technology to identify genetic variants associated with these disorders (McCarroll and Hyman, 2013). The resulting data sets have revealed an extremely complex genetic architecture, including many genes that each contribute to a fraction of cases (Chen et al., 2015). To sift through this complexity, a growing number of studies have taken genetic variants identified in human patients with ASDs, and introduced corresponding mutations into the genome of laboratory mice. Mice carrying genetic variants associated with neuropsychiatric disease provide an opportunity to probe brain function in a highly specific fashion, and explore underlying mechanisms in a manner that can inform the rational design of therapeutics (Fuccillo et al., 2016). In terms of modeling complex disorders like ASDs, a significant strength of this

approach is the construct validity provided by studying genetic variants in mice with known ASD association in humans (Nestler and Hyman, 2010). Mice carrying ASD-associated genetic mutations also exhibit behavioral phenotypes that map onto primary ASD symptom domains, including reduced social interaction and repetitive patterns of behavior (Silverman et al., 2010), providing an additional degree of face validity. Some of these behavioral phenotypes can be corrected by drugs approved for treatment of ASDs in humans (e.g., Peñagarikano et al., 2011), although the limited number of effective medications precludes more rigorous evaluation of predictive validity.

Many of the genes associated with ASDs play a role in regulating synaptic transmission between neurons (Zoghbi and Bear, 2012), including synaptic cell adhesion molecules like neurexins and neuroligins (Südhof, 2008), as well as postsynaptic scaffolding molecules like SHANK (Jiang and Ehlers, 2013). The generation of mouse lines carrying these ASD-associated genetic mutations has provided opportunities to evaluate changes in synaptic transmission across a variety of brain regions. The emerging synaptic architecture of ASDs is nearly as complex as its genetic architecture, with little consistency when phenotypes are compared across different brain regions or different ASD-associated mutations. For instance, the same genetic mutation can produce distinct functional changes at different synapses (Etherton et al., 2011; Földy et al., 2013), and the same synaptic process can be oppositely affected by different mutations (Auerbach et al., 2011). These perplexing results highlight the importance of winnowing down the essential synaptic circuits that contribute to ASD pathogenesis.

One such critical pathway may involve the striatum and interconnected basal ganglia nuclei, which have been implicated by a number of recent mouse studies, and exhibit functional and structural changes in human patients with ASDs that often correlate with symptom severity (e.g., Sears et al., 1999; Hollander et al., 2005; Rojas et al., 2006; Voelbel et al., 2006; Langen et al., 2009, 2014; Delmonte et al., 2012; Abrams et al., 2013). This growing literature on striatal dysfunction in ASDs has, rather surprisingly, implicated pathways and circuit elements known to play a role in drug addiction. The development and progression of addiction have long been tied to a number of striatal neurochemical systems (e.g., Wise, 1987; Koob and Bloom, 1988; Sarnyai and Kovacs, 1994). More recently, chronic drug exposure has been shown to cause changes in the structure and function of striatal synapses (Russo et al., 2010; Grueter et al., 2012), and these forms of drug-evoked synaptic plasticity contribute to a variety of addiction-related behaviors in rodents. Several recent studies suggest genes and molecules canonically associated with ASDs function in the striatum to regulate drug-evoked synaptic and behavioral plasticity in addiction models - another surprising connection between these seemingly distinct disorders.

The purpose of this article is to review the emerging intersection between ASDs and addiction in the striatum, and consider potential implications for the pathophysiology and treatment of both disorders. Many of the topics covered below relate recent publications in the realm of ASDs to longstanding or established concepts in addiction research, though recent examples of addiction research are included when appropriate. The topics and references are drawn from personal familiarity with both fields of research, as well as manual review of literature searches including autism and addiction, autism and striatum, or autism and each of the various signaling pathways discussed below. The manuscript is organized on the basis of emerging common themes, and thus represents an integrative review of select literature that highlights areas of overlap and potential shared mechanisms, rather than a comprehensive or systematic review of research on either ASDs or addiction (Whittemore et al., 2014).

STRIATAL PATHWAYS IN ASDs

The striatum serves as a gateway to the basal ganglia, receiving synaptic input from numerous cortical, thalamic, and limbic brain regions, and relaying information to downstream processing stations in the basal ganglia (Sesack and Grace, 2010; Nelson and Kreitzer, 2014). In humans and primates, the striatal complex includes the caudate nucleus, the putamen, and a ventral striatal region known as the nucleus accumbens. In rodents, the nucleus accumbens also occupies the ventral portion of striatum, while the caudate and putamen roughly correspond to medial and lateral subregions of the dorsal striatum, respectively (Graybiel, 2008). Of these striatal subregions, the nucleus accumbens is most closely associated with reward-related behavioral functions (Carlezon and Thomas, 2009; Sesack and Grace, 2010). Many of these functions pertain to learning reward-related associations, either in terms of cues that predict delivery of reward (classical/Pavlovian conditioning), or actions that must be completed to obtain reward (operant/instrumental conditioning). In human brain imaging studies, normal reward-related activation of the nucleus accumbens is disturbed in patients with ASDs (e.g., Scott-Van Zeeland et al., 2010; Delmonte et al., 2012; Dichter et al., 2012; Kohls et al., 2013; Richey et al., 2014).

Dorsal striatal subregions also play a role in processing reward, particularly in terms of the movements and actions that must be learned and executed in order to obtain reward (Balleine et al., 2007). A number of important dissociations have been reported in the behavioral functions of dorsomedial and dorsolateral striatum in rodents (Yin and Knowlton, 2006; Balleine and O'Doherty, 2010). The model emerging from these studies suggests that dorsomedial striatum is important for behaviors that are flexible and sensitive to outcome, which is often the case early in learning. As actions are repeated many times and become streamlined and automatic, they also become less sensitive to outcome, and this late stage of learning involves dorsolateral striatum. These inflexible and ingrained patterns of behavior are considered "habitual," and the process of habit formation could contribute to some of the repetitive and stereotyped routines and rituals observed in patients with ASDs. Indeed, many studies have reported structural and functional alterations in the caudate and putamen of human patients with ASDs (e.g., Sears et al., 1999; Eliez et al., 2001; Levitt et al., 2003; Hollander et al., 2005; Haznedar et al., 2006; Silk et al., 2006; Turner et al., 2006; Voelbel

et al., 2006; Langen et al., 2007, 2012; Takarae et al., 2007; Di Martino et al., 2011).

Many robust behavioral assays for striatum-dependent reward processing have been developed in rodents. One example is the place conditioning assay, which involves the association between a rewarding stimulus and a distinct set of contextual cues (e.g., floor texture, wall pattern, or chamber odor). The choice to spend time in the presence of these cues vs. a neutral set of cues is operationally defined as a conditioned place preference (CPP), and most drugs of abuse produce CPP (Tzschentke, 1998). Rodents will also develop CPP for contextual cues associated with social interaction with conspecifics, relative to cues experienced during social isolation (Panksepp and Lahvis, 2007; Trezza et al., 2009). This preference for cues associated with social interactions (i.e., "social CPP") is likely related to the preference for pair-bonded partners exhibited by monogamous species like prairie voles (Carter et al., 1995) and titi monkeys (Carp et al., 2015). Many of the same striatal pathways contribute to social behavior as well as drug reward, suggesting neurochemical systems that originally evolved to mediate social attachment may be hijacked by drugs of abuse (Insel, 2003; Burkett and Young, 2012). Recent studies of mice carrying ASD-associated genetic mutations point to dysfunction of these same striatal systems, which will be reviewed below in terms of both basic function as well as dysfunction in ASDs.

Oxytocin

The peptide hormone oxytocin contributes to a myriad of social behaviors across many mammalian species (Anacker and Beery, 2013). The pro-social effects of oxytocin have generated substantial interest in its use as a treatment for social deficits associated with ASDs, and there is also some evidence for genetic polymorphisms in the oxytocin receptor associated with ASDs (reviewed by Yamasue et al., 2012). The monogamous behavior of prairie voles is associated with a high density of oxytocin receptors in the nucleus accumbens, and pharmacological blockade of these receptors prevents pair bond formation (reviewed by Insel and Young, 2001), pointing to a key role for oxytocin signaling in the nucleus accumbens in social behavior.

A recent study by Dölen et al. (2013) found that pharmacological antagonism of oxytocin receptors in the nucleus accumbens also blocks social CPP in mice. This result was somewhat surprising, as mice were previously reported to have a relatively low density of oxytocin receptors in the nucleus accumbens compared to other rodent species (Olazábal and Young, 2006). However, conditional genetic deletion of the oxytocin receptors in the nucleus accumbens itself did not impair social CPP. Instead, the oxytocin receptors that mediate social CPP appeared to be expressed on the axon terminals of serotonergic fibers that originate in the dorsal raphe nucleus, and pharmacological antagonism of serotonin 5HT1B receptors in the nucleus accumbens also blocked social CPP (Dölen et al., 2013).

Synaptic plasticity in the nucleus accumbens may be important for encoding the association between social interaction and contextual cues that leads to social CPP. In acute brain slice preparations, stimulation of either oxytocin or serotonin 5HT1B receptors in the nucleus accumbens produced a long-term depression (LTD) of excitatory synapses onto MSNs (Dölen et al., 2013). This reduction of excitatory synaptic drive was associated with a decrease in the probability of presynaptic glutamate release, and provides a plausible synaptic mechanism that may contribute to social reward. Other forms of nucleus accumbens LTD that involve presynaptic changes in glutamate release are impaired by addictive drug exposure (e.g., Fourgeaud et al., 2004; Grueter et al., 2010). This occlusion could contribute to decrements in social behavior caused by drug exposure, like the impairment of social bonding in prairie voles caused by amphetamine exposure (Liu et al., 2010). This impairment can be reversed by oxytocin administration (Young et al., 2014), and oxytocin can attenuate other behavioral effects of psychostimulant administration (reviewed by Sarnyai and Kovács, 2014), clearly demonstrating an interaction between drug effects and the oxytocin system. However, the neurobiological substrata of social and drug reward are at least partially separable, because pharmacological antagonism of oxytocin receptors does not block cocaine CPP (Dölen et al., 2013).

Dopamine Release

Dopaminergic input to the nucleus accumbens originates from dopamine neurons in the ventral tegmental area of the midbrain, and this "mesolimbic" dopamine pathway is closely tied to motivation, reward, and the development of addiction (for reviews, see Wise, 2004; Berridge, 2007; Salamone and Correa, 2012). Mesolimbic dopamine is also important for social behavior in rodents, including pair bond formation in prairie voles (reviewed by Curtis et al., 2006). In mice, activity of the mesolimbic dopamine pathway corresponds to social behavior in real time, and optogenetic manipulations of this pathway affect social interaction (Gunaydin et al., 2014). Given these critical functions of dopamine in social behavior and reward, it is perhaps not surprising that genetic polymorphisms in dopamine signaling genes are also associated with ASDs (e.g., Comings et al., 1991; Hettinger et al., 2008, 2012; De Krom et al., 2009; Hamilton et al., 2013; Bowton et al., 2014; Staal et al., 2015).

A recent report by Karayannis et al. (2014) examined the function of the mesolimbic dopamine system in mice following genetic deletion of *Cntnap4* (also known as *Caspr4*), the gene encoding contactin-associated protein-like 4 (Cntnap4). Cntnap4 is a transmembrane protein that belongs to the neurexin superfamily of cell adhesion molecules, which interact with presynaptic proteins involved in neurotransmitter release (Spiegel et al., 2002). Genetic mutations in other members of this family of molecules have been previously reported in patients with ASDs, and Karayannis et al. (2014) report several new ASD probands with *CNTNAP4* gene disruptions. In mice, they found that expression of Cntnap4 was enriched in midbrain dopamine neurons, as well as inhibitory interneurons in the cerebral cortex, and therefore examined the release of dopamine and GABA from these populations of brain cells. While the release of GABA from cortical interneurons was substantially reduced in *Cntnap4* mutant mice, the release of dopamine in the nucleus

accumbens was significantly increased. These divergent effects on release of two different neurotransmitters further underscore the heterogeneous effects that a single ASD-associated genetic mutation can have on different types of synapses.

Cntnap4 mutant mice also exhibited a variety of aberrant behavioral phenotypes (Karayannis et al., 2014). Most striking was hair loss on the snout, face, and body, which was caused by over-grooming of offspring by parents carrying the *Cntnap4* mutation. This excessive grooming phenotype was reversed by chronic treatment with haloperidol, a dopamine D2 receptor antagonist, suggesting over-grooming was caused by excessive dopamine signaling. Social behavior was not assessed by Karayannis et al. (2014) in *Cntnap4* mutant mice, though it is disrupted by mutations of *Cntnap2*, another member of this protein family (Peñagarikano et al., 2011; Burkett et al., 2015). It will be fascinating to see whether excessive dopamine signaling in the nucleus accumbens of *Cntnap4* mutant mice alters either social reward or behavioral responses to addictive drug exposure.

Dopamine-Sensitive Medium Spiny Neurons

A variety of dopamine receptors are expressed by different cell types in the striatum (Gerfen and Surmeier, 2011). While a small fraction of striatal cells are interneurons that release acetylcholine or GABA, the vast majority of striatal cells are medium spiny projection neurons (MSNs). MSNs are the principal neurons of the striatum and relay information to downstream processing stations in the basal ganglia, including the substantia nigra pars reticulata and the globus pallidus. In the dorsal striatum, striatonigral and striatopallidal MSNs represent two discrete subpopulations that differ in expression of D1 vs. D2 dopamine receptors, as well as a variety of other properties (Gerfen and Surmeier, 2011). The nucleus accumbens also contains discrete populations of D1-MSNs and D2-MSNs, but D1-MSNs in the nucleus accumbens project to both the ventral mesencephalon and ventral pallidum, whereas D2-MSNs project only to the ventral pallidum (Kupchik et al., 2015).

The ability to identify and manipulate specific MSN subtypes was dramatically advanced by the development of bacterial artificial chromosome (BAC) transgenic mice (Heintz, 2001), which allow cell type-specific expression of fluorescent proteins (Gong et al., 2003; Shuen et al., 2008) as well as Cre recombinase (Gong et al., 2007; Durieux et al., 2009). The application of these tools to research on drug addiction has revealed that activation of D1-MSNs promotes addiction-related behaviors, whereas activation of D2-MSNs tends to inhibit the same behaviors (Lobo et al., 2010; Bock et al., 2013; Pascoli et al., 2014; reviewed by Smith et al., 2013). These divergent effects are consistent with classic models of basal ganglia function, in which the direct pathway formed by D1-MSNs and the indirect pathway formed by D2-MSNs exert opposite influences on overall basal ganglia output (Albin et al., 1989; DeLong, 1990).

Striatal MSNs show enriched expression of genes associated with ASDs (Chang et al., 2015), and recent studies have begun to explore how ASD-associated mutations affect specific MSN subtypes. One study focused on ASD-associated mutations in neuroligin-3 (*Nlgn3*), a synaptic cell adhesion molecule that plays important roles in shaping the functional properties of synaptic transmission. Loss-of-function genetic mutations in *Nlgn3* caused a specific impairment of inhibitory synaptic transmission onto nucleus accumbens D1-MSNs (Rothwell et al., 2014). This reduction of inhibitory synaptic transmission is intriguing because human patients with ASDs have been reported to have decreased GABA receptor binding in the nucleus accumbens (Mendez et al., 2013). In mice, nucleus accumbens D1-MSNs appeared to be selectively vulnerable to genetic deletion of *Nlgn3* because it is expressed at a relatively high level compared to neighboring D2-MSNs, or MSNs in the dorsal striatum. Conditional genetic deletion of *Nlgn3* from nucleus accumbens D1-MSNs also caused the development of a more repetitive motor routine on the accelerating rotarod task (Rothwell et al., 2014). This behavioral phenotype has also been reported in several other mouse lines carrying ASD-associated genetic mutations (Kwon et al., 2006; Etherton et al., 2009; Nakatani et al., 2009), and thus might have some relevance to the repetitive and stereotyped movements and routines associated with ASDs in human patients.

Altered striatal structure and function have also been reported in mice lacking the genes normally found on human chromosome 16p11.2 that are deleted in some human patients with ASDs (Weiss et al., 2008). These 16p11 mutant mice have an increased number of striatal cells expressing markers of D2-MSNs, as well as more MSNs co-expressing both D1 and D2 markers, but no change in the number of D1-MSNs (Portmann et al., 2014). These changes in cell number were associated with a relative enlargement in the size of the nucleus accumbens as well as the globus pallidus, which receives synaptic input from D2-MSNs. In 16p11 mutant mice, D2-MSNs also appeared to send ectopic projections to the medial globus pallidus, which is normally only targeted by D1-MSNs. Nucleus accumbens MSNs of 16p11 mutant mice had altered excitatory synaptic properties, including a presynaptic increase in the probability of glutamate release and changes in postsynaptic glutamate receptor complement. Mutant mice also exhibited behavioral phenotypes that included hyperactivity, circling, and a lack of habituation to novelty.

Opioids

Striatal opioid systems play an important role in the rewarding aspects of social interaction (Burkett et al., 2011; Trezza et al., 2011; Resendez et al., 2013), as well as the rewarding properties of opiate narcotics and other abused drugs (Befort, 2015). The mu opioid receptor (MOR) is a particularly critical mediator of the rewarding effects of opiates like morphine (Matthes et al., 1996). Juvenile mice lacking the MOR gene (*Oprm1*) exhibit a diminished behavioral response to separation from their mothers, suggesting that interaction with the mother may be less rewarding (Moles et al., 2004). Adult *Oprm1* knockout mice also exhibit other ASD-related behavioral phenotypes, including decreased sociability and stereotyped behaviors (Becker et al., 2014). These behavioral changes were associated with dramatic gene expression changes in the nucleus accumbens and dorsal striatum, including a number of genes involved in excitatory synaptic signaling. One of these genes (*Grm4*) encodes the

metabotropic glutamate receptor mGluR4, and administration of an mGluR4 positive allosteric modulator effectively relieved many of the behavioral phenotypes exhibited by MOR knockout mice.

Endogenous opioids may interact with other neuromodulatory systems in the nucleus accumbens. MOR knockout mice exhibit a dramatic decrease in mRNA levels for oxytocin in the NAc (Becker et al., 2014). Conversely, oxytocin receptor levels in the NAc are increased (Gigliucci et al., 2014), which could represent a homeostatic response to compensate for loss of endogenous ligand. Intranasal administration of oxytocin to adult male MOR-KO mice led to an increase in the number of ultrasonic vocalizations emitted during interaction with a female mouse (Gigliucci et al., 2014). This interaction between opioid and oxytocin signaling in the NAc suggests a convergence of multiple signaling pathways that may contribute to ASD pathophysiology.

Endocannabinoids

Brain cannabinoid receptors are activated by both exogenous and endogenous cannabinoids, and play an important role in drug reward (Befort, 2015). Endogenous cannabinoid systems in the nucleus accumbens also regulate social behavior, as local blockade of endocannabinoid degradation enhances social play (Trezza et al., 2012). Social interaction increases levels of anandamide (an endogenous cannabinoid) in the nucleus accumbens, an effect that is stimulated by oxytocin and may contribute to social reward (Wei et al., 2015). Endocannabinoids are important for regulating synaptic function in a variety of ways, including a key role in many forms of synaptic plasticity. Mice lacking FMRP have an impairment of endocannabinoid-dependent LTD of excitatory synapses in the NAc (Jung et al., 2012). This impairment is due to a mislocalization of diacylglycerol lipase-α (DGL-α), a key enzyme in the biosynthetic pathway for the endocannabinoid 2-arachidonoyl-sn-glycerol (2-AG). In the absence of FMRP, DGL-α is localized farther away from the postsynaptic density and is not robustly activated by stimulation of mGluR5. However, this signaling deficit could be rescued by inhibiting the degradation of 2-AG, leading to a normalization of LTD in the NAc as well as some behavioral phenotypes of FMRP knockout mice. ASD-associated genetic mutations in *Nlgn3* did not appear to affect endocannabinoid-dependent LTD in the nucleus accumbens (Rothwell et al., 2014), though these mutations do alter endocannabinoid signaling in other parts of the brain (Földy et al., 2013). In the dorsal striatum, exaggerated LTD has been reported in mice overexpressing eukaryotic translation initiation factor 4E (Santini et al., 2013), which is encoded by the ASD candidate gene *Eif3e* (Neves-Pereira et al., 2009).

Pathways to Dorsal Striatum

The progression of drug abuse is thought to involve a transition in the control of behavior from ventral to dorsal striatum (for a recent review, see Everitt and Robbins, 2016). This transition corresponds with a shift in the motivation to take drugs, which becomes less goal-directed and more compulsive or habitual (i.e., insensitive to outcome), leading to continued use despite adverse consequences. This transition in behavioral control may involve polysynaptic pathways that link ventral striatum with dorsal striatum, as neurons in the nucleus accumbens project to dopaminergic centers in the midbrain that subsequently project to more dorsal striatal subregions (Haber et al., 2000; Ikemoto, 2007). This ascending spiral of connectivity may be engaged over the course of chronic drug use and contribute to habitual patterns of addiction (Belin and Everitt, 2008).

Changes in the nucleus accumbens caused by ASD-associated genetic variants could accelerate the transition of behavioral control to more dorsal striatal subregions, leading to more repetitive, stereotyped, or habitual patterns of behavior. However, changes that directly affect the dorsal striatum may also produce similar behavioral consequences: for example, the exaggeration of dorsal striatal LTD in *Eif3e* mutant mice could potentially affect the process of habit formation (Santini et al., 2013). Dysfunction in the dorsal striatum has also been reported in mice carrying gene deletions of *Shank3* (Peca et al., 2011), which encodes a synaptic scaffolding protein and is disrupted in Phelan-McDermid syndrome (an autism spectrum disorder) as well as idiopathic ASDs (reviewed by Jiang and Ehlers, 2013). These *Shank3* mutant mice exhibit reduced social interaction and excessive self-grooming to the point of skin lesions (Peca et al., 2011). MSNs in the dorsal striatum exhibited abnormal morphology and impaired excitatory synaptic transmission, although it is presently unclear if D1- or D2-MSNs were preferentially affected.

ASD MOLECULES IN ADDICTION

Genes and molecules that have been extensively studied in the realm of ASDs have recently emerged as potential mediators of addiction-related behavior. These include the genes known to cause Rett syndrome (MECP2—Amir et al., 1999) and fragile X syndrome (FMR1—Pieretti et al., 1991), which have been topics of sustained research since their initial identification. MeCP2 and FMRP play important roles in the regulation of gene transcription and translation, respectively. In mice, genetic mutations in *Mecp2* and *Fmr1* cause a variety of deficits in synaptic transmission and plasticity throughout the brain, which are thought to contribute to the ASD-related symptoms frequently observed in patients with these syndromes (Zoghbi and Bear, 2012).

These ASD-associated molecules have been shown to regulate multiple aspects of striatal function. For example, dopamine signaling in the striatum is regulated by both MeCP2 (Su et al., 2015) and FMRP (Wang et al., 2008). Genetic deletion of *Fmr1* also affects synaptic structure, function, and plasticity in the nucleus accumbens (Jung et al., 2012; Smith et al., 2014; Neuhofer et al., 2015) as well as the dorsal striatum (Centonze et al., 2008; Maccarrone et al., 2010). The development of drug addiction is strongly tied to dopamine signaling and synaptic remodeling in the striatum, motivating several recent investigations of how genetic mutations in *Mecp2* and *Fmr1* affect behavioral responses to addictive drugs. These studies focused on common behavioral outcomes in addiction research, including CPP as a measure of drug reward, as well as performance an instrumental

response to receive contingent delivery of the drug (i.e., drug self-administration). These studies also examined psychomotor activation following acute drug administration, as well as the psychomotor sensitization that occurs following repeated drug exposure, as these behavioral processes are closely tied to striatal dopamine release and remodeling of striatal circuitry (for reviews, see Vanderschuren and Kalivas, 2000; Grueter et al., 2012).

Methyl CpG-Binding Protein-2 (MeCP2)

Psychostimulant administration induces MeCP2 phosphorylation in the nucleus accumbens (Deng et al., 2010, 2014). This effect is not seen in MSNs, but is instead selective for fast-spiking GABAergic interneurons that express parvalbumin and GAD67. Behavioral responses to amphetamine administration are altered by a constitutive hypomorphic mutation in *Mecp2* (Deng et al., 2010), as well as a Ser421Ala mutation in Mecp2 that prevents psychostimulant-induced phosphorylation (Deng et al., 2014). Enhanced behavioral sensitivity to psychostimulants is also observed following restricted knockdown of *Mecp2* expression in the nucleus accumbens. These findings suggest phosphorylation of MeCP2 in the nucleus accumbens plays a role in constraining behavioral responses to psychostimulants. However, the role of MeCP2 may be different in the dorsal striatum, as a separate study found cocaine self-administration was reduced following restricted knockdown of *Mecp2* expression in the dorsal striatum (Im et al., 2010).

Fragile X Mental Retardation Protein (FMRP)

Constitutive genetic deletion of *Fmr1* did not alter the acute locomotor response to cocaine, but decreased locomotor sensitization over course of repeated cocaine exposure, and also attenuated cocaine CPP (Smith et al., 2014). Conditional genetic deletion of *Fmr1* in the nucleus accumbens also decreased locomotor sensitization, though it did not affect cocaine CPP. Cocaine-evoked changes in the structure and function of excitatory synapses onto nucleus accumbens MSNs were also enhanced in *Fmr1* constitutive knockout mice. These data suggest that FMRP serves as a negative regulator of the structural and functional changes at nucleus accumbens synapses caused by chronic drug exposure, similar to MeCP2 (Deng et al., 2014).

OUTLOOK AND OPEN QUESTIONS

How do these emerging parallels between ASDs and addiction (**Table 1**) inform our understanding of the pathophysiology of each disorder in relationship to the striatum? Despite these parallels, these are clearly two distinct disorders, as evidenced not only by distinct symptomatology but also the lack of comorbidity. Nevertheless, ongoing research may benefit from more careful consideration of neurobiological and psychological process that may be commonly affected in both disorders. This approach is reminiscent the Research Domain Criterion (RDoC) initiative by the U.S. National Institute of Mental Health (Insel et al., 2010; Insel and Cuthbert, 2015), which aims to establish a new framework for psychiatric research that moves away from specific clinical diagnoses and toward dimensions or constructs with well-defined neurobiological substrata that may be impaired across multiple mental disorders. Below, I consider two behavioral dimensions that may be commonly affected in both ASDs and addiction, as well as potential neural mechanisms and therapeutic implications raised by the shared and distinct elements of these disorders.

Repetitive Behavioral Patterns as a Common Dimension of Dysfunction

One hallmark of both ASDs and addiction is the repetition of specific patterns of behavior, sometimes in the absence of an obvious goal or even in the face of adverse consequences. Indeed, the transition from casual to compulsive or habitual drug use is one of the defining features of addiction. Conversely, ASDs are associated with behavioral patterns that are performed in a stereotyped fashion and are resistant the change. This can include simple movements like hand flapping, as well as more complex routines and rituals. While somewhat different terminology is used to describe these behavioral patterns (e.g., "compulsive" or "habitual" for addiction, "repetitive" or "stereotyped" for ASDs), all may be characterized by a failure to inhibit the repetition of behavioral patterns. This lack of inhibitory control over repetitive behavior may also contribute to other mental disorders, such as obsessive-compulsive disorder, which is also linked to striatal dysfunction (Burguière et al., 2015).

Rodent models of ASDs and addiction obviously lack the nuance and specificity of the clinical condition, but some interesting similarities in repetitive behavioral patterns seem to suggest common striatal circuitry may be affected in both disorders. For example, stereotyped patterns of rotational behavior caused by ASD-associated genetic mutations (Portmann et al., 2014; Rothwell et al., 2014) are also observed following exposure to psychostimulants (Fowler et al., 2001). Similarly, the orofacial stereotypy caused by repeated exposure to a high dose of cocaine is exacerbated in *Fmr1* knockout mice (Smith et al., 2014). While it is obviously difficult to extrapolate these simple rodent behaviors to the symptoms of complex human disorders likes ASDs and addiction, these examples at least illustrate that some elementary forms of repetitive behavior can be similarly affected by addictive drugs and ASD-associated genetic mutations.

Aberrant Reward Processing as a Common Dimension of Dysfunction

The processing of reward-related information represents another behavioral dimension that may be affected in both ASDs and addiction. The concept of reward figures prominently in addiction, where decades of research have led to the identification and dissection of distinct components of reward (reviewed by Berridge et al., 2009). One important distinction is between the hedonic impact or "liking" of reward, and the incentive salience or "wanting" associated with rewards and related environmental stimuli. Repeated drug exposure is thought to

TABLE 1 | Striatal signaling pathways and molecules commonly implicated in both addiction and autism spectrum disorders (ASDs); see text for details.

Pathway/Molecule	Relevance to addiction	Relevance to ASDs
Oxytocin	Attenuates behavioral effects of psychostimulant administration (Sarnyai and Kovács, 2014)	Social reward involves actions of oxytocin in the nucleus accumbens (Dölen et al., 2013)
Dopamine	Important for reward and attribution of incentive salience (Wise, 2004; Berridge, 2007)	Increased release in *Cntnap4* mutant mice (Karayannis et al., 2014)
Opioids	Contribute to rewarding effects of exogenous opiates and other drugs (Befort, 2015)	Autism-related behavioral phenotypes in *Oprm1* mutant mice (Moles et al., 2004; Becker et al., 2014)
Endocannabinoids	Contribute to drug reward and synaptic plasticity (Befort, 2015)	Altered striatal synaptic plasticity in *Fmr1* and *Eif3e* mutant mice (Jung et al., 2012; Santini et al., 2013)
Methyl CpG-Binding Protein-2 (MECP2)	Altered behavioral responses to psychostimulant administration (Deng et al., 2010, 2014; Im et al., 2010)	Deletions cause Rett syndrome (Amir et al., 1999)
Fragile X Mental Retardation Protein (FMRP)	Altered behavioral and synaptic responses to cocaine exposure (Smith et al., 2014)	Deletions cause fragile X syndrome (Pieretti et al., 1991)

selectively exaggerate or sensitize the incentive salience attributed to drugs and drug-related cues, a process that is closely linked to dopamine transmission (for a recent review, see Robinson and Berridge, 2008).

In ASD research, aberrant "reward processing" often refers to altered patterns of brain activity in response to social stimuli or monetary reward (e.g., Scott-Van Zeeland et al., 2010; Delmonte et al., 2012; Dichter et al., 2012; Kohls et al., 2013; Richey et al., 2014). Reduced sensitivity to social stimuli may contribute to the deficits in social behavior associated with ASDs, but the precise nature of this aberrant reward processing remains unclear. For instance, are social deficits in ASDs due to decreased hedonic impact or "liking" of social interaction, or rather to a decrease in the incentive salience or "wanting" associated with social stimuli? An alternative possibility (discussed in more detail below) is that ASDs are associated with indiscriminate attribution of incentive salience to inappropriate stimuli in the environment, which could lead to fixation on these stimuli at the expense of social interaction. Rodents carrying ASD-associated genetic mutations may provide a tractable model to begin disentangling these possibilities, through behavioral assays and direct manipulations of brain function needed to fractionate the different components of reward (Berridge et al., 2009).

Neural Mechanisms for Common and Distinct Dimensions of Dysfunction

In light of the putative behavioral dimensions that are commonly impaired in ASDs and addiction, an obvious question arises regarding the elements of striatal circuitry that could commonly contribute to both disorders. One intriguing possibility involves D1-MSNs in the nucleus accumbens, as these cells appear to be a key locus for structural and functional synaptic changes caused by chronic drug exposure (Grueter et al., 2012; Smith et al., 2013). Drug-evoked plasticity at excitatory synapses on D1-MSNs in the nucleus accumbens appears to drive multiple forms of addiction-related behavior (Pascoli et al., 2012, 2014). Furthermore, direct activation of D1-MSNs in the nucleus accumbens enhances both drug reward and the development of psychomotor sensitization (Lobo et al., 2010; Koo et al., 2014), whereas inhibition of these cells reduces the same behavioral effects (Hikida et al., 2010; Ferguson et al., 2011).

Given this established link between addiction and D1-MSNs in the nucleus accumbens, it was both surprising and fascinating that recent reports linked these same cells to social behavior in wild-type mice (Gunaydin et al., 2014), as well as repetitive behavior in mice carrying ASD-associated mutations in *Nlgn3* (Rothwell et al., 2014). The former study suggests dopamine release and activation of D1-MSNs are both important for the expression of social behavior in mice (Gunaydin et al., 2014), whereas the latter study suggests synaptic disinhibition of D1-MSNs leads to repetitive behavior (Rothwell et al., 2014). These two patterns of results may appear contradictory in relation to ASDs, which are associated with less social behavior and more repetitive behavior. One potential explanation for this apparent contradiction (considered below) also provides insight into how the altered activity of one cell type could lead to both common and distinct dimensions of dysfunction.

Continuous synaptic disinhibition of D1-MSNs may lead to chronic elevation of baseline activity in these cells, thereby occluding phasic increases in activity that encode normal social behavior. Chronic elevation of baseline activity in D1-MSNs may also influence dopamine levels in the striatum, as axonal projections from these cells to the ventral tegmental area appear to synapse preferentially onto interneurons, which in turn inhibit dopamine neurons (Xia et al., 2011; Bocklisch et al., 2013). Disinhibition of D1-MSNs may therefore translate into disinhibition of dopamine neurons and increase dopamine release in the back into the striatum. This continuous increase in dopamine release would resemble the effects of ASD-associated genetic mutations in *Cntnap4* (Karayannis et al., 2014), and could lead to indiscriminate attribution of incentive salience to inappropriate stimuli in the environment. This "non-specific" attribution of incentive salience would be fundamentally different from the attribution of incentive salience in addiction, which is excessively large in magnitude but still specifically attributed to drugs and drug-related cues. Thus, increased activation of D1-MSNs and excessive dopamine release may be a common feature of ASDs and addiction, but there may be differences in how this cellular change is manifested in the clinical features of each disorder.

It is also becoming increasingly apparent that D1-MSNs in the nucleus accumbens exhibit heterogeneity in terms of function,

structure, and neurochemistry. D1-MSNs specifically co-express the neuropeptide dynorphin, and activation of dynorphinergic cells in different subregions of the nucleus accumbens can lead to opposite behavioral effects (Al-Hasani et al., 2015). D1-MSNs in the nucleus accumbens also send axonal projections to both the ventral mesencephalon and ventral pallidum, and therefore contribute to both the direct and indirect pathways through the basal ganglia (Kupchik et al., 2015). Furthermore, a small population of MSNs in the nucleus accumbens appears to express both D1 and D2 dopamine receptor subtypes (Perreault et al., 2011). Subtle differences in how ASDs and addiction impact different subpopulations of D1-MSNs could also contribute to distinct clinical manifestations of these disorders.

Therapeutic Implications

Parallel dissection of striatal circuitry in ASDs and addiction may enable therapeutic advances in one field to inform progress in the other. For example, in the realm of addiction, the key role for D1-MSNs in promoting the behavioral effects of cocaine recently inspired an intersectional approach to reversing cocaine-evoked synaptic plasticity in mice, through the combination of deep brain stimulation of the nucleus accumbens and a pharmacological manipulation that selectively targets D1-MSNs (Creed et al., 2015). This type of intersectional strategy may prove beneficial as deep brain stimulation and other forms of neuromodulation are developed for clinical treatment of patients with ASDs (Sturm et al., 2012; Enticott et al., 2014; Sinha et al., 2015). Other types of intersectional therapeutic strategies could involve simultaneous manipulation of multiple neuromodulatory systems that intersect in the nucleus accumbens, such as oxytocin and serotonin (Dölen et al., 2013), or opioids and cannabinoids (Befort, 2015). The opioid and cannabinoid systems may be particularly tractable therapeutic targets, given their rich and diverse pharmacology as well as active drug development efforts for treatment of pain.

ASDs and drug addiction are complicated disorders that likely involve many parts of the brain, but the literature reviewed here highlights a central role for the striatum and basal ganglia in both disorders. In the striatum, these disorders impact a variety of neuromodulatory systems converging on multiple postsynaptic cells types. This intrinsic complexity makes it challenging to study striatal circuits in the context of disease, but also increases the number of potential therapeutic targets as well as the possibility of developing interventions that specifically affect individual circuit elements. It is quite likely that some common elements of this circuitry (like D1-MSNs in the nucleus accumbens) contribute to the pathophysiology of both ASDs and addiction, while other elements within or beyond the striatum are uniquely involved in only one disorder. A growing knowledge of both common and distinct dimensions of dysfunction will help guide the development of interventions that could be broadly useful for normalizing neural circuit dysfunction that contributes to behavioral deficits in both ASDs and addiction.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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Striatal Circuits as a Common Node for Autism Pathophysiology

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Autism spectrum disorders (ASD) are characterized by two seemingly unrelated symptom domains—deficits in social interactions and restrictive, repetitive patterns of behavioral output. Whether the diverse nature of ASD symptomatology represents distributed dysfunction of brain networks or abnormalities within specific neural circuits is unclear. Striatal dysfunction is postulated to underlie the repetitive motor behaviors seen in ASD, and neurological and brain-imaging studies have supported this assumption. However, as our appreciation of striatal function expands to include regulation of behavioral flexibility, motivational state, goal-directed learning, and attention, we consider whether alterations in striatal physiology are a central node mediating a range of autism-associated behaviors, including social and cognitive deficits that are hallmarks of the disease. This review investigates multiple genetic mouse models of ASD to explore whether abnormalities in striatal circuits constitute a common pathophysiological mechanism in the development of autism-related behaviors. Despite the heterogeneity of genetic insult investigated, numerous genetic ASD models display alterations in the structure and function of striatal circuits, as well as abnormal behaviors including repetitive grooming, stereotypic motor routines, deficits in social interaction and decision-making. Comparative analysis in rodents provides a unique opportunity to leverage growing genetic association data to reveal canonical neural circuits whose dysfunction directly contributes to discrete aspects of ASD symptomatology. The description of such circuits could provide both organizing principles for understanding the complex genetic etiology of ASD as well as novel treatment routes. Furthermore, this focus on striatal mechanisms of behavioral regulation may also prove useful for exploring the pathogenesis of other neuropsychiatric diseases, which display overlapping behavioral deficits with ASD.

Keywords: autism spectrum disorders, dorsal striatum, nucleus accumbens (NAcc), mouse models, synaptic transmission, circuit

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A CIRCUIT HYPOTHESIS FOR AUTISM SPECTRUM DISORDER PATHOPHYSIOLOGY

Moving from a Clinical to Molecular Characterization of Autism Spectrum Disorders

The earliest clinical descriptions of autism highlighted two symptom domains, focusing on social behaviors and the regulation of motor output. Kanner's seminal article "Autistic Disturbances of Affective Contact" carefully described the profound social deficits of his patients, concluding "these children have come into the world with innate inability to form the usual biologically

provided affective contact with people…" (Kanner, 1943). Shortly thereafter, in his "'Autistic Psychopathy' in childhood," Asperger documented a range of abnormalities in behavioral control ("most conspicuous were his stereotyped movements: he would suddenly start to beat rhythmically on his thighs, bang loudly on the table, hit the wall…"), motor performance (when trying a "particular physical exercise, his movements would be ugly and angular") and goal-directed actions ("…drives and instincts are often severely perturbed. This is shown in the failure of instinctive situational adaptation…"; Asperger, 1944). The detailed clinical observations from these papers highlighted the diversity of behavioral presentations in autism and demonstrated the extensive comorbidity between symptoms in the social and motor control domains. Nearly 70 years later, the fruits of the genetic revolution are beginning to reveal molecular abnormalities contributing to the behaviors originally observed by Kanner and Asperger (Krumm et al., 2014; Willsey and State, 2015). In an attempt to generate a coherent pathophysiological hypothesis of autism spectrum disorders (ASD) that considers both the diversity of implicated proteins as well as the range of observed behavioral phenotypes, I will focus on deficits within striatal circuitry. First I will examine how diverse theoretical concepts of striatal function may relate to key ASD symptom domains. Next, I provide evidence from both the clinical and experimental literature that suggests a pattern of core striatal dysfunction in ASDs. Finally, I will explore why the striatum might occupy such a central place in autism pathophysiology and how we might use this information to refocus our treatment endeavors.

The Implications of Studying Circuit Dysfunction in Neuropsychiatric Disease

From the outset, it is worthwhile considering the utility of exploring nervous system disease pathophysiology from the vantage of neural circuit dysfunction. This approach seeks to uncover alterations in defined, reproducibly interconnected sets of neurons that are responsible for discrete behavioral phenomenon seen in neuropsychiatric diseases such as schizophrenia (Spellman and Gordon, 2015), obsessive-compulsive disorder (Ahmari and Dougherty, 2015; Monteiro and Feng, 2016), and mood disorders (Fox and Kalin, 2014; Lammel et al., 2014). It does not attempt to link any of these complex diseases to a single region, as the mammalian brain is a massively interconnected structure whose full functional output certainly relies on coordinated and parallel processing between multiple areas. Nonetheless, the approach does seek to uncover specific circuit nodes for disease pathophysiology—neuronal connections that are uniquely vulnerable to genetic or environmental insult which also have a key role in regulating behavioral output. Alterations in these nodes may represent an initiating event that triggers subsequent downstream adaptations that together become the driver of abnormal behavior. My focus on the involvement of striatal dysfunction in this review by no means precludes the involvement of other brain regions. Rather, given the intersection between motor and cognitive abnormalities seen in ASD, the prefrontal cortex and cerebellum may represent equally susceptible terrain for the physiological alterations that drive behavioral changes (Fatemi et al., 2012; Martinez-Sanchis, 2014; Wang et al., 2014; Bicks et al., 2015; Chmielewski and Beste, 2015). The potential importance of these systems and their interactions with basal ganglia circuits will be considered in detail later.

The Basal Ganglia: an Evolutionarily Conserved Neural Circuit for Weighing Costs

The striatum is the input structure of the basal ganglia, a series of interconnected subcortical nuclei first appearing in the vertebrate lineage approximately 530 million years ago (Murray et al., 2011). While the overall anatomical organization and immunohistochemical composition of this region has remained largely unchanged dating back to anamniotes, connectivity with cortical circuitry has been significantly enhanced in mammalian lineages (Medina and Reiner, 1995; Reiner et al., 1998). From its inception, the basal ganglia has likely served as an essential intermediary between an organism and its outside environment. However, what began as a relatively simple structure linking incoming sensory information to regulation of motor output, has evolved into a complex circuitry capable of computing "cost-benefit" algorithms and selecting optimally efficient actions based upon incoming sensory information, previous memories, expectations and current motivational state (Hikosaka, 1998; Daw et al., 2006; Floresco et al., 2008). The proposed functions of basal ganglia circuits are in part derived from its extraordinary anatomical organization. The striatum receives a wealth of convergent excitatory projection inputs from motor and sensory cortex, hetero-modal association areas, thalamic nuclei, hippocampus, prefrontal cortical regions, insula, and amygdala (**Figure 1A**; Kelley et al., 1982; Gerfen, 1984; Malach and Graybiel, 1986; Voorn et al., 2004; Pan et al., 2010). These excitatory projections diffusely synapse onto D1 dopamine receptor expressing (D1R+) and D2 dopamine receptor expressing (D2R+) medium spiny neurons (MSNs) of the dorsal striatum, which differentially project via the direct pathway to the substantia nigra pars reticulata (SNr)/internal segment of the globus pallidus (GPi or entopeduncular nucleus in rodents) and via the indirect pathway to the external segment of the globus palliudus (GPe), respectively (**Figure 1B**; Gerfen et al., 1990; Surmeier et al., 1993; Kreitzer and Malenka, 2008; Gerfen and Surmeier, 2011). While both circuits eventually target motor regions of thalamus, the presence of an additional inhibitory connection in the indirect pathway is thought to account for the opposing effects of striatal medium spiny neuron subtype on thalamic output. Activation of direct pathway MSNs relieves thalamic inhibition and promotes motor output while activation of indirect pathway MSNs maintains pallidal inhibition of thalamus, reducing motor output. This long-standing model for the dichotomous function of striatal MSNs on movement, based originally on clinical observations (Albin et al., 1989), has recently been confirmed by cell type-specific optogenetic interrogation (Kravitz et al., 2010). Following modulation by midbrain nuclei, thalamic neurons project back to the same regions of cortex that initially targeted striatum, providing

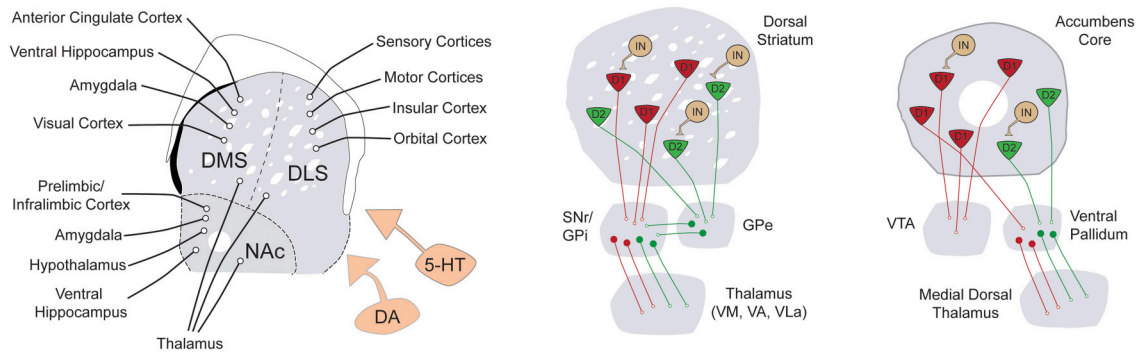

FIGURE 1 | Simplified schematic of the input and local connectivity of the mammalian basal ganglia. (A) Diagram of known excitatory inputs to distinct striatal sub-regions including dorsomedial striatum (DMS), dorsolateral striatum (DLS), and nucleus accumbens (NAc). All striatal sub-regions are under dopaminergic and serotonergic neuromodulatory control (orange). **(B)** Diagram of major dorsal striatal cell types and their downstream connectivity within basal ganglia circuits. In contrast to striatal inputs, nearly all cell types within the basal ganglia are inhibitory. The striatum is comprised of D1R+ (red) and D2R+ (green) medium spiny neurons (MSNs), as well as a smaller population of local circuit interneurons (tan). The direct pathway projection of D1R+ MSNs is diagrammed in red and the indirect pathway projection of D2R+ MSNs is drawn in green. **(C)** Diagram of the major cell types and connectivity of the nucleus accumbens core. Note that in contrast to the strict divergence of D1R+ and D2R+ MSNs in the dorsal striatum, D1R+ MSNs of the nucleus accumbens project both to the ventral tegmental area and the ventral pallidum.

sensory feedback control of ongoing behaviors (Bosch-Bouju et al., 2013). However, the seemingly "closed-loop" nature of these circuits is interrupted and expanded, perhaps through non-reciprocal cortico-thalamic pathways (McFarland and Haber, 2002) and spiraling dopaminergic inputs (Haber et al., 2000), to allow for iterative stages of cortico-striato-thalamic processing. In this manner, information from higher cortical areas involved in the cognitive aspects of action is transmitted to primary motor areas for the execution of specific motor output (McFarland and Haber, 2002). The functions of the basal ganglia are numerous and exist along a continuum framed on one end by sensorimotor control and on the other, by the generation of motivated and intentioned behaviors. While it is still largely speculative, extensive overlap between ASD symptomatology and striatal function seems apparent across this entire range (**Figure 2**).

FROM MOTOR CONTROL TO MOTIVATED BEHAVIORS: DIVERSITY OF BASAL GANGLIA FUNCTION AND ITS RELATION TO ASD SYMPTOMS

Sensory Control

While early vertebrate lineages utilized a simple neural network to transform sensory information into direct motor responses, vertebrate evolution has selected circuits of increasing complexity that extract more information from the environment and effectively use it to guide motor behaviors (Murray et al., 2011). Basal ganglia circuits, developing in parallel with pallial structures and midbrain dopamine nuclei, comprise a core computational unit enabling this more sophisticated control of motor output (Stephenson-Jones et al., 2011). To achieve this, the basal ganglia had to address two areas of increasing complexity in higher vertebrates—(1) the growth of incoming information from more specialized sensory systems (sensory-selection), and (2) the expanding number of motor responses that were possible in response (action-selection). The increasing diversity and convergence of sensory inputs in developing vertebrates necessitated a robust mechanism of sensory selection to extract context-relevant information (Hikosaka, 1998). Studies on the regulation of memory-guided saccades in primate suggest the basal ganglia serves this purpose by using its inhibitory connections to gate which sensory inputs regulate collicular output, and thereby select the environmental information guiding memory-based saccades (Hikosaka and Wurtz, 1985). Alternative examples of basal ganglia-mediated sensory control may be on display when animals dynamically adjust their level of attention to salient features of the environment. In a provocative model by Krauzlis and colleagues, attention does not occur via filtering of sensory representations within the neocortex, but rather as a byproduct of neural computations made by the basal ganglia to correctly determine internal state (Krauzlis et al., 2014). Here, the basal ganglia match motivational drives and previous task history with sensory information from the external environment, in hopes of achieving the most accurate assessment of current reality from which to make future decisions. The abrogation of ongoing motor activities by salient sensory stimuli may also be mediated through basal ganglia circuits. Specifically, thalamic projections to striatal cholinergic interneurons produce a brief period of reduced cortical drive to both MSN subtypes, followed by a period of enhanced sensitivity of the indirect pathway—a key mediator of motor suppression (Ding et al., 2010). Abnormalities in sensory processing are a widespread clinical feature of ASD and frequently manifest as abnormal reactivity to sensory aspects of the environment (Gomot et al., 2006; Kwakye et al., 2011; Elwin et al., 2013). In addition, patients

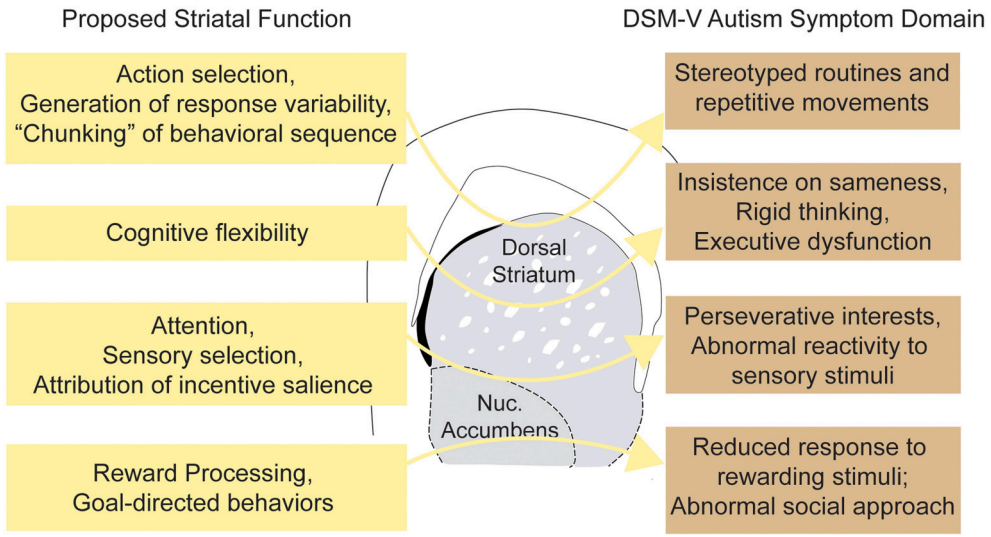

FIGURE 2 | Striatal dysfunction and major autism spectrum disorder symptom domains. Schematic illustrating hypothetical connections between abnormalities of striatal function and the varied clinical phenotypes observed in ASDs.

with autism exhibit robust deficits in the voluntary control of saccades (Minshew et al., 1999) and may use fronto-striatal neural circuits, typically reserved for higher cognitive processes, to compensate for broad sensorimotor deficiencies (Takarae et al., 2007). Finally, it will be worthwhile to explore whether dysfunction in regulating attentiveness to sensory stimuli may provide a substrate for the development of highly focused, fixated interests seen in ASD.

Action Selection and Motor Patterns

Basal ganglia circuits have also been hypothesized to function in action-selection, whereby a single behavioral output is selected and executed from a range of motor programs. The neural mechanisms mediating this selection process remain controversial but *in vivo* (Samejima et al., 2005; Kimchi and Laubach, 2009; Seo et al., 2012; Tai et al., 2012) and computational studies (Humphries et al., 2006; Lisman, 2014; Gurney et al., 2015) suggest the striatum and downstream basal ganglia nuclei have a central function. One hypothesis posits that distributed, synchronized extra-striatal excitation recruits specific MSN populations that subsequently release downstream basal ganglia pathways to initiate select motor programs. There is extensive convergence at the level of excitatory inputs to the striatum (roughly 10:1) as compared with downstream pallidal and thalamic nuclei, implying that initial processing for action selection occurs at striatal synaptic connections (Zheng and Wilson, 2002; Yim et al., 2011). Despite the large portion of striatal volume covered by many cortico-striatal axons, adjacent MSNs seem to sample unique excitatory inputs, thereby creating sparse striatal representations of cortical firing (Kincaid et al., 1998). Lateral inhibition from recurrent MSN collaterals and feed-forward inhibition from inhibitory interneurons may play a significant role in shaping the activity of neighboring striatal ensembles encoding alternative behaviors (Gage et al., 2010; Chuhma et al., 2011; Yim et al., 2011). Finally, interactions between downstream basal ganglia circuits may further reinforce the striatal selection process (Gittis et al., 2014).

Another proposed function of basal ganglia circuits, and the dorsal striatum in particular, is to encode short motor programs (so-called "chunking" of action repertoires), which can prevent excessive computational demands on cortical structures (Graybiel, 1998). These short motor programs can then be linked together in the dorsal striatum to increase the complexity of motor output (Yin, 2010). When functioning properly, the aforementioned systems should permit efficient selection and assembly of motor programs. However, when dysfunctional, these same networks may be prone to driving the repetitious, automated behavioral patterns frequently observed in ASD. Despite an array of documented striatal morphological abnormalities (see following Section Clinical Indications for Striatal Involvement in ASD), human imaging studies can only suggest a correlation between restricted, repetitive motor output and striatal changes. A small supporting body of evidence comes from two structural magnetic resonance imaging (MRI) studies—the first highlighted a correlation between growth of the caudate nucleus and repetitive behaviors (specifically "resistance to change") in a longitudinal study of preschool-age children (Langen et al., 2014) and another correlating caudate and putamen volumes with global repetitive behavior metrics (Hollander et al., 2005). Further, research will be needed to assess whether other autism-relevant repetitive symptoms, including motor stereotypies or speech abnormalities, are associated with alterations in basal ganglia morphology.

Reward-Guided Behaviors

The midbrain dopamine system has developed together with the sensorimotor circuitry of the basal ganglia to dramatically enhance the manner in which rewards bias an animal's

behavior (Murray et al., 2011). Dopamine signaling can increase behavioral efficiency both through its actions on sensory-selection mechanisms (detecting pertinent cues; Berridge, 2007) and action-selection mechanisms (selecting previously rewarded behaviors; Schultz, 2013), although the specific neuronal mechanisms remain controversial. One hypothesis is that striatal interactions with the dopamine system selectively reinforce associations between an environmental cue, a specific response and an outcome to create an internal representation of an animal's action and its consequences. This template could then be used for guiding adaptive behaviors when contingencies change or as a foundation upon which commonly rewarded activities could become automated (Liljeholm and O'Doherty, 2012; Rueda-Orozco and Robbe, 2015).

In rodents, the striatal systems mediating these functions are thought to be segregated, with the dorsal medial striatum (roughly analogous to the caudate in humans) supporting goal-directed behavioral responding, the dorsal lateral striatum (analogous to the putamen) supporting automated behaviors and the nucleus accumbens mediating motivational states and reward processing (Yin and Knowlton, 2006; Balleine and O'Doherty, 2010; Floresco, 2015). In addition, both the dorsal medial striatum and nucleus accumbens are key neural circuits for maintaining flexible behavioral responding under changing reward contingencies (Kehagia et al., 2010). Each striatal domain receives discrete excitatory projections (Pan et al., 2010) and dopaminergic innervation (Lerner et al., 2015) believed to support its specific processing functions, and abnormal coordination between these domains is believed to underlie behavioral control deficits in several neuropsychiatric diseases, including OCD and substance abuse (Voorn et al., 2004; Pan et al., 2010; Russo et al., 2010; van den Heuvel et al., 2010; Ahmari et al., 2013; Burguière et al., 2015). Imaging studies and psychological testing have documented discrete reward-processing deficits in ASD patients, both for social and monetary rewards (Kohls et al., 2013). These abnormalities may contribute to the widespread deficits in motivation and incentive-based learning that are observed clinically (Kohls et al., 2012). Furthermore, a range of deficits in executive function have been observed in high-functioning autistic patients, including alterations in response inhibition, planning, and behavioral flexibility (Pennington and Ozonoff, 1996; Geurts et al., 2004; Hill, 2004; Shafritz et al., 2008). In contrast, other striatal-based paradigms, such as the acquisition of basic operant performance and the ability to coordinate goal-directed and habitual behavioral control seem unchanged (Geurts and de Wit, 2014). Taken together, it seems likely that the profound deficits in social approach and rigid behavioral patterns that typify ASD may stem in part from specific abnormalities in striatal-based reward processing.

The Creation and Modulation of Behavioral Variability

One final consideration with particular relevance to ASD symptomatology is the proposed function of the striatum as a generator of behavioral variation. A wealth of information on the development and context-dependent modulation of highly stereotyped motor output has come from work on a dedicated cortico-basal ganglia circuit that regulates bird-song variability, the anterior forebrain pathway (Fee and Goldberg, 2011). During "practice singing" in isolation, a male's exploration of different song renditions is mediated by variable basal ganglia firing downstream of synchronized striatal output (Woolley et al., 2014). In contrast, song directed at potential female mates is precise—a byproduct of a more stereotyped basal ganglia firing pattern, which may result from cue-dependent increased dopamine release within striatum (Gale and Perkel, 2005; Leblois et al., 2010). While it is currently unclear if the mammalian striatum is similarly involved in the regulation of behavioral variability, it is easy to see how deficits in this function could contribute to the restricted behavioral output observed in ASD.

CLINICAL INDICATIONS FOR STRIATAL INVOLVEMENT IN ASD

Early clinical evidence for striatal involvement in ASD came from widespread "disturbances of motility" noted in neurological testing (Damasio and Maurer, 1978; Maurer and Damasio, 1982). Autistic patients displayed classical neurologic signs of basal ganglia dysfunction including dystonia of the extremities and "striatal toes"—a Babinski-like spontaneous reflex. In addition to involuntary choreoathetoid movements and postural changes, bradykinetic abnormalities were also common, resulting in significant delays in the initiation, modulation and halting of motor output (Maurer and Damasio, 1982). In a cohort of 154 children with ASD from ages 2 through 7, the prevalence of motor abnormalities was substantial, with 51% exhibiting dystonia and 34% motor apraxia (Ming et al., 2007). Magnetic resonance imaging (MRI) studies exploring disease-related changes in striatal volume found evidence for alterations in caudate size both in children and adult ASD patients (Sears et al., 1999; Langen et al., 2007, 2009; Estes et al., 2011). An unbiased meta-analysis of voxel-based morphometric studies taken from the current autism literature has similarly highlighted the basal ganglia as a brain region with consistent structural alteration (Nickl-Jockschat et al., 2012). Alternative approaches have employed functional-MRI to investigate task-specific patterns of striatal activity in ASD patients and uncovered decreased responsiveness during paradigms assaying social reward processing (Delmonte et al., 2012; Kohls et al., 2013) and cognitive flexibility (Shafritz et al., 2008). With regard to the functional connectivity of ASD brains, there is evidence suggesting an increased connectivity between the caudate nucleus and a range of autism-relevant cortical areas, including prefrontal, premotor and parietal areas, observed both in resting-state and task-specific paradigms (Turner et al., 2006; Di Martino et al., 2011).

The abundance of clinical and imaging evidence, when considered together with the diversity of striatal function, presents a compelling argument for a central role of striatal circuits in ASD pathophysiology (see **Table 1** for summary). Nonetheless, our understanding of pathophysiological mechanisms ultimately relies on the ability to manipulate

TABLE 1 | Brief summary of clinical evidence for the involvement of striatal circuits in ASD pathophysiology.

References	Population	Methodology	Conclusion
Damasio and Maurer, 1978; Maurer and Damasio, 1982	unclear	Neurologic assessment	Classical neurologic signs of basal ganglia dysfunction, including "striatal toes," choreoathetoid movements, postural changes, and bradykinesia
Sears et al., 1999	12-29 YO	Structural MRI	Enlargement of caudate observed in autism patients; caudate volume associated with compulsions and rituals
Geurts et al., 2004	6-12 YO	Cognitive testing	Children with high-functioning autism exhibited deficits across multiple executive function domains
Turner et al., 2006	15-39 YO males	Functional connectivity MRI	Autism cases displayed decreased functional connectivity in caudate circuits, despite diffusely enhanced connectivity in pericentral regions
Langen et al., 2007	Children and adolescents	Volumetric MRI	Caudate enlargement observed in medication-naïve subjects
Langen et al., 2007	6-25 YO	Structural MRI	Caudate increased in volume with age in autism
Shafritz et al., 2008	Young adult	fMRI	High-functioning autistics had reduced activation in frontal, striatal and parietal regions, as well as lower accuracy on response-shift trials
Estes et al., 2011	3-4 YO	MRI	Enlargement of left and right putamen, left caudate observed in ASD cases
Di Martino et al., 2011	7-13 YO	Functional connectivity MRI	Children with ASD exhibited enhanced striatal connectivity with heteromodal associative and limbic cortices
Delmonte et al., 2012	Teenage males	fMRI	ASD cases showed reduced activation in the dorsal striatum during the receipt of social rewards but normal activation for the receipt of monetary rewards
Nickl-Jockschat et al., 2012	Meta-analysis	MRI	Unbiased meta-analysis of brain structure changes across multiple MRI studies using voxel-based morphometry shows that basal ganglia is significantly affected
Kohls et al., 2013	Teenage males	fMRI	NAc hypo-activation for monetary but not social reward; Amygdala and anterior cingulate cortex hypoactivation for both types of reward
Langen et al., 2014	Preschool children	Structural MRI	Correlation between growth of caudate nucleus and repetitive behaviors (measured as "resistance to change")

systems to test causality. The modeling of ASDs in rodents has largely been pursued through environmental and genetic models, with a focus on construct validity (the disease relevance of how a model was generated) and face validity (how the model recapitulates disease behaviors and pathology; Nestler and Hyman, 2010). Environmental models have been essential in the generation of experimental ASD rodents while the discovery of causal genetic factors was still on the horizon. For example, both prenatal exposure to valproic acid and models of maternal infection can cause social and motor phenotypes that are consistent with abnormalities seen in ASD patients (Arndt et al., 2005). However, our limited understanding of environmental contributions to ASD pathogenesis has severely limited the construct validity of these approaches (McOmish et al., 2014). Genetic modeling in mice has provided alternative disease models whose construct validity rests largely on the quality of genetic association data that serves as the starting point for functional analysis.

STRIATAL DYSFUNCTION IN MOUSE GENETIC MODELS OF ASD

The parallel revolutions in genetic sequencing technologies and genome editing have provided an unparalleled opportunity to explore causality between mutations and aberrant behaviors in genetically tractable systems such as mice. Examinations of copy number variations (CNVs), together with whole-exome and genome sequencing, has demonstrated a substantial amount of genetic heterogeneity underlying ASD etiology, with some estimates predicting that 300–800 loci will eventually be associated with increased risk for ASD (O'Roak et al., 2012). The diversity of documented ASD-associated mutations includes syndromic mutations, rare alleles of larger effect size, de-novo CNVs and more common mutant alleles of smaller phenotypic penetrance (Krumm et al., 2014; Willsey and State, 2015). Genetic modeling of ASD in mice has largely focused on syndromic mutations and rare alleles, although the generation of CNV models has recently gained traction (Nakatani et al., 2009; Horev et al., 2011; Portmann et al., 2014). My goal here is not to exhaustively review each mouse ASD model, but instead provide an overview of the physiological and behavioral phenotypes resulting from mutations in a range of genes with solid genetic association to ASD. When considered together, I believe these data begin to make a strong case for primary striatal deficits in the pathogenesis of ASD (**Figure 3**).

Fragile-X Mental Retardation Protein

The Fragile-X Mental Retardation Protein (FMRP), a key repressor of translation at central synapses, has been a central

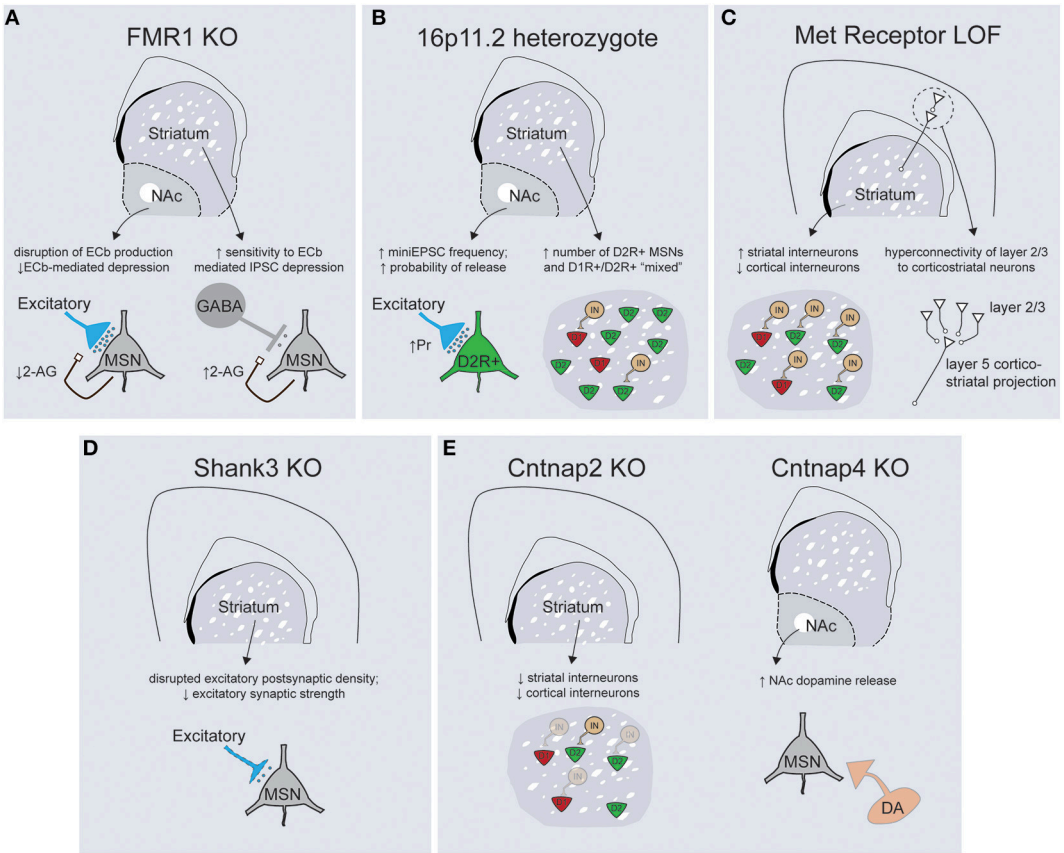

FIGURE 3 | Mouse models of ASD-associated genes display abnormalities in striatal structure and function. (A) FMR1 KO mice display abnormalities in the regulation of endocannabinoid signaling within the striatum, with decreased 2-arachidonylglycerol (2-AG) production in the NAc leading to altered plasticity of excitatory inputs (left) and increased 2-AG production in the dorsal striatum enhancing depression of inhibitory transmission onto dorsal striatal MSNs (right). **(B)** 16p11.2 heterozygotes exhibit an increase in excitatory synaptic transmission onto D2R+ MSNs of the NAc (left) while both the dorsal and ventral striatum have larger overall numbers of D2R+ MSNs (right). **(C)** Loss of Met receptor function within ventral telencephalic progenitors leads to an increase in the number of parvalbumin and somatostatin-positive striatal interneurons at the expense of cortical interneuron populations (left). Met receptor KO mice display enhanced connectivity of superficial layer cortical neurons that synapse on corticostriatal projection neurons (right). **(D)** Shank3 KO mice exhibit gross abnormalities in synaptic structure, alterations in the protein architecture of synapses and a decrease in general excitatory synaptic strength within the dorsal striatum. (**E**, left) Cntnap2 KO mice exhibit deficits in migration of interneuron progenitors such that striatal interneuron populations are decreased. Cntnap4 KO mice demonstrate enhanced release of dopamine specifically within the nucleus accumbens (right).

model for exploring the pathogenesis of mental retardation and accompanying developmental disorders including autism (Danish-Belgium Fragile-X Consortium, 1994; Reiss et al., 1995; Bear et al., 2004). While this protein exerts widespread control of mRNA translation, many of the neuropsychiatric-related symptoms seen in Fmr1 KO mice result from abnormal activation of the mGluR5 metabotropic glutamate receptor, as evidenced by the striking behavioral rescue achieved in Fmr1 KO; mGluR5 heterozygote mice (Dölen et al., 2007). While initial studies described a role for Fmr1 in regulating long-term synaptic plasticity at hippocampal synapses (Huber et al., 2002), it has subsequently been implicated in synaptic dysfunction across multiple brain regions, including cingulate cortex, amygdala, and neocortex (Patel et al., 2013; Martin et al., 2014; Koga et al., 2015). The idea that fronto-striatal circuit dysfunction is critical to specific domains of behavioral dysfunction seen in Fragile X patients is suggested by deficits of response inhibition and abnormal patterns of task-related activity in anterior cingulate cortex and striatum (Menon et al., 2004). Interestingly, this study also noted that responses in the ventrolateral prefrontal cortex and the striatum were correlated with the levels of Fmr1 gene expression. The importance of fronto-striatal dysfunction has received additional support from recent cognitive studies in the Fmr1 mouse model demonstrating abnormalities in visuospatial discrimination and extinction of instrumental responses (Krueger et al., 2011; Sidorov et al., 2014). Consistent with the importance of fronto-striatal circuits, synaptic analysis of Fmr1 KO mice has demonstrated alterations in both excitatory and inhibitory synaptic transmission within the ventral and dorsal striatum, respectively (**Figure 3A**; Centonze et al., 2008; Jung et al., 2012). In the dorsal striatum Fmr1 KOs display an increased sensitivity to endocannabinoid-mediated depression of inhibitory transmission onto MSNs, while in the nucleus accumbens a form of endocannabinoid-mediated

depression of excitatory transmission is disrupted (Centonze et al., 2008; Jung et al., 2012). These studies illustrate how a common mutation may yield similar net circuit effects (less inhibition of striatal MSNs) through region-specific physiological mechanisms. Nevertheless, the widespread nature of physiological dysfunction that occurs downstream of perturbations to this global regulator of translation makes it currently unclear what specific role striatal dysfunction might play in the diverse behavioral changes documented in Fmr1 KO mice.

Mouse Models of the 16p11.2 Human CNV

Copy number variations on chromosome 16p11.2 are one of the most common sequence abnormalities associated with ASD (Weiss et al., 2008). Deletions of this region cause a range of phenotypes in addition to autism, including language delay, seizures, cognitive impairments and attention-deficit hyperactivity disorder, while duplications are associated with schizophrenia. The 16p11.2 chromosomal region contains 26 genes whose orientation in humans is perfectly conserved on chromosome 7 in mice, allowing faithful genetic modeling (Horev et al., 2011; Portmann et al., 2014). Consistent with the large size of genetic insult, 16p11.2 heterozygote mice displayed gross abnormalities in brain morphology and size. In particular, rostral striatum, nucleus accumbens, globus pallidus, medial cortical structures, and thalamus all exhibited enlargement, suggestive of a coordinated increase in the morphological footprint of basal ganglia circuitry. Detailed anatomical analysis revealed evidence for changes in medium spiny neuron specification, with increased overall numbers of D2R+ MSNs as well as a larger fraction of spiny neurons with "mixed" D1R+ and D2R+ phenotypes. Furthermore, there was a dramatic increase in the net excitatory strength onto D2R+ MSNs secondary to a cell-type specific increase in the presynaptic probability of neurotransmitter release (Portmann et al., 2014). All together, these changes should act to enhance output from the indirect pathway in both the dorsal and ventral striatum, although the resulting changes at the circuit level should be interpreted with caution in the face of what seems to be significant abnormalities in neural specification and development (**Figure 3B**). Further work is necessary to conclude whether these striatal circuit alterations play a causal role in the numerous motor behaviors documented by this study, as 16p11.2 deletions have also been associated with cortical dysplasia and aberrant hippocampal mGluR signaling (Pucilowska et al., 2015; Tian et al., 2015).

Met Receptor Signaling

The Met receptor tyrosine kinase binds its ligand, hepatocyte growth factor, and mediates numerous signaling events essential for development of epithelial populations including proliferation, differentiation, and trophic support. In the developing nervous system, Met is expressed in proliferative progenitor zones and maintained during neuronal migration and integration, suggesting Met signaling is essential for early neuronal specification (Powell et al., 2001). Consistent with this, mutations in the Met receptor have been associated with both autism and Tourette's syndrome (Martins et al., 2011; Peng et al., 2013). The role that Met dysfunction plays in the pathophysiology of these disorders is unclear, but targeted genetic dissection has demonstrated abnormalities at multiple levels of cortico-striatal circuitry (**Figure 3C**). Using two-photon glutamate uncaging to explore circuit-specific synaptic connectivity, it was shown that cortical disruption of Met function caused hyper connectivity of cortical layer 2/3 neurons specifically onto layer 5 cortical neurons that projected into the striatum (Qiu et al., 2011). Cortical-specific Met disruption also resulted in a non-cell autonomous increase in the total dendritic arbor length and spine volume of striatal MSNs (Smith et al., 2012). An alternative approach that removed Met signaling from ventral neural progenitors produced abnormalities in the proper distribution of inhibitory interneurons, such that the number of parvalbumin- and somatostatin-positive interneuron subtypes was increased in the striatum at the expense of cortical populations (Martins et al., 2011). It is difficult to estimate what the net circuit effect of these seemingly opposing alterations might be—while cortical Met disruption should enhance cortico-striatal network output, too little is known about the putative functions of striatal interneurons to extrapolate the result of enhanced local inhibition on overall striatal processing (Gage et al., 2010; Yim et al., 2011). Nonetheless, further work is necessary to directly attribute alterations in corticostriatal pathways to the deficits in procedural and reversal learning seen with Met genetic loss-of-function (Martins et al., 2011).

The Shank Gene Family

Shank proteins are a central component of the postsynaptic density that function to scaffold the large protein networks associated with excitatory synapses. While all three Shank family members are associated with ASD, the strongest genetic evidence exists for Shank-3, whose location maps to the critical region for Phelan-McDermid syndrome—a disorder characterized by intellectual disability, autistic behaviors and hypotonia (Phelan, 2008). Understanding how Shank-3 mutations cause neuropsychiatric disease has been complicated by the molecule's complex structure, which employs multiple transcriptional start sites to generate proteins with numerous adhesion domains (Jiang and Ehlers, 2013). Genetic alterations have been detected throughout the Shank-3 gene and the variability of resulting phenotypes provides insight into the relative importance of the specific adhesion domains that are disrupted. For example, mice in which the upstream ankyrin repeats are affected have specific reductions in the Shank3α protein isoform and relatively mild behavioral phenotypes compared to mutations in the downstream PDZ domain, which eliminate the α, β, and γ isoforms and produce significant increases in grooming behaviors and reductions in social interactions (Peca et al., 2011). Biochemical and physiological experiments focused on striatum because of the severe grooming phenotype and the high levels of Shank-3 mRNA expression selectively within this structure. These analyses demonstrated truncated postsynaptic density structure along with broad reductions in key scaffolding proteins and neurotransmitter receptors (Peca et al., 2011). Consistent with this, Shank-3 KOs have large reductions in cortico-striatal excitatory synaptic transmission, although whether there is

cell-type or input specificity to this deficit remains unclear (**Figure 3D**). Despite the smaller number of Shank-2 mutations associated with ASD, two distinct mouse models of Shank-2 display social deficits and abnormalities of motor output despite exhibiting opposite hippocampal synaptic phenotypes (Schmeisser et al., 2012; Won et al., 2012).

Contactin Associated Proteins

Contactin associated proteins are transmembrane molecules of the neurexin superfamily that have been linked to ASD and epilepsy through numerous human genetic approaches (Strauss et al., 2006; Alarcón et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008). Contactin associated protein-like 2 (Cntnap2) is implicated in neuron-glial interactions and clustering of potassium channels at the nodes of myelinated axons (Poliak et al., 1999). Recent work in cultured cortical neurons has also demonstrated a potential developmental role in the elaboration of dendritic arbors and development of synaptic spines (Anderson et al., 2012). Mouse models of Cntnap2 loss-of-function display stereotypic movements, behavioral inflexibility, social and communication deficits, as well as seizures (Peñagarikano et al., 2011). While the underlying neural mechanisms of these behaviors remain unclear, there are widespread abnormalities in the migration of inhibitory interneurons, leading to a decrease in both cortical and striatal interneuron populations (**Figure 3E**; Peñagarikano et al., 2011). Given the phenotypes of the Met receptor (Martins et al., 2011) and Cntnap2 KOs, further studies are needed to explore whether alterations in striatal interneuron development represent a common causal factor for ASD pathogenesis. Cntnap4 is a closely related family member that has been associated with neuropsychiatric disease whose expression is restricted to parvalbumin-positive interneurons and tyrosine hydroxylase positive midbrain dopamine neurons in the substantia nigra pars compacta and ventral tegmental area (Karayannis et al., 2014). Cntnap4 protein is expressed presynaptically and KO mice display diverse synaptic phenotypes including a reduction in cortical GABAergic tone and an increase in release of dopamine specifically within the nucleus accumbens (**Figure 3E**). The perseverative grooming displayed by Cntnap4 KOs is lessened by systemic administration of the dopamine D2 receptor antagonist haloperidol, suggesting that increased dopaminergic tone is in part responsible for the observed motor control abnormalities (Karayannis et al., 2014). Taken together, functional data from mutations in the Cntnap family suggest widespread abnormalities in inhibitory function and focal changes in local dopaminergic release. Whether, these changes converge at the level of striatal circuits is an interesting future question.

DIRECTLY EXPLORING THE LINK BETWEEN GENE DYSFUNCTION, CIRCUIT ABNORMALITIES AND ASD-RELEVANT BEHAVIORS

Taken together, these studies demonstrate that the introduction of ASD-associated mutations into mice causes dysfunction of striatal structure and function. However, an equally compelling case could be made for ASD-associated abnormalities in hippocampal CA1 neurons, a cell type in which many autism-associated mutations have been screened due to their well-characterized connectivity and anatomy (Etherton et al., 2011; Peñagarikano et al., 2011). In fact, most of the aforementioned genes produce physiological changes in multiple brain regions. To further our understanding of ASD pathophysiology, it is necessary to move beyond the basic concept that ASD-associated genes cause physiological abnormalities to instead explore which behaviorally relevant neural circuits are changed and how those networks function to normally regulate behavior (Fuccillo et al., 2016). Two recent studies have tackled this issue by employing viral and genetic dissection of gene function toward the goal of a circuit-based understanding of ASD-relevant behavioral abnormalities (**Figure 4**; discussed in Section Striatal Oxytocin Function in Social Reward and Striatal Neuroligin-3 Dysfunction Boosts Repetitive Behaviors; Dölen et al., 2013; Rothwell et al., 2014).

Striatal Oxytocin Function in Social Reward

Abnormalities in reward processing likely contribute to the widespread deficits in social engagement and communication seen in ASD patients. In support of this, human imaging studies have demonstrated reduced neural activity throughout corticostriatal circuits in response to a variety of social rewards (Kohls et al., 2012). To better understand the circuit mechanisms of social behaviors, a conditioned place preference assay (similar to that used to examine the rewarding properties of drugs of abuse) was employed to quantify a preference for contexts associated with grouped vs. isolation housing (Panksepp and Lahvis, 2007; Dölen et al., 2013). Using this approach, the function of oxytocin, an ancestral neuropeptide known to regulate affiliative behavior across many species, was mechanistically examined. Alterations in the oxytocin promoter have been associated with ASD and intra-nasal oxytocin delivery is currently being explored as a treatment option to enhance pro-social behaviors (Yamasue et al., 2012). Through a combination of acute slice electrophysiology and behavioral pharmacology, two interesting but seemingly disconnected effects of oxytocin were demonstrated in mice—(1) bath application of oxytocin induced a long lasting depression of excitatory synaptic transmission onto MSNs of the nucleus accumbens, and (2) blockade of oxytocin signaling in the NAc inhibited the formation of a preference for social cues (Dölen et al., 2013). Viral-mediated circuit dissection of oxytocin receptor (Oxtr) function demonstrated that both the synaptic plasticity and the social preference behavior depended on oxytocin-ergic signaling in dorsal raphe inputs to the NAc, a major source of striatal serotonin.

Through a series of experiments, a unifying mechanism emerged in which oxytocin signaling regulated the release of serotonin in the nucleus accumbens, which subsequently modulated excitatory synaptic strength by acting at presynaptic serotonergic receptors on excitatory afferent fibers (**Figure 4A**). Despite the complexity of interactions across several brain regions, it is clear that the nucleus accumbens is the final locus at which this modulation of social reward occurs, as both oxytocin and serotonin-1B (Htr1b) receptor blockade in the accumbens abrogate the social preference *in vivo*. Questions remain

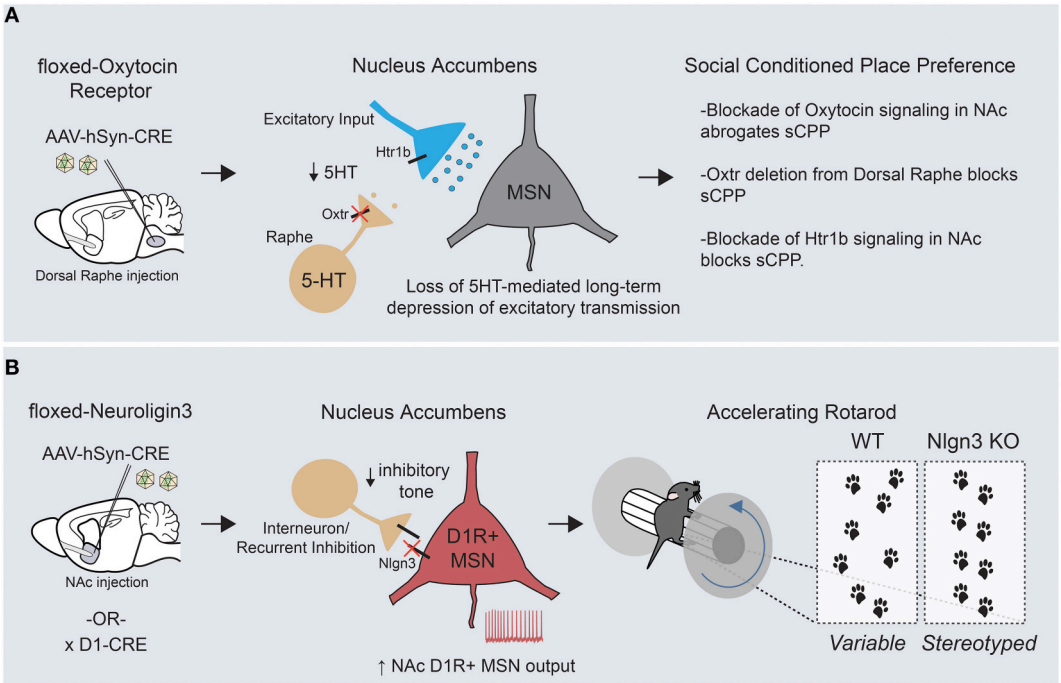

FIGURE 4 | Circuit-specific analysis of ASD-related behaviors points toward an underlying striatal deficit. (A) Targeted removal of oxytocin receptor within the dorsal raphe demonstrates a key role for this molecule in formation of social conditioned place preference, a measure of social reward. Oxytocin receptor functions on dorsal raphe terminals within the nucleus accumbens to regulate the release of serotonin, which can hetero-synaptically modulate excitatory transmission onto MSNs through presynaptic Htr1b receptors. **(B)** Removal of Neuroligin-3, a synaptic adhesion molecule associated with ASD, from D1R+ MSNs of the nucleus accumbens is sufficient to drive the enhanced formation of repetitive motor routines, as assayed by learning on the accelerating rotarod (KOs display an increased and earlier stereotyped pattern of foot placements compared with WTs). In addition, Neuroligin-3 KO mice have a cell type- specific deficit in inhibitory transmission onto accumbens D1R+ MSNs, which presumably leads to an increase in output from this circuit.

regarding how these long-term changes in accumbal excitatory transmission ultimately regulate social reward. Nonetheless, these experiments have made the first inroads into understanding the synaptic basis of social reward processing and furthermore offer potential mechanistic clues to the function of two ASD-associated genes (Oxtr and Htr1b) in the development of ASD-associated behaviors.

Striatal Neuroligin-3 Dysfunction Boosts Repetitive Behaviors

Applying a similar "circuit dissection" approach, we attempted to explore how ASD-associated mutations could promote development of the restricted and repetitive behaviors so frequently observed in ASD patients (Rothwell et al., 2014). To do so, we employed the accelerating rotating rod (rotarod) as a potential behavioral endophenotype for the formation of motor routines. To assess the validity of this approach, we used video-capture foot tracking of mice during standard rotarod training to demonstrate that improved motor performance was tightly linked to increasingly stereotyped location and timing of hind-paw placement. We then investigated this behavior in two distinct mouse lines mutant for Neuroligin-3, a synaptic adhesion molecule associated with ASD (Jamain et al., 2003; Sanders et al., 2011). Interestingly, both Neuroligin-3 KO and Neuroligin-3 R451C point mutant mice demonstrated enhanced learning on the rotating rod and a more rapid stereotyping of their paw placement. This finding is of great significance given that several genetic ASD models have a similar enhancement of rotarod performance (Kwon et al., 2006; Nakatani et al., 2009; Etherton et al., 2011; Peñagarikano et al., 2011). Using a series of viral and genetic approaches, Neuroligin-3 was removed from discrete circuits and cell types in an attempt to delineate where Neuroligin-3 dysfunction was crucial for enhancing rotorod learning and other stereotyped behaviors. Surprisingly, our data suggested that deletion of Neuroligin-3 in D1R+ MSNs of the mature nucleus accumbens was sufficient to generate the behavioral phenotypes seen in two separate Neuroligin-3 autism models. Complementary acute slice analyses demonstrated a deficit in inhibitory synaptic transmission specifically onto D1R+ MSNs of the accumbens, which altered the neuronal balance between excitation and inhibition in this circuit element (**Figure 4B**).

To prove that altered nucleus accumbens MSN output can modulate rotarod acquisition, we employed cell type-specific suppression of neuronal activity through targeted stereotaxic injection of a Cre-dependent virus expressing the inwardly-rectifying potassium channel, Kir2.1. These experiments demonstrated that decreasing excitability of D1R+ and D2R+ MSNs could bi-directionally modulate rotarod learning, and strongly suggests that the synaptic dis-inhibition of accumbal

D1R+ MSNs is a causal physiological event in the promotion of repetitive motor behaviors in Neuroligin-3 mutant mice. How exactly changes in nucleus accumbens D1R+ MSN output help to organize motor patterns is intriguing given that these programs are likely executed elsewhere within cortico-striatal circuits. Along these lines, it is important to note the critical differences in downstream connectivity recently reported between medium spiny neuron subtypes of the dorsal vs. ventral striatum (Kupchik et al., 2015; see **Figure 1C**). Whereas D1R+ MSNs of the dorsal striatum project exclusively through the direct pathway to the midbrain, D1R+ MSNs of the nucleus accumbens send inhibitory projections to both mesencephalic dopamine centers and the ventral pallidum, the main target site of D2R+ accumbal MSNs. This distinction suggests that there are two possible downstream pathways through which alterations in D1R+ MSN output could shape repetitive behaviors. Together with the aforementioned oxytocin study, these results begin to show a clustering of causal ASD-related neural dysfunction within the striatum. Further work will be necessary on both fronts to better understand how these circuit abnormalities interact with global brain function to alter behavior.

CAN UNDERSTANDING STRIATAL CIRCUIT REGULATION OF BEHAVIOR HELP GENERATE HYPOTHESES OF ASD PATHOPHYSIOLOGY?

Given the recent evidence that striatal dysfunction has a causal role in ASD-related behaviors, a more comprehensive understanding of how specific striatal circuits mediate behavioral control may aid our attempts to forge mutation-behavior correlates in rodent disease models. Lesion studies, pharmacological manipulations and more recently, optogenetic/pharmacogenetic interrogation of striatal circuits has begun to create a cellular and synaptic understanding for the selection and reinforcement of particular behavioral patterns.

Striatal Regulation of Flexible and Automatic Behavioral Responses

Striatal function is believed to support both flexible, goal-directed behaviors as well as more automated responding, in an attempt to enhance overall behavioral efficiency. How these two systems interact to shape behavior is of considerable importance given their widespread dysfunction across multiple neuropsychiatric disorders. Lesion studies in rodents have attributed goal-directed responding to dorsomedial striatal function, as disruptions of this territory generate rigid behavioral patterns that are insensitive to reward devaluation (Yin et al., 2005). Furthermore, acquisition of goal-directed actions is correlated with bidirectional synaptic plasticity within the dorsomedial striatum, with enhanced AMPA receptor mediated synaptic transmission onto D1R+ MSNs and decreased excitatory synaptic drive onto D2R+ MSNs (Shan et al., 2014). These data reinforce a basic circuit logic whereby enhanced drive onto D1R+ MSNs serves to boost selected behaviors while decreased activation of D2R+ MSNs allows for the removal of an inhibitory brake. Finally, dorsomedial striatal MSNs also seem to have a role in encoding the net expected return of a given task, and modulating response vigor accordingly (Wang et al., 2013). This regulation of task effort may prove integral to the function of the dorsomedial striatum in shaping aspects of reward-sensitive associative learning.

Lesion studies of the rodent dorsolateral striatum suggest that this domain is necessary for the formation of habits—automated, sensory-driven responses that are insensitive to changes in reward value or contingency (Yin et al., 2004; Yin and Knowlton, 2006). In addition, inactivation of the dorsolateral striatum after the establishment of habitual responding causes a reversion to more reward-sensitive behavioral output, suggesting either parallel or antagonistic interactions between these two systems. The neural mechanisms that coordinate reward-directed flexibility and fixed motor responding have received little attention but are likely of significant importance to ASD pathology. Unfortunately, a strict anatomical segregation of these two processes is unlikely, as evidenced by state-related MSN activity in both dorsomedial and dorsolateral striatal compartments during goal-directed and habitual responding (Gremel and Costa, 2013).

Intrinsic Striatal Circuits and Goal-Directed Learning

Traditional models of striatal function are grounded in the proposed dichotomy of striatal medium spiny neuron subtypes, with D1R+ MSN activity important for initiating movement and D2R+ MSNs essential for suppressing actions. While the precise temporal sequence and coordination of MSN subtypes during activity remains unclear (Cui et al., 2013), this theoretical framework has been a powerful tool for progress in understanding striatal motor function (Kravitz et al., 2010). Recent work has attempted to explore whether these striatal cell types have analogous functions in the regulation of reinforced actions. Mice expressing channelrhodopsin in either direct or indirect MSNs of the dorsomedial striatum were allowed to lever press to receive optogenetic activation of either circuit component (Kravitz et al., 2012). In this optogenetic variant of self-stimulation, it was noted that stimulation of D1R+ MSNs resulted in persistent operant reinforcement that was maintained across training session, while D2R+ MSN stimulation caused a transient aversive state within each session. Surprisingly, both cell-type specific modulations of behavior occurred in the presence of systemic dopamine antagonists, suggesting that the behavioral plasticity was occurring through alternative mechanisms. Another study focused specifically on direct pathway neurons of the dorsomedial striatum, employing DREADDs technology to increase or decrease G-protein coupled signaling in this cell type during the acquisition of a high vs. low reward-discrimination task (Ferguson et al., 2013). While these manipulations had no real-time effect on reward preference or task acquisition, they were able to bi-directionally modulate the encoding of strategies for subsequent trials. A comparison between these optogenetic and pharmocogenetic manipulations of MSN activity demonstrates how distinct temporal windows of

activity within the same neural circuit can mediate diverse aspects of behavioral control.

Similar cell type-specific functions have been proposed for MSN cell types within the nucleus accumbens, as demonstrated by a cell type-specific block of synaptic transmission through regulated viral expression of tetanus toxin (Hikida et al., 2010). Using locomotor sensitization and conditioned-place preference behaviors to explore MSN contributions to psycho-stimulant exposure, the authors suggest that D1R+ MSNs mediate a broad set of associative reward functions. In contrast, D2R+ MSNs were essential for the aversive response to foot-shocks seen in the inhibitory avoidance task. A separate study exploring the transition to compulsive cocaine use during self-administration paradigms suggests that increased synaptic strength onto D2R+ MSNs of the nucleus accumbens was associated with resilience to chronic cocaine use (Bock et al., 2013). These results argue that the indirect pathway may shape behaviors by curtailing unwanted or maladaptive efforts. In fact, the interpretation that D2R+ MSN activity encodes aversive states may reflect this cell type's function in suppression of action associated with non-rewarded or aversive contexts. When taken together, the data thus far suggest that D1R+ MSNs in the NAc and associative striatum have a key role in goal-directed actions and learning, while D2R+ MSNs in these regions may encode either aversive states or mediate the inhibition of specific behaviors associated with these states.

Striatal Circuit Modulation and Flexible Behavioral Responding

Current theories of striatal function highlight the importance of afferent projection neurons and neuromodulatory populations in controlling the final output of basal ganglia circuits, however few studies have functionally tested these assumptions (Mathur and Lovinger, 2012; Tritsch and Sabatini, 2012; Wall et al., 2013; Guo et al., 2015). Recent lesion experiments have demonstrated how the central nucleus of the amygdala (CeA) may interact with the dorsolateral striatum to regulate the balance between goal-directed and habitual responding (Lingawi and Balleine, 2012). Rats with asymmetrical lesions of the CeA and dorsolateral striatum demonstrated an inability to form habitual responding (defined as becoming insensitive to reward devaluation), despite extensive training. How communication between these regions regulates a switch to habitual responding is unclear, although other amygdalar regions such as the basolateral nucleus have been shown to gate the plasticity of cortico-striatal synapses through NMDA-dependent mechanisms (Popescu et al., 2007). Another fascinating demonstration of striatal afferent-mediated regulation of behavioral control comes from attempts to use optogenetics to ameliorate compulsive grooming behaviors seen with deletion of Sapap-3, a synaptic scaffolding gene (Burguière et al., 2013). These mutant mice display behavioral control abnormalities in a cued-grooming task that likely results from dysfunctional inhibition of MSN activity. Surprisingly, this impulse-control deficit could be improved by optogenetic activation of excitatory projections from the lateral orbitofrontal cortex to the dorsal striatum. Increased recruitment of this circuit was able to compensate for striatal inhibitory deficits and restore MSN inhibition, suggesting that cortical regulation of local striatal inhibition is a potentially powerful mechanism for regulating normal behavioral output.

THE SEARCH FOR ASD-RELEVANT CIRCUIT DYSFUNCTION

The growing catalog of abnormalities—including structural and functional changes observed with imaging of autistic patients and physiological abnormalities documented in mouse genetic models for autism—highlights the importance of discerning commonalities of circuit dysfunction as a path toward understanding disease pathophysiology. While I have focused on striatal dysfunction, a growing body of evidence has accumulated implicating other regions in ASD pathology, including the cerebellum and cortex (Zikopoulos and Barbas, 2010, 2013; Wang et al., 2014). The recurrent phenotypes exhibited by patients and genetic mouse models begs the question as to why particular circuits would be intimately associated with ASD. One possibility is that these circuits have some specific molecular vulnerability rendering them more likely to become dysfunctional in response to a given genetic insult. An alternative hypothesis would be that despite ASD-relevant mutations having widespread effects on physiology, ASD-associated circuits occupy central and convergent points of processing which are uniquely sensitive to neuronal dysfunction and regulate motor and cognitive behaviors typically associated with ASD. Preliminary evidence exists for both of these concepts.

Do Particular Circuits Exhibit a Unique Molecular Vulnerability?

One can imagine several related mechanisms that would create a circuit-specific vulnerability to genetic insult—(1) neurons either uniquely or more highly express ASD-associated genes such that loss-of-function is more acutely sensed, or (2) neurons do not express alternative family members of ASD-associated genes that would typically allow for genetic compensation. Using a computational approach that analyzed autism genetic datasets to correlate mutations to known biological networks, it was found that autism-associated mutations are preferentially found in genes whose expression levels are enriched in both populations of striatal medium spiny neuron, as well as cortical inhibitory and projection populations, deep cerebellar nuclei and layer 6 corticothalamic neurons (Chang et al., 2015). Another study employing high-confidence autism genes as a "seed" from which to build co-expression networks implicated specific brain region and developmental windows, including mid-fetal cortical development, as well as postnatal thalamic and cerebellar nuclei, in disease pathogenesis (Willsey et al., 2013). Together, these data provide a relatively straightforward explanation for the observed bias toward cortico-striato-thalamic and cerebellar circuit dysfunction seen in ASD patients. In addition to being enriched for ASD-associated genes, it is currently unclear whether lack of molecular redundancy in these circuits also contributes to increased vulnerability to genetic insult. Single neuron transcriptional profiling, with its ability to quantitatively

assess mRNA levels across multiple families of ASD-associated genes will be an essential tool in further exploring the molecular vulnerability of neural circuits (Fuccillo et al., 2015).

Beyond possessing a molecular susceptibility for dysfunction, it is interesting to consider whether the evolutionary history of particular neural circuits has contributed to their predisposition for involvement in both ASD and neuropsychiatric disease more broadly. Anatomical and physiological analysis of the basal ganglia in lamprey, the oldest vertebrate lineage, shows a surprising degree of circuit and cell type conservation (Stephenson-Jones et al., 2011). The authors concluded that this reflects a process of "exaptation," wherein a core ancestral unit is repurposed by natural selection into a structure with altered function (Gould and Vrba, 1982). In this context, the lamprey basal ganglia, which functioned to control basic motor output has been co-opted over time for use by higher vertebrates in the processing of complex cognitive and emotionally driven actions. If true, the evolution of striatal circuits represents a parsimonious solution to enhance the range of behavioral output. However, this constant increase in the complexity of striatal integration may have come at a price. Might more molecules be required in striatal MSNs to integrate these new inputs? Would more spatially precise dendritic targeting of these proteins be required? Does this type of repurposing render the newer striatal functionalities of cognitive and emotional behavioral control less stable than the original motor control circuits? Might this hypothesis hold true for other highly conserved structures such as the cerebellum? Answers to these fascinating questions will require both technical advances and rigorous comparisons between the circuit connectivity of lower vertebrates and their human ancestors.

Are Abnormalities in Striatal Circuits likely to Cause Widespread Behavioral Dysfunction?

An alternative hypothesis for the importance of cortico-striato-thalamic and cerebellar abnormalities in ASD pathophysiology is not that these circuits are uniquely sensitive to genetic mutations, but rather their physiological dysfunction consistently produces robust deficits in social behavior and motor control. As previously discussed, striatal circuits occupy an intersection between internal representations of sensory input, prior experience, motivational state and motor control, and thus are capable of regulating behaviors that are typically linked to ASD. These striatal circuits further serve to regulate the output of the midbrain dopamine system, which extends a widespread neuromodulatory influence throughout the brain (Kupchik et al., 2015; Lerner et al., 2015). Dopamine signaling has been proposed to subserve multiple behavioral functions, depending both on the locus and timescale of its action (Schultz, 2007). Given this, the disruption in striatal function seen in ASD may serve to initiate disparate behavioral changes through brain region-specific dysregulation of dopamine release. In this model, motor control deficits would result from altered dopamine signaling in dorsal striatal structures while abnormalities in reward processing would be secondary to abnormalities in ventral striatal or prefrontal cortical dopamine signaling. Careful mechanistic studies in genetic mouse models will be necessary to further develop these concepts.

How Might Cerebellar and Cortical Circuit Dysfunction Produce ASD-associated Behaviors?

Are there other neural circuits as centrally placed as the striatum for the regulation of behavioral output? Cerebellar and prefrontal cortical circuits also appear as key candidates with influence on both the cognitive and motor aspects of ASD pathology. Given the importance of the cerebellum in motor control and balance, its perhaps unsurprising that the initial discovery of vermal hypoplasia in autistic patients (Courchesne et al., 1988) has been followed by studies linking autism with changes in cerebellar function during basic motor tasks, adaptation of saccades and feedback/feedforward regulation of grasping (Allen et al., 2004; Mosconi et al., 2013, 2015). However, the cerebellum exhibits significant connectivity with cognitive and affective brain regions and its function in language processing and aspects of social cognition may also contribute to autism symptomatology (Reeber et al., 2013; Wang et al., 2014). The extensive connectivity between the cortex and the cerebellum, together with the significant effect of early cerebellar lesions on cognition and social function as opposed to motor control, have led some to hypothesize the existence of a critical period for cerebellar function (Wang et al., 2014). Disruptions during this period may perturb the plasticity and development of cortical regions or block the early-stage learning of basic motor patterns and skills. A related hypothesis posits the cerebellum as an iterative processing unit essential for all motor and cognitive tasks, either through its general regulation of timing or orchestration of widespread neuronal adaptations for skilled motor output (for detailed review, see Strick et al., 2009). Although beyond the scope of this review, it would be interesting to explore the potential for interactions between the cerebellar and striatal systems with regard to autism pathophysiology. Tracing studies have highlighted two interesting points of intersection—(1) the connection of the deep cerebellar nuclei with the dorsal striatum, through a di-synaptic thalamic relay (Ichinohe et al., 2000; Hoshi et al., 2005), and (2) the mono-synaptic projection of deep cerebellar nuclei to the ventral tegmental area (Phillipson, 1979).

Cortical regions, particularly domains within the prefrontal cortex, have also been proposed as sites of dysfunction in ASDs. In rodents, the prefrontal cortex is a heterogeneous anatomical structure comprised of the orbital, cingulate, prelimbic, infralimbic, and agranular cortices (Heidbreder and Groenewegen, 2003). Tracing studies have revealed an extensive connectivity of prefrontal cortex, suggesting it functions to integrate input from sensory, limbic, and autonomic systems (Groenewegen and Uylings, 2000). Prefrontal dysfunction has been proposed to account for abnormalities of social cognition, action control and multi-sensory integration seen in autistic patients (see Martinez-Sanchis, 2014; Bicks et al., 2015; Chmielewski and Beste, 2015 for detailed review). Social cognition and action control both rely upon the integration

of motivational states, knowledge of specific environmental or social contexts and the flexible adjustment of behavior. The circuit bases of such wide-ranging functions remains fuzzy and warrant greater study. However, it seems likely that interactions between prefrontal circuits, the striatum and the thalamus will play a key role.

FUTURE PROGRESS IN UNDERSTANDING ASD PATHOPHYSIOLOGY

An integrated picture of ASD pathophysiology will clearly require further research studies that probe, in a non-biased manner, for circuit dysfunctions mediating discrete aspects of ASD symptomatology. Animal models, which allow for direct tests of causality, can provide mechanistic hypotheses that can be fully explored via functional imaging during human psychological testing.

Making Progress with Striatal-Based Models of ASD

Multiple lines of evidence from clinical imaging and rodent disease models have converged to suggest that striatal dysfunction is intimately associated with the etiology and pathophysiology of ASD. This knowledge provides an initial foothold into understanding how genetic abnormalities perturb neuronal and circuit function to generate the complex range of behavioral abnormalities seen in ASD. An important next step will be to assess whether the extreme genetic diversity of ASD-associated genes can be distilled down to a smaller number of circuit or cell type-specific deficits. Comparative physiological and behavioral analyses between pre-existing ASD model mice should aim to discern common behavioral deficits and ascertain whether they are attributable to conserved striatal abnormalities. Elucidation of a recurrent cell type, synaptic or circuit-specific deficit contributing to ASD-related behaviors would dramatically help in focusing future treatment endeavors. This information could then be integrated with the molecular profiles of affected circuit components in search of novel targets for disease amelioration.

Another key step in exploring ASD pathophysiology is to increase the biological relevance of rodent-based disease studies so that their results can be translated and built upon in the pre-clinical setting. To date, a large portion of disease modeling in rodents has focused on mice that are homozygous for functionally null alleles of ASD-associated genes. While this approach has no doubt improved our ability to detect abnormalities, it does so within a biological context that lies far outside the physiological range. The analysis of mutations in a more physiological setting can be achieved through numerous approaches—(1) examining heterozygous mutations, disease-associated point mutations or genetic modifiers, (2) exploration of environmental interactions with mutations (3) physiological analysis of behaviorally relevant circuits through selective optogenetic recruitment and (4) highly quantitative analysis to detect subtle changes in discrete components of complex behaviors. Even with these improvements, it is important to acknowledge the intra-species differences in brain complexity, genomic regulation and behavioral repertoire, which may place limits on the generalizability of rodent-based research findings (Fuccillo et al., 2016).

Striatal Dysfunction in the Broader Context of Neuropsychiatric Disease

Given the evidence provided here, I believe that a greater understanding of how normal striatal circuit function is perturbed in the presence of ASD-associated mutations will yield great returns. It is worth noting that many neuropsychiatric disorders demonstrate partially overlapping symptom domains that suggest striatal dysfunction. Obsessive-compulsive disorder is marked by deficiencies in reward processing and behavioral control, while schizophrenia, and major depressive disorder both exhibit profound psychomotor retardation, deficits in attention and decreased goal-directed action. Therefore, a deeper exploration of striatal function, employing current viral and genetic technologies to gain access to discrete components of striatal circuitry, may well shed light on a wide range of neuropsychiatric disorders.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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The role of cerebellar circuitry alterations in the pathophysiology of autism spectrum disorders

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The cerebellum has been repeatedly implicated in gene expression, rodent model and post-mortem studies of autism spectrum disorder (ASD). How cellular and molecular anomalies of the cerebellum relate to clinical manifestations of ASD remains unclear. Separate circuits of the cerebellum control different sensorimotor behaviors, such as maintaining balance, walking, making eye movements, reaching, and grasping. Each of these behaviors has been found to be impaired in ASD, suggesting that multiple distinct circuits of the cerebellum may be involved in the pathogenesis of patients' sensorimotor impairments. We will review evidence that the development of these circuits is disrupted in individuals with ASD and that their study may help elucidate the pathophysiology of sensorimotor deficits and core symptoms of the disorder. Preclinical studies of monogenetic conditions associated with ASD also have identified selective defects of the cerebellum and documented behavioral rescues when the cerebellum is targeted. Based on these findings, we propose that cerebellar circuits may prove to be promising targets for therapeutic development aimed at rescuing sensorimotor and other clinical symptoms of different forms of ASD.

Keywords: autism spectrum disorder, cerebellum, sensorimotor, genetics, pathophysiology, oculomotor, precision grip, gait

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Cerebellar Pathology in Autism Spectrum Disorder

The majority of *in vivo* brain studies of individuals with autism spectrum disorder (ASD) have focused on neural networks involved in social behavior, language, and behavioral and cognitive flexibility—the defining features of the disorder (American Psychiatric Association, 2013). Still, the full extent of neural systems impacted by ASD is not yet well understood, and pathophysiological mechanisms associated with the disorder remain elusive. There are multiple factors that have limited progress toward identifying brain mechanisms in ASD including the complexity of the psychological/behavioral constructs that have been most systematically investigated (e.g., theory of mind processing), limited knowledge about their neural underpinnings, clinical, and neurobiological heterogeneity across the autism spectrum, a lack of integration of knowledge about the developmental neurobiology of relevant brain systems, and failures to link *in vivo* case-control studies of psychological dimensions with what is known about histopathological and molecular mechanisms associated with ASD.

The cerebellum remains an understudied area in clinical investigations of ASD. It is perhaps the most consistently implicated brain region in post-mortem studies. Reports have indicated 35–95% fewer cerebellar Purkinje cells in ASD brains compared to controls (Bauman and Kemper, 1985; Arin et al., 1991; Bailey et al., 1998; Whitney et al., 2008, 2009; Wegiel et al., 2014), and remaining cells appear to be reduced in size (Fatemi et al., 2002a). The majority of cases studied to date (30/45) show reduced Purkinje cell density in posterior lateral hemispheres of the cerebellum, but fewer studies have found these anomalies in the vermis. A recent examination of eight patients found that Purkinje cell density reductions were more severe in Crus I–II, but that they were still present in lobules IV–VI and lobule X as well (Skefos et al., 2014). Deep cerebellar nuclear cells to which Purkinje cells project also are abnormal in ASD showing enlargement during childhood and subsequent reductions in size and number during adolescence and adulthood (Bauman, 1991). Therefore, patterns of cerebellar pathology may be regionally specific as well as variable across development. Levels of glutamic acid decarboxylase (GAD) 65 and 67 proteins involved in converting glutamate to GABA are reduced in the cerebella of individuals with ASD (Fatemi et al., 2002b; Yip et al., 2007, 2008). Reductions in $GABA_A\alpha1$ protein levels and $GABA_B$ R1 receptor density in cerebella of ASD patients also have been documented (Fatemi et al., 2010).

Studies of etiopathologic mechanisms associated with ASD have consistently implicated the cerebellum as well. Computational studies have found that ASD susceptibility genes are co-expressed in human cerebellum between the neonatal period and age 6 years (Willsey et al., 2013), particularly within the granule cell layer (Menashe et al., 2013). Further, many syndromic forms of ASD involve cerebellar alterations including Phelan-McDermid Syndrome, Fragile X Syndrome (FXS), Tuberous Sclerosis (TSC), and patients with 15q11 duplication syndrome (Abrahams and Geschwind, 2010; Mosconi et al., 2011; Kloth et al., 2015). Cerebellar alterations also appear to be specifically associated with ASD features. For example, while FXS is associated with ASD and involves disruptions of multiple brain systems, posterior vermis lobules VI–VII are affected only in individuals with FXS with comorbid ASD (Kaufmann et al., 2003). In the context of structural MRI studies showing that posterior vermis lobules VI–VII also are reduced in volume in idiopathic ASD, these findings provide strong evidence that posterior vermal alterations may be uniquely associated with ASD (see Stanfield et al., 2008 for a meta-analysis). Additional evidence for cerebellar anomalies being selectively involved in ASD comes from studies of individuals with TSC, a genetic disorder caused by mutations of either TSC1 or TSC2 genes and characterized by hamartomas in the brain and other organs. Approximately 40% of individuals with TSC also are diagnosed with ASD, and those individuals with TSC and cerebellar lesions have more severe ASD features than those with lesions affecting other brain regions (Eluvathingal et al., 2006). Studies of children who have experienced perinatal cerebellar injuries further support a central role of the cerebellum in the development of ASD. These children experience a 36-fold increased risk of developing ASD, making perinatal cerebellar damage the greatest known non-genetic risk factor associated with the disorder (Bolduc and Limperopoulos, 2009; Limperopoulos et al., 2009; Bolduc et al., 2011; Wang et al., 2014).

Despite evidence for a primary role of the cerebellum in the pathophysiology of ASD, the literatures describing the cerebellar circuitries that are affected in patients and how they relate to clinical impairments remain in their infancy. Afferent processes to pontine nuclei originate from widespread regions of neocortex and are relayed to different lobules of cerebellar cortex via mossy fiber inputs (Eccles et al., 1967). These inputs arrive from motor, sensory, posterior parietal, prefrontal, cingulate, orbitofrontal, and temporal cortices as well as basal ganglia nuclei (Dum and Strick, 2003). Output from the deep nuclei of the cerebellum (dentate, interpositus, and fastigial) innervate different subdivisions of ventrolateral thalamus (Percheron et al., 1996) and then project to multiple neocortical areas (Leiner et al., 1991, 1993). These cortical-pontine-cerebellar-thalamic-cortical loops are highly segregated and support distinct behavioral and cognitive functions including sensorimotor, language, affective, and executive abilities (Habas et al., 2009; Krienen and Buckner, 2009). Defects of the cerebellum thus could have a pervasive impact on behavioral and cognitive development while increasing risk for ASD by disrupting the maturation and function of these cortical-cerebellar loops. If pathology in the cerebellum is localized, cerebellar anomalies could have a selective impact on different circuitries and thus contribute to symptom heterogeneity in ASD.

There is accumulating evidence that multiple cortical-cerebellar circuits are anatomically and functionally abnormal in patients with ASD (**Table 1**). Diffusion tensor imaging (DTI) studies have identified white matter alterations of the primary output pathway from the cerebellum, the superior cerebellar peduncle, and the primary cortical input pathway to the cerebellum, the middle cerebellar peduncle (Catani et al., 2008; Shukla et al., 2010; Sivaswamy et al., 2010). While these studies suggest that cerebellar input and output processes connecting it to neocortical areas are atypical in ASD, current DTI methods are not able to discern the extent to which these anomalies selectively involve different cortical-cerebellar loops.

A recent meta-analysis highlights a unique profile of volumetric reductions in cerebellar gray matter of individuals with ASD that is distinct from alterations found in attention deficit-hyperactivity disorder (ADHD) and developmental dyslexia (Stoodley, 2014). ASD-specific reductions in volume were found in inferior lobule IX, left lobule VIIIB, and Crus I. These regions also showed functional connectivity with frontoparietal, default mode, somatomotor, and limbic areas, consistent with the idea that different forms of cerebellar pathology may differentially impact multiple brain networks and cause varying developmental dysfunctions. Accordingly, atypical patterns of cortical-cerebellar activation and functional connectivity have been demonstrated in ASD during tests of simple motor skills (Mostofsky et al., 2009), language (Hodge et al., 2010; Verly et al., 2014), and emotion processing (Critchley et al., 2000). Importantly, a recent resting state fMRI study of individuals with ASD documented overconnectivity of cortical-cerebellar circuits involved in sensorimotor control, as well as

TABLE 1 | Summary of findings from prior ASD studies of different sensorimotor cerebellar circuits.

	Oculomotor circuits	Upper limb circuits	Gait/Posture circuits
Histopathology	• Minority of cases show reduced PC density in vermis, though not as prominent as in hemispheres	• Consistent reports of reduced PC density in lob. IV–VI extending into lateral areas including Crus I–II	• Reduced PC density in spinocerebellum, though less prominent than in anterior or lateral lobs
MRI anatomy	• Findings of vermal hypoplasia involving lobules VI–VII	• Consistent findings of overgrowth throughout hemispheres	• Reduced volumes found in inferior lob. IX
Sensorimotor behavior	• Increased amplitude of saccadic intrusions during gaze fixation • Reduced saccade accuracy • Increased saccade amplitude variability • Reduced rates of saccade adaptation • Reduced closed-loop smooth pursuit gain • Reduced gain of rightward eye movements during open-loop phase of smooth pursuit (first 100 ms)	• Atypical reaching kinematics • Slower, less smooth reach-to-grasp movements • Reduced coordination of grip and lift forces during grasping • Excess grip force during initial gripping • Increased force variability during sustained gripping • Increased reliance on proprioceptive feedback during motor learning processes • Impairments in visual feedback processing during gripping and motor learning	• Reduced anterior postural adjustments during self-timed loading/unloading • Increased postural sway, esp. in mediolateral directions, during quiet stance • Reduced postural sway when trying to initiate and maintain a dynamic stance • Increased stride width, decreased stride length, atypical walking kinematics
Functional imaging	• Reduced BOLD activation during saccades and smooth pursuit eye movements	• Reduced and increased anterior and lateral cerebellar BOLD activity found during finger tapping and button pressing sequencing tasks	• No studies reported

PC, Purkinje cells; ms, milliseconds; lob, lobule(s).

underconnectivity of cerebellar circuits involved in cognitive and higher-order operations (Khan et al., 2015). These findings provide important functional evidence that while cerebellar pathology in ASD may affect multiple cortical and deep nuclear circuits, distinct cortical-cerebellar loops or circuits may be altered in different ways.

Studies of sensorimotor behaviors offer perhaps the most direct approach for understanding the functional integrity of different cerebellar circuits in ASD patients. Sensorimotor tasks are highly translational, precisely quantifiable in both spatial and temporal domains, and readily studied across wide age ranges and developmental levels. The cerebellar networks supporting sensorimotor development are relatively well defined and include motor and parietal cortices as well as basal ganglia nuclei (Gazzaniga and Mangun, 2014)—regions that have been repeatedly implicated in ASD (Sears et al., 1999; Stanfield et al., 2008; Mostofsky et al., 2009; Wolff et al., 2013).

Sensorimotor control is understudied in ASD, but there is a growing literature showing that cerebellar-dependent sensorimotor behaviors are compromised in ASD patients. Sensorimotor deficits are found in the majority of individuals with ASD (Fournier et al., 2010a), and these impairments can emerge and be detected as early as infancy (Brian et al., 2008; Lebarton and Iverson, 2013; Ben-Sasson and Gill, 2014; Leonard et al., 2014; Ozonoff et al., 2014; Sacrey et al., 2015). They also appear to be familial, suggesting that they may serve as useful endophenotypes for advancing gene discovery (Mosconi et al., 2010). In the present paper, we will describe what is known about the distinct cerebellar circuitries involved in sensorimotor behaviors and their functional integrity in ASD. Approaches for understanding these circuitries in animal models of ASD and determining their utility as targets for treatment development also will be discussed.

Cerebellar Circuits Supporting Sensorimotor Behaviors

More than half of all mature neurons in the brain are located in the cerebellum (Butts et al., 2012), and many of the circuits formed by these cells are involved in various aspects of motor control (Ito, 1984). The precise role of these circuits in controlling motor behavior remains debated, as various zones of the cerebellum appear to make distinct contributions including controlling the timing of movements, simultaneously coordinating the movement dynamics of different effectors (e.g., shoulder, elbow, and wrist joints during reaching), and integrating multiple cortical signals and sensory inputs (Holmes, 1917; Bower, 2002; Jacobson et al., 2008; D'angelo and De Zeeuw, 2009; De Zeeuw et al., 2011). One prominent and unifying framework hypothesizes that the cerebellum serves as a forward controller of motor and cognitive activity (Ito, 1983, 2008; Miall and Reckess, 2002). According to this model, the cerebellum provides forward models used to predict the position and motion of body parts based on an internal model of the dynamics required to complete a given task. Forward control allows for

rapid action as sensory feedback processes often occur too slowly to guide initial or dynamic movements. After a motor command is generated by the motor cortex, a copy of this command is sent to the cerebellum (efference copy). The cerebellum then uses its forward model to predict the sensory consequences of the action (corollary discharge). The sensory predictions are subsequently compared to actual sensory feedback and the cerebellum generates corrective commands to refine the ongoing movement (Wolpert et al., 1998). When the action repeatedly deviates from the expected outcome, the forward model of the cerebellum undergoes refinement to ensure the accuracy of subsequent output (Scudder, 2002; Izawa et al., 2012a; Herzfeld et al., 2014).

A unifying framework for the cerebellum appears plausible in the context of its relatively invariant cellular architecture that contrasts the diversity of cellular composition across different neocortical areas. The cerebellar cortex consists of numerous "microcomplexes" that are structured similarly across lobules and different subregions (Ito, 2008) (**Figure 1**). These microcomplexes are comprised of mossy fiber inputs, primarily originating from pontine nuclei but also from vestibular nuclei, the spinal cord, and reticular formation. Mossy fibers innervate Purkinje cells of the cerebellar cortex via granular cells and parallel fibers as well as feedback collaterals from deep cerebellar nuclei. Excitatory mossy fiber inputs provide information from neocortical regions as well as the spinal cord (Vogel et al., 1996; Ramnani, 2006; Geborek et al., 2014). Climbing fibers originating in the inferior olive communicate instructional or "teaching" signals directly to Purkinje cells and initiate a process of long-term depression (LTD) that selectively prunes parallel fiber-Purkinje cell synapses and modifies the strength of inhibitory output from Purkinje cells to deep nuclei (Nguyen-Vu et al., 2013). This process is the basis of cerebellar learning and it allows the cerebellum to consolidate and then modify internal models of action that are used to predictively control motor behavior (Wolpert et al., 1998).

Despite the similarity of these learning units across the cerebellar hemispheres and vermis, there is considerable anatomical specificity for different types of movements and different aspects of movement control (**Figure 2**). Within the cerebellum, somatic representations similar to those localized in motor cortex have been demonstrated both in the cerebellar cortex and in the deep nuclei (see Manni and Petrosini, 2004 for a review). Microzones consisting of parasagittally aligned Purkinje cell populations form functional units that innervate discrete areas of deep cerebellar nuclei and receive segregated projections from the inferior olive (Cerminara, 2010; Oberdick and Sillitoe, 2011). Thus, the geometry of the cerebellum is largely invariant at the cellular level, but highly specialized and segregated functional units are found at an intermediate level and at the level of cortical-cerebellar networks.

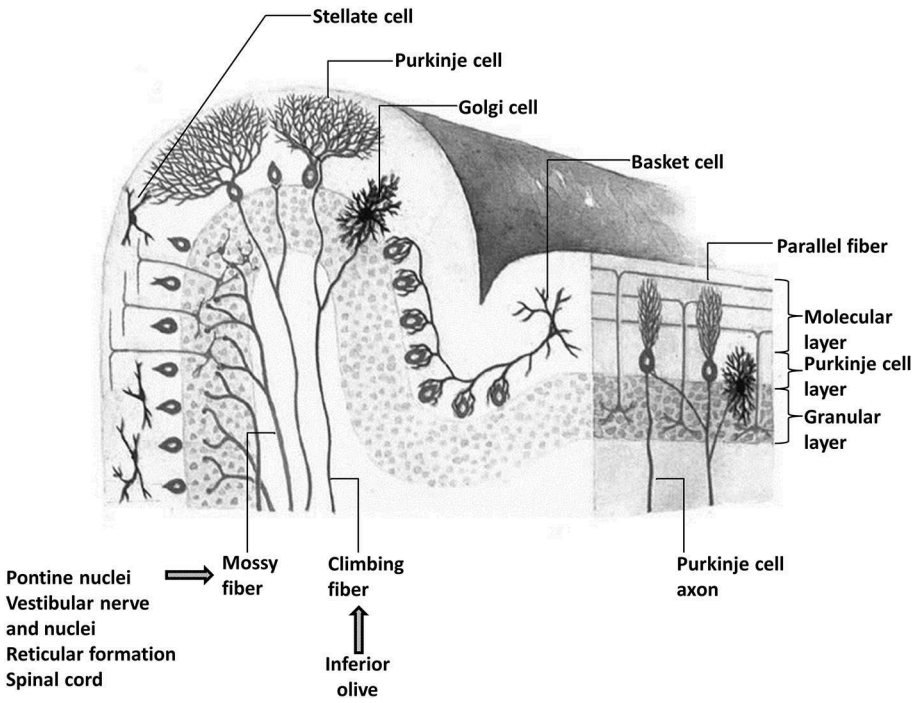

FIGURE 1 | The microstructural organization of the cerebellar cortex showing the presence of three layers and the relative position of Purkinje, basket, stellate, and Golgi cells and the main inputs (mossy and climbing fibers) of the cerebellum. There are two main afferents to the cerebellar cortex: climbing fibers, which make direct excitatory contact with the Purkinje cells, and mossy fibers, which terminate in the granular layer and make excitatory synaptic contacts primarily with granule cells, but also with Golgi cells. The ascending axons of the granule cells branch in a T-shaped manner to form the parallel fibers, which, in turn, make excitatory synaptic contacts with Purkinje cells and molecular layer interneurons including stellate and basket cells.

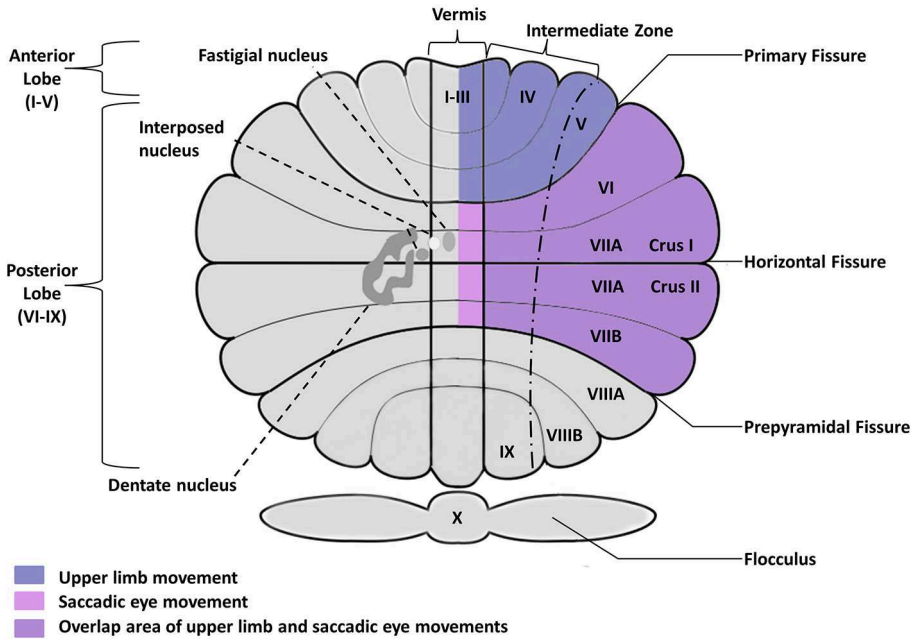

FIGURE 2 | Posterior view of the human cerebellum, showing the cerebellar fissure, lobular organization, and deep nuclei embedded within the cerebellar cortex. Deep nuclei are located bilaterally but shown only in the left hemisphere for clarity purposes. Saccadic and smooth pursuit eye movements are controlled by the oculomotor vermis including posterior lobules VI–VII, Crus I–II of the ansiform lobule, and their outputs in caudal fastigial nuclei. Upper limb movements primarily involve anterior lobules I–V as well as more lateral areas of lobules V–VI extending into Crus I–II. Cerebellar circuits involved in controlling balance and gait have been identified in the vermis and intermediate cerebellum (not shown).

The distinct cerebellar regions that support different types of motor behavior have been well described in human imaging and lesion studies as well as single-cell recordings of non-human primates. These studies have identified discrete circuits supporting eye movements, limb movements, and posture/gait. Saccadic eye movements, or rapid shifts in eye gaze, as well as smooth pursuit eye movements are controlled by the oculomotor vermis including posterior lobules VI–VII, Crus I–II of the ansiform lobule, and their outputs in caudal fastigial nuclei (Takagi et al., 1998; Alahyane et al., 2008; Panouillères et al., 2012). Crus I–II of ansiform lobule, the flocculus and paraflocculus, uvula and nodulus are critically involved in steady gaze fixation, smooth pursuit eye movements, and the vestibular-ocular reflex (VOR) that is used to maintain fixation during head rotation (Robinson et al., 1993; Hashimoto and Ohtsuka, 1995; Baier et al., 2009). Upper limb movements are under the supervision of the intermediate and lateral zones of the cerebellar cortex and their targets in the interposed and dentate nuclei (Thach et al., 1992; Thach, 1997; Kuper et al., 2012; Maderwald et al., 2012; Stefanescu et al., 2013). Circuits located more medial in the vermis and intermediate cerebellum receive neocortical input as well as direct spinal input to control balance and gait (Brooks and Thach, 1981; Sullivan et al., 2010; Vassar and Rose, 2014). Further, there appear to be distinct subregions within these circuits to control different aspects of motor output. For example, Neely et al. (2013) found that cerebellar areas controlling initial manual motor output based on internal action representations appear to be anterior to those involved in continuous control of motor behavior based on visual feedback. The high degree of functional specialization of these distinct circuits suggests that their study in ASD may provide key insights into the developmental neurobiology of this disorder and the pathogenesis of sensorimotor issues and perhaps broader behavioral and cognitive deficits.

Oculomotor Control in ASD

Studies of oculomotor control may be highly informative regarding cerebellar function in ASD owing to their well-defined neurophysiological substrates, quantitative nature, high degree of heritability (Bell et al., 1994), and stability over time (Yee et al., 1998; Reilly et al., 2005; Irving et al., 2006; Lencer et al., 2008). Abnormalities of eye gaze also are part of the diagnostic criteria for ASD, and while these deficits have been well studied during social interactions, it is possible that more fundamental and earlier emerging alterations of oculomotor control could contribute to atypical patterns of eye gaze coordination among affected children (Bryson et al., 2007; Elison et al., 2013).

Gaze fixation is an active process used to stabilize the fovea on an image or object. While the eye undergoes naturally occurring drift during the process of visual fixation, the oculomotor system generates microsaccades to counter this drift and maintain fixation (Zuber et al., 1965; Epelboim and Kowler, 1993). Visual fixation is supported by the reciprocal balance of excitatory burst and inhibitory omnipause neurons within the pons of the brainstem as well as inputs from the frontal eye fields and

superior colliculus that actively suppress saccades away from the object of interest (Leigh and Zee, 2006). Pontine nuclei innervate Purkinje cells of cerebellar vermis lobules VI–VII, and inhibitory output from the oculomotor vermis helps suppress unwanted eye movements and maintain an image on the fovea (Kase et al., 1980). Abnormalities during visual fixation including slow, large amplitude ocular drift, square wave saccadic intrusions, and gaze-evoked nystagmus (repetitive, to-and-fro movements of the eyes) each have been documented in patients with cerebellar lesions (Jeong et al., 2007; Serra et al., 2008; Shaikh et al., 2009; Baier and Dieterich, 2011).

Structural MRI and post-mortem studies have documented abnormalities of the pons (Gaffney et al., 1988; Hashimoto et al., 1991, 1993; Hashimoto and Ohtsuka, 1995; Bailey et al., 1998; Jou et al., 2013) and cerebellar vermal lobules VI–VII in ASD (Courchesne et al., 1988; Murakami et al., 1989; Hashimoto et al., 1995; Fatemi et al., 2002a; Stanfield et al., 2008). Studies of visual fixation in ASD have demonstrated increased amplitude and reduced inter-saccade intervals of square-wave jerk saccades relative to healthy controls (Nowinski et al., 2005; Aitkin et al., 2013) suggesting increased excitation in ponto-cerebellar circuitry in patients.

Saccades, or rapid shifts in eye gaze, are controlled by highly specialized cortical-cerebellar circuits that also involve posterior vermis and caudal regions of fastigial nuclei. The initiation of saccades relies on the inverse process of visual fixation control via the pons described above. In order for a saccade to be initiated, the tonic inhibition of pontine burst cells must be simultaneously released by omnipause cells while also being driven by excitatory signals from the superior colliculus (Robinson, 1975; Leigh and Zee, 2006). The dynamics of saccadic eye movements are directly related to the firing rates of the burst cells and their interactions with cerebellar output that predictively controls the amplitude and accuracy of the movement (Luschei and Fuchs, 1972; Van Gisbergen et al., 1981; Yoshida et al., 1999).

Reduced accuracy (Rosenhall et al., 1988; Takarae et al., 2004b; Luna et al., 2007; Johnson et al., 2012; Schmitt et al., 2014) and increased trial-to-trial accuracy variability (Takarae et al., 2004b; Stanley-Cary et al., 2011; Johnson et al., 2012; Schmitt et al., 2014) of saccadic eye movements have been repeatedly documented in individuals with ASD. These results implicate forward control mechanisms of the oculomotor vermis in ASD and a reduced ability to precisely update internal representations used for forward control. This profile of deficits in saccade control is similar to what is seen in non-human primates following ablation of the oculomotor vermis (Takagi et al., 1998) and patients with spinocerebellar (Federighi et al., 2011) and Friedreich's Ataxia (Kirkham et al., 1979). Studies of saccade dynamics in ASD have identified reduced velocities (Johnson et al., 2012; Schmitt et al., 2014), increased duration (Rosenhall et al., 1988; Stanley-Cary et al., 2011; Schmitt et al., 2014), and prolonged periods of movement acceleration during the saccade (Schmitt et al., 2014) suggesting an imbalance of pontine excitatory and inhibitory processes that reciprocally interact with the cerebellum.

In the only known ASD study to directly examine brain systems underlying saccade control, Takarae et al. (2007) used fMRI to show reduced activation of frontal eye fields, posterior parietal cortex, and cerebellar vermis and hemisphere lobules VI–VII in patients making visually guided saccades. Individuals with ASD also demonstrated increased activation within frontal-striatal regions including the thalamus, caudate nucleus, dorsolateral prefrontal cortex, and anterior cingulate cortex. These results provide direct evidence for cortical-cerebellar dysfunctions during eye movements in ASD, and also suggest that frontostriatal systems typically dedicated to higher-order cognitive processes may become more involved in simple motor actions to compensate for cortical-cerebellar alterations in ASD patients.

The oculomotor vermis also is involved in controlling smooth pursuit eye movements used to track slowly moving targets. Smooth visual pursuit relies on the rapid and temporally precise integration of information from multiple brain regions including extrastriate areas of the visual cortex responsible for processing visual motion, cortical eye fields and the cerebellum responsible for translating sensory information into motor commands, and the striatum and brainstem responsible for initiating motor commands (Lisberger et al., 1987; Keller and Heinen, 1991; Ilg, 1997; Berman et al., 1999; Rosano et al., 2002). The initial phase of visual pursuit is open-loop (typically defined as the first 100 ms of pursuit) and is driven solely by feedforward mechanisms. The latter phase of smooth pursuit is closed-loop (occurring after 100 ms of pursuit) and is defined by online refinements of eye velocity and position based on sensory feedback processes encoded in the striate cortices and projected to posterior parietal cortices and then to medioposterior cerebellar lobules VI–VII (Ritchie, 1976; Fuchs et al., 1985; Stein, 1986; Kawato et al., 1987; Noda et al., 1990; Takagi et al., 1998; Chen-Harris et al., 2008).

Reduced pursuit accuracy has been documented in ASD during both the open-loop (Takarae et al., 2004a) and closed-loop phases (Takarae et al., 2004a; Aitkin et al., 2013) implicating both forward control and visual feedback processes. Deficits in open-loop pursuit were lateralized in ASD affecting only rightward movements. While suggesting a lateralized deficit, this finding (also present in unaffected parents) (Mosconi et al., 2010) is broadly consistent with diverse evidence indicating that hemispheric specialization for motor functions may be disrupted in patients (Escalante-Mead et al., 2003; Lindell and Hudry, 2013; Seery et al., 2013; Forrester et al., 2014). Further, the amplitude of saccades made to "catch up" to moving targets during pursuit also appears to be increased in ASD (Takarae et al., 2004a; Aitkin et al., 2013). However, it should be noted that some studies have found no differences in pursuit accuracy in ASD (Scharre and Creedon, 1992; Kemner et al., 2004), which may be related to findings that older individuals with ASD demonstrate more similar closed-loop tracking accuracy compared to healthy controls (Takarae et al., 2004a). Still, cortical-cerebellar dysfunctions appear to persist in adulthood as demonstrated by an fMRI study of smooth pursuit eye movements that found reduced activation in frontal eye fields, posterior partietal cortex, cingulate motor area, pre-supplemental motor cortex, and cerebellar lobules VI–VII in individuals with ASD (Takarae et al., 2007).

Perhaps the most sensitive probe of cerebellar circuits supporting oculomotor control is to systematically induce error into the system and then quantify the extent and rate at

which the system adapts in order to evaluate plasticity in forward control mechanisms. Tests of saccadic adaptation have been used to assess cerebellar motor learning in non-human primates and patients with cerebellar lesions. In prototypical saccade adaptation tests, the visual target used to elicit a saccade is displaced by a consistent amplitude close to the time of movement initiation. Due to saccadic suppression of visual information during the movement, target displacement is seldom detected by the subject, but the amplitude of the saccade is adjusted over subsequent trials to land more closely to the displaced target rather than the original location. Adaptation mechanisms have been localized to the oculomotor vermis during this test (Desmurget et al., 1998; Barash et al., 1999), and reduced rates of adaptation and increased variability of saccade amplitudes have been found in individuals with cerebellar lesions that include the vermis (Golla et al., 2008; Xu-Wilson et al., 2009). Importantly, patients with non-vermal cerebellar damage show spared adaptation abilities.

Two recent ASD studies revealed reduced rates of saccade adaptation in affected individuals (Johnson et al., 2013; Mosconi et al., 2013). In addition, increased variability of saccade accuracy (Mosconi et al., 2013) and reduced time to peak saccade velocity (Johnson et al., 2013) also were reported. Importantly, Mosconi et al. (2013) found that 27% of subjects with ASD failed to show any level of adaptation compared to only 6% of controls, suggesting that a subset of patients may show more severe defects in cerebellar learning processes.

Studies of oculomotor control in ASD thus suggest alterations within cortical-ponto-cerebellar circuits involving the posterior vermis. These dysfunctions appear to be familial. Mosconi et al. (2010) demonstrated that unaffected family members of individuals with ASD show profiles of eye movement abnormalities similar to those described in individuals with ASD. Specifically, this study reported increased saccade error and saccade error variability and reduced pursuit accuracy during both closed- and open-loop phases suggesting that defects of cortical-cerebellar circuits involved in oculomotor control may contribute to the pathophysiology of ASD. Studies assessing the extent to which these deficits co-segregate within different families will be important for determining their utility as endophenotypes in family genetic studies. Similarly, studies showing direct linkages between these sensorimotor alterations and pathology in discrete cerebellar circuits may help sort out heterogeneity in the syndrome of autism based on biological parameters for which there is mechanistic understanding.

Upper Limb and Manual Motor Control in ASD

Control of upper limb movement and force generation is supported by frontoparietal cortices and their targets in the cerebellar cortex and deep nuclei. The circuits that control various body parts are segregated at the levels of neocortex, cerebellar cortex, and within the deep nuclei (Grodd et al., 2001). Upper limb movements primarily involve anterior lobules I–V as well as more lateral areas of lobules V–VI extending into Crus I–II (Vaillancourt et al., 2006). Within these circuits, separate zones have been found to be differentially involved in controlling the amplitude, duration, and timing of movements (Mai et al., 1988; Spraker et al., 2012; Neely et al., 2013).

The most prominent feature of upper limb movements and manual motor control in patients with cerebellar lesions is dysmetria which frequently is characterized by overshooting of the target (Flament and Hore, 1986; Goodkin et al., 1993). Patients also show increased accuracy variability from trial to trial, impaired timing of their movements, overall slowness, and increased curvature of movement trajectories (Hallett et al., 1975; Bares et al., 2010). Upper limb and manual motor deficits are associated with atrophy of the intermediate and lateral cerebellum. Upper limb ataxia is found in patients with lesions of lobules IV–VI, whereas lower limb ataxia appears to result from defects in lobules III–IV (Schoch et al., 2006). Limb ataxia also is correlated with lesions affecting the interposed or dorsomedial dentate nuclei.

Limb movement abnormalities consistent with those seen in cerebellar patients have been found in individuals with ASD. When reaching toward targets, arm movements of individuals with ASD show increased temporal and spatial variability as well as atypical kinematic profiles characterized by reduced velocities and rates of acceleration as well as increased latencies of peak velocity (Glazebrook et al., 2006, 2009). The authors hypothesized that individuals with ASD compensate for deficits in forward control by slowing their reach and allowing more time for sensory feedback control processes to help guide the movement. Similar deficits in forward control have been shown for children with ASD when reaching for objects on large (easy) or small (hard) targets (Fabbri-Destro et al., 2009). While control subjects slowed the speed of their movement when reaching for smaller objects (in the more difficult condition), individuals with ASD failed to modulate their movement speed to account for the increased difficulty of the task. This finding suggests a compromised ability to appropriately modulate the action plan according to different task conditions.

Deficits in the sustained control of reaching movements also have been found in ASD. Glazebrook et al. (2009) found that reaching movements of individuals with ASD were more severely affected when they required greater visual-proprioceptive integration. These results suggest that individuals with ASD show a reduced ability to simultaneously process and integrate multisensory information, a process involving posterior cerebellar circuits that translate sensory feedback information into refined motor commands (Stein and Glickstein, 1992). Further, Gowen and Miall (2005) showed that individuals with ASD do not benefit from increased movement time in terms of their end-point accuracy during rapid targeted pointing. A more recent analysis of sinusoidal arm movements in ASD similarly found that patients show atypical kinematic profiles characterized by decreased movement smoothness (Cook et al., 2013). Unlike targeted movements, these oscillatory movements of individuals with ASD were increased in velocity and rate of acceleration. One possible explanation for the reduced smoothness of patients' movements is a failure to anticipate the point at which they must change the direction of their movement,

or difficulties using predictive mechanisms to modulate action kinematics. Further, it is possible that patients were not able to precisely and consistently modulate the timing of the onset and offset of agonist and antagonist muscles across joints to facilitate smoother movements (Vilis and Hore, 1980; Nowak et al., 2004).

Prehension

Prehension involves the coordinated act of reaching and grasping, and it is central to many daily living activities that are difficult for individuals with ASD (Brisson et al., 2012; Mulligan and White, 2012; Libertus et al., 2014). The ballistic acts of reaching and positioning the hand, affected by upper arm and forearm musculature, are largely independent from mechanisms subserving grasping actions, i.e., hand opening and then closing upon the object (Jeannerod, 1981, 1984). The two neural channels are assumed to be activated in parallel so that they can be functionally coupled during the act of reaching to grasp (Jeannerod, 1981, 1984). The "arm reaching" channel is believed to extract information about the spatial location of the object for transformation into motor patterns that bring the hand appropriately toward the object. The "grasp" channel extracts information about the intrinsic properties of the object for the determination of a suitable grasping position and appropriate level of force generation.

Slower reach-to-grasp movements have been found in individuals with ASD and comorbid intellectual disability (Mari et al., 2003). These movements were characterized by longer durations, greater temporal delay between peak reach velocity and peak grip aperture, a prolonged deceleration phase, reduced reaching velocity and prolonged time to maximum hand aperture. However, these deficits in forward control and movement coordination were not evident in individuals with ASD whose IQ was in the average range, suggesting that cerebellar dysfunctions may vary across the autism spectrum in relation to general cognitive ability. Still, others have found that individuals with ASD decompose reaching and grasping movements, and that these deficits are evident across the autism and IQ spectra (Cattaneo et al., 2007; Fabbri-Destro et al., 2009). These findings suggest that instead of translating movement goals into a chain of smoothly synchronized motor acts, individuals with ASD independently execute each component of the goal in a more sequential manner (Mari et al., 2003; Nazarali et al., 2009).

Studies of the grasping component of prehension provide a unique opportunity to investigate distinct motor control mechanisms in ASD that are linked to different circuits of the cerebellum. Neely et al. (2013) identified separate cerebellar circuits associated with dynamic aspects of gripping and sustained feedback control of grip. Spraker et al. (2012) also found that cerebellar regions that scaled with force amplitude could be segregated from those associated with the duration of force output. By examining different components of precision grip control in ASD, it thus may be possible to localize cerebellar circuit dysfunctions.

Prior studies of patients with cerebellar lesions have documented patterns of deficit during precision gripping including excess initial force output, increased sustained force variability, and decreased rates of force relaxation (Mai et al., 1988; Müller and Dichgans, 1994; Serrien and Wiesendanger, 1999; Fellows et al., 2001; Nowak et al., 2002, 2004). Forward control deficits would limit individuals' ability to form accurate initial movements, and sensory feedback processes would need to compensate to steer the movement back to the intended goal following afferent delays. Given the delays in sensory feedback, movement corrections can never be optimal (increased movement variability), because they are always computed for a portion of the trajectory that occurred in the past. Thus, a strategy emphasizing feedback over forward control processes may contribute to reduced precision or increased variability of motor output (Bastian, 2006).

Precision gripping studies have been performed to analyze forward control and feedback mechanisms. When an object is lifted using a pinch grip, grip force (against the object) increases simultaneously with load force (lifting of the object) prior to picking up the object (Johansson and Westling, 1984, 1988; Westling and Johansson, 1984). The rate of grip force increase and the grip force amplitude each depend on the object's weight and its surface texture. Therefore, grip force must be timed correctly with respect to the *anticipated* object load. During a test of precision gripping, Johansson and Westling (1984) found that participants' final grip force was greater when lifting heavier or more slippery objects suggesting that increases in grip force during the loading phase are planned in anticipation of the properties of the object.

Assessing grip and load force timing in individuals with ASD, David et al. (2009, 2012) found significant increases in grip to load force onset intervals suggesting temporal dyscoordination of these component processes. The authors also documented increased grip force at load force onset that may reflect either patients' inability to correctly time the grip force with respect to the anticipated load, or a compromised ability to use prior experience to correctly predict the required load force. In contrast, Gowen and Miall (2005) failed to identify forward control deficits in adults with ASD during precision gripping. Differences between these findings could reflect the non-overlapping age distributions across the studies. Gowen and Miall studied an older cohort (ages 18–49 years), and recent work has suggested that deficits in forward control of precision gripping may become less pronounced with age in ASD (Mosconi et al., 2015). Further, the nature of the different tasks used in these studies could have led to the discrepant findings. Gowen and Miall (2005) had subjects lift an object repeatedly allowing them to have greater experience with the load force required and thus providing sufficient information for individuals with ASD to adjust their force output. This would suggest that patients may be able to accurately calibrate forward controllers, but that they may need more practice than healthy individuals.

Using a novel analysis approach adopted from prior reaching studies, Wang et al. (2015) recently found that individuals with ASD show distinct patterns of initial gripping strategies. By measuring the derivatives of individual force traces, the authors identified inflections in force output reflecting changes to the initial motor plan putatively based on visual, somatosensory, and proprioceptive feedback inputs (Novak et al., 2000; Wisleder and Dounskaia, 2007; Grafton and Tunik, 2011). Three different types

of feedforward control processes were identified: Type 1 pulses were associated with target overshooting and were characterized by rapid increases in force followed by a rapid force reduction to match the target; Type 2 pulses were defined by a more gradual increase in force followed by a pause and then a secondary force increase to reach the target; Type 3 pulses were distinguished by a series of temporally overlapping pulses used to reach the target force level. While controls showed a bias toward Type 1 pulses at low target force levels and trials that were shorter in duration, they utilized the more efficient Type 2 pulses to gradually reach their target levels when the target force or trial duration were increased. Individuals with ASD, in contrast, did not adapt their primary force strategy as flexibly, showing a persistent bias toward Type 1 pulses at both higher force levels and during trials of longer duration. These findings suggest that the internal representations used to predictively guide initial motor output are more stereotyped for individuals with ASD and thus may limit their ability to adapt motor skills to new and more complex task demands.

Further evidence for deficits in anticipatory control of motor output in ASD is seen in an analysis of force relaxation during precision gripping (Wang et al., 2015). The authors found that individuals with ASD show reduced rates of force relaxation after receiving visual cues that they should release their force level. To initiate grip relaxation, antagonist, and agonist muscles of the fingers must be synchronously activated and deactivated, respectively, within a rapid time frame. Reduced rates of grip force relaxation in ASD may reflect impairments in generating or executing coordinated muscle activities as seen in patients with cerebellar lesions (Küper et al., 2011). Studies directly examining muscle activities during precision gripping using electromyography (EMG) will be important for elucidating the mechanisms contributing to deficits in anticipatory motor processes in ASD.

Feedback control of sustained precision grip also appears to be disrupted in ASD. During sustained control of grip, visual feedback information from visual cortex is projected to posterior parietal cortex, and then anterior to premotor and primary cortices (Glickstein and Stein, 1991). A more efficient subcortical route through posterior cerebellum also is used to translate visual-spatial feedback information into a more precise motor command executed by motor cortex (Glickstein and Stein, 1991; Stein and Glickstein, 1992). Mosconi et al. (2015) and Wang et al. (2015) each found that individuals with ASD show increased sustained force variability during a precision gripping test in which they receive online visual feedback about their performance (**Figure 3**). In these studies, participants pressed on two opposing load cells with their thumb and index finger while a horizontal force bar moved upwards with increased force. They were instructed to press on the load cells so that the force bar reached the same level as a static target bar, and then to keep it there as steadily as possible. Individuals with ASD showed increased variability of their force output over time that became more severe at higher force levels and in relation to the gain of visual feedback (Mosconi et al., 2015). Elevations in force variability were evident both at the lowest and highest visual gains, suggesting that individuals with ASD have a reduced ability to process highly degraded and highly magnified visual feedback information.

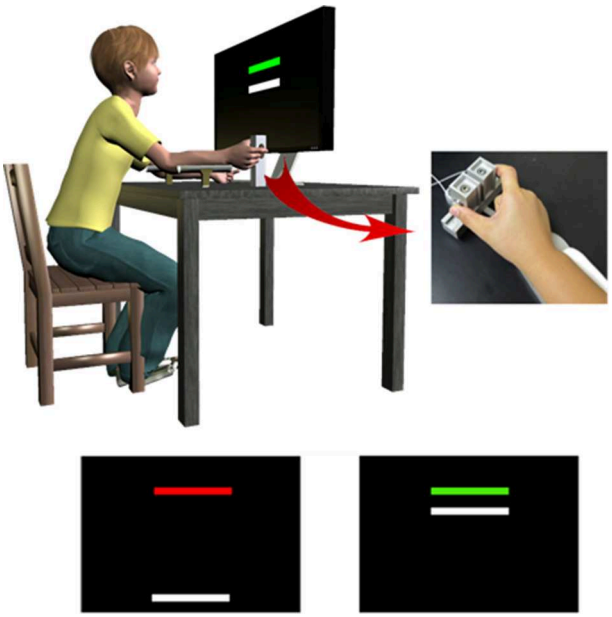

FIGURE 3 | To assess precision gripping control during rise, sustained, and relaxation phases, individuals pressed against two opposing load cells while receiving visual feedback from the monitor in front of them. Individuals viewed two horizontal bars: a red/green target bar and a white force bar. The white force bar moved upward with increased force, and individuals were instructed to press on the load cells as quickly as possible when the target bar turned green so that the force bar reached the height of the target bar. They also were instructed to keep the force bar as close as possible to the target bar until the target bar turned red again, and then to release the load cells as fast as possible. Adapted with permission from Wang et al. (2015).

Mosconi et al. (2015) also found that individuals with ASD showed less complexity in the time-dependent structure of their force output, suggesting a failure to utilize the multiple control processes required to rapidly and precisely adjust motor behavior, including visual, proprioceptive, somatosensory, and forward mechanisms. Analyses of spectral profiles identified increased power in the 0–4 Hz range for individuals with ASD and relatively decreased power at higher frequencies (4–12 Hz) indicating an overreliance on slower feedback mechanisms. This is an inefficient strategy during large force productions for which rapid corrections are necessary to reduce larger errors in behavioral outputs. As the time delay of the motor response is increased, there would be a greater grip force deviation from the target if slower mechanisms are used exclusively. Therefore, individuals with ASD appear to show central deficits in integrating sensory feedback information and dynamically adjusting motor output consistent with defects of neocortical-posterior cerebellar circuitry. Thus, deficits in feedback control processes supporting online motor adjustments also appear to be present in ASD.

Motor Learning

Cerebellar processes involved in learning and updating internal action representations of upper limb movements appear to

be compromised in ASD. During adaptation, error signals relayed via climbing fiber inputs to Purkinje cells invoke LTD that modifies the strength of population firing of GABAergic output from zones of Purkinje cells (Antunes and De Schutter, 2012). Initial studies of adaptation suggested that cerebellar learning occurs at a similar rate in ASD relative to healthy controls (Mostofsky et al., 2004; Gidley Larson et al., 2008). However, subsequent studies have indicated that individuals with ASD acquire new motor skills differently than controls. In a series of studies, Mostofsky and colleagues had participants complete a reaching adaptation task in which they moved a robotic handle toward a target. During this test, participants' moving arm was shielded from view, but they received visual feedback on a screen in front of them about the location of the handle and the target. Force perturbations were introduced perpendicular to the moving arm, and subjects thus changed the trajectory of their movement to counteract these forces and move as directly as possible to the target on subsequent trials. Participants then completed trials without force perturbations in which they demonstrated their ability to generalize their learned movement trajectories using an identical joint rotation (proprioceptive feedback) or hand motion (visual feedback) as the training movements. Haswell et al. (2009) and Izawa et al. (2012b) each found that individuals with ASD generalized their movements in proprioceptive coordinates to a greater extent than controls suggesting an overreliance on proprioceptive feedback information. Izawa et al. (2012b) and Marko et al. (2015) also found that individuals with ASD showed weaker generalization in visual space compared to controls implicating a reduced ability to integrate visual feedback information during motor learning. The latter study showed that reduced learning rates in ASD in visual coordinates were related to reduced volumes of anterior cerebellar lobules extending into lobules VI and VIII. Further, patients' over-reliance on proprioceptive feedback in acquiring new motor skills was associated with social and imitation impairments suggesting that fundamental deficits in motor control and learning may contribute to deficits in more complex social-motor skills in ASD (Haswell et al., 2009; Cook et al., 2013).

In summary, studies of upper limb movements, manual motor control, and motor learning implicate defects of forward control, sensory feedback control, and cerebellar dependent learning in ASD. These processes are supported by distinct zones of the cerebellum and their interactions with frontal and parietal cortices. Few functional MRI studies have examined cortical-cerebellar contributions to upper limb control. Allen and Courchesne (2003), Allen et al. (2004) each found increased anterior cerebellar activation and atypical contralateral and posterior cerebellar activation in subjects with ASD during a self-paced finger tapping test. During finger tapping tests in which participants follow a visual prompt, anterior, and ipsilateral cerebellar lobules show reduced activation in ASD (Müller et al., 2001; Mostofsky et al., 2009). Further, reduced activation was seen in thalamic and motor cortical targets in ASD and reduced functional connectivity between these motor regions also was documented (Müller et al., 2001; Mostofsky et al., 2009). Anterior cerebellar lobules IV–VI and their connections with frontal and parietal motor regions thus appear to be compromised in ASD. These effects may disrupt control and learning of upper limb and manual motor actions, and they could impact the development of more complex social motor skills that are central to the disorder.

Gait and Postural Control in ASD

Cerebellar circuits involved in controlling balance and gait have been identified in the vermis and intermediate cerebellum (Haines and Mihailoff, 2002; Apps and Garwicz, 2005; Ramnani, 2006). These regions receive afferent input both from motor and parietal cortices as well as direct innervation from the spinal cord (Apps and Garwicz, 2005). Afferent relays to the spinocerebellum originate from interneurons in the spinal gray matter that terminate as mossy fiber inputs in the vermis or intermediate cortex (Apps and Garwicz, 2005; Ramnani, 2006). Spinocerebellar inputs provide rapid proprioceptive feedback information that can be integrated with somatosensory, visual and vestibular feedback to maintain postural stability. Based on these inputs, individuals are able to align the projection of their body's center of mass within their base of support by actively manipulating the center of pressure under their feet while standing (Riccio, 1993; Winter, 1995; Horak, 2006). During walking, ventral spinocerebellar tracks carry internally generated information about movement rate and trajectories as well as the rhythmic discharge of somatic receptors to the cerebellum whereas the dorsal track provides sensory feedback information during the movements (Jahn et al., 2004; Hoellinger et al., 2013).

During walking, patients with spinocerebellar atrophy show an increased postural sway path along the anterior-posterior axis (Diener et al., 1985). Increased stride width and stride length variability are hallmark signs of ataxia evident in the majority of cerebellar patients during walking (Cavallari et al., 2013; Kafri et al., 2013). Patients with anterior cerebellar lesions also demonstrate issues when trying to maintain postural control while standing. In response to externally triggered perturbations, patients with alcohol-induced anterior cerebellar lobe syndrome showed increases in the magnitude of their EMG responses and overshooting of their initial postural compensation suggesting imprecision of forward control mechanisms (Horak and Diener, 1994). Abnormal EMG latencies recorded from anterior tibial and triceps surae muscles also have been observed in patients with anterior lobe atrophy as well as those with vestibulo-cerebellar lesions and Friedreich's ataxia (primarily affecting the spinocerebellar pathway) in response to unexpected rotations of a platform on which they were standing (Diener et al., 1984). These results suggest alterations in sensory feedback control processes that reactively adjust muscular forces used to maintain postural stability. These deficits differ somewhat from those reported in patients with basal ganglia or motor cortical dysfunctions suggesting that the cerebellum plays a highly specialized role in forward and feedback control of postural and balance mechanisms, and in coordinating the timing and amplitude of movements during walking.

General balance preservation when standing is a continuous process driven primarily by lower limb muscular reflexes (a feedback control process) that involve minimal effort or attention

(Woollacott and Shumway-Cook, 2002). When self-initiated (e.g., leaning forward) or externally triggered perturbations to the system are predicted, forward control processes are invoked to make anticipatory postural adjustments (APAs) and dynamically ensure balance (for details, see reviews by Massion, 1992; Aruin, 2002). For example, APAs allow an individual to release an object without falling over by initiating a compensatory backward sway through deactivation of postural flexor muscles (i.e., erector spinae and soleus) and activation of extensor muscles (i.e., rectus abdominis, rectus femoris, tibialis anterior), respectively (Aruin and Latash, 1995). Comparatively, if the load is slowly taken away by the experimenter, the backward postural sway and muscle activities will be attenuated or absent.

Few studies have examined APA mechanisms in ASD. Studying a small sample of children with ASD, Schmitz et al. (2003) found a unique pattern of atypical muscle activation/deactivation during a task of postural control. During this test, participants were seated in a chair with their left elbow flexed at 90° while their forearm rested on a support attached to the chair. A load was attached to a bracelet on the participant's left wrist. In trials of self-initiated unloading, participants unloaded the bracelet using their right hand when they felt ready. To stabilize their left forearm after unloading, healthy controls showed anticipatory adjustments involving activity in the biceps brachii 15 ms *before* unloading the weight. This change was followed by a stretch reflex of triceps brachii after the onset of unloading. In contrast, children with ASD showed delayed biceps brachii activation with its onset 58 ms *after* unloading and triceps activity attenuation throughout the trial. Results thus indicate that individuals with ASD are less able to use APA mechanisms to maintain postural control during self-initiated activities.

The neurophysiological substrates of APA have been examined in ASD using electroencephalogram (EEG) during imposed and self-initiated unloading tasks (Martineau et al., 2004). While EEG provides high frequency information on neocortical activity, it is less suitable for directly studying cerebellar mechanisms. However, given the known effects of cerebellar activity on motor and parietal cortical activity, analysis of EEG data during tests of APA and postural control may offer some insight into cortical-cerebellar functions in ASD (Manto et al., 2012).

During an externally-imposed unloading task, decreased power density at 6–8 Hz was observed after unloading corresponding to the cortical responses recorded during involuntary arm reflexes. This cortical response was identified bilaterally over motor cortices (i.e., C3 and C4) in both control children and children with ASD suggesting that unloading reflexes are intact in ASD (Schmitz et al., 2003). During a self-initiated bimanual unloading task, control children showed a significant decrease in power density above C3 and C4 400–500 ms before the onset of the action. However, children with ASD did not show corresponding anticipatory decreases in power density suggesting deficits in cortical systems involved in forward control mechanisms supporting APAs as well as possibly their cerebellar targets. However, this pattern of deficit appears to be different from what has been reported for patients with cerebellar lesions (Horak and Diener, 1994). While individuals with ASD show antagonist muscle activations that are largely delayed and depressed, postural corrections in patients with cerebellar lesions show a pattern of overshooting. Determining the mechanisms contributing to deficits in forward control processes involved in postural stability will be important for identifying abnormal circuits in ASD. This information may be particularly important in the context of findings that postural stability provides a critical foundation for a range of more complex and fine-grained motor behaviors.

During studies of balance, individuals with ASD have been found to show increased postural sway across the lifespan (Molloy et al., 2003; Minshew et al., 2004; Chang et al., 2010; Fournier et al., 2010a; Radonovich et al., 2013; Memari et al., 2014). An important observation from these studies is that children with ASD showed greater medio-lateral as opposed to anterior-posterior postural sway, and that their medio-lateral range of motion was greater than that for typically developing controls (Kohen-Raz et al., 1992; Chang et al., 2010; Fournier et al., 2010a; Memari et al., 2014). Increased postural sway in medio-lateral directions during quiet stance is commonly observed in young children under the age of 10 years who show a reduced ability to maintain balance in the anterior-posterior direction causing them to increase their base of support in medio-lateral directions to compensate from internal perturbations (Maki et al., 1990; Riach and Starkes, 1994; Slobounov and Newell, 1994; Prieto, 1996). Studies documenting postural instability in ASD thus suggest that this system remains immature throughout development (Minshew et al., 2004).

When required to stand as still as possible under naturalistic conditions, healthy controls primarily rely on somatosensory information, followed by vestibular and then visual feedback (Peterka, 2002; Horak, 2006). Increased weighting toward vestibular and visual information can be induced by reducing the stability of the standing surface and thus limiting somatosensory feedback (Massion, 1994; Peterka, 2002; Horak, 2006). When asked to stand on a foam board to provide an unstable surface, individuals with ASD showed increased variability of their center of pressure over time and a reduction of postural stability compared to healthy controls (Molloy et al., 2003). This deficit became more severe when participants kept their eyes shut suggesting a greater reliance on visual feedback control processes and a reduced ability to utilize vestibular information to help preserve balance.

During tests of dynamic standing in which participants attempt to maintain balance in a virtual environment with oscillatory visual feedback information, healthy controls are able to optimize the frequency of their postural sway to match that of visual feedback (Slobounov et al., 1997, 1998). In contrast, individuals with ASD showed attenuated postural sway at 0.2 Hz (Gepner et al., 1995; Gepner and Mestre, 2002). Greffou et al. (2012) also found that younger participants with ASD (ages 12–15 years old as opposed to 16–33 years old) displayed significantly less postural sway than controls when visual stimuli oscillated at 0.5 Hz. These findings indicate that visual feedback mechanisms used to help dynamically support postural control are compromised in individuals with ASD.

Gait

At the initiation of walking, there is a purposeful uncoupling of the center of pressure and center of mass (Winter, 1983, 1995; Remelius et al., 2014). The center of pressure shifts posteriorly to generate forward momentum and propel the center of mass forward. The center of pressure also shifts initially toward the swing leg as a result of the unloading of the stance leg generating the initial lateral acceleration of the center of mass toward the stance leg (Winter, 1995). Studying gait initiation, (Fournier et al., 2010b) found that individuals with ASD showed intact center of pressure posterior shifts but a significantly reduced center of pressure lateral range of motion indicating a reduced capacity to generate lateral momentum and propel the upper body from side-to-side.

Walking has been described as a "throw-catch up" process during which the body propels the center of mass into an unstable state beyond the anterior limit of the base of support in what is often called a "controlled fall" (Winter, 1983, 1995; Remelius et al., 2014). The catch up process then involves taking a step forward to slow momentum and create a new base of support. Early studies of gait in ASD identified Parkinsonian features including dystonia, involuntary dyskinesia of the extremities, abnormalities in muscle tone, rigidity, hypertonia, and decreased coordination of arm and leg movements. Studying individuals with ASD, Vilensky et al. (1981) observed reduced stride length, increased stance duration, increased hip joint flexion at "toe-off" and reduced knee extension and ankle dorsiflexion at initial contact. Additional features reported in a series of observational studies included toe-striking, strides in which the whole foot was simultaneously placed on the ground at the phase of initial contact rather than as a heel-strike, "striatal" toes (i.e., spontaneous upward movement of the big toes similar to the Babinski reflex), claw toe and hand posture (Walker and Coleman, 1976; Folstein and Rutter, 1977; Damasio and Maurer, 1978; Teitelbaum et al., 1998; Esposito and Venuti, 2008). Vernazza-Martin et al. (2005) reported significant increases in ASD patients' head, shoulder, trunk, and hip angular motion that were associated with an increased variability in the trajectories of their walking paths. Increased stride length and stride width variability also have been commonly observed in individuals with ASD (Blin et al., 1990; Vernazza-Martin et al., 2005; Rinehart et al., 2006).

The mechanisms that contribute to walking abnormalities remain unclear as patterns do not cleanly fit with models of ataxia, basal ganglia dysfunction, or other neurological disorders. However, sensory processing and integration disturbances appear to play a significant role in the walking disturbances observed in individuals with ASD. Rinehart et al. (2006) studied participants with ASD who were instructed to walk either at their own preferred speed (stride length) or with strides that were 20% greater than their average stride length. For the longer stride condition, participants walked either utilizing visual cues indicating how long to stride, or without visual cues. In contrast to healthy controls, individuals with ASD showed significant increases in stride-length variability for both preferred and visually cued conditions indicating an impaired ability to consistently anticipate the amplitude of the targeted movement or to use visual feedback to guide the amplitude of the movement.

In summary, studies of posture, gait initiation and walking in ASD implicate gross motor impairments consistent with deficits of both forward control and sensory feedback mechanisms involving medial and intermediate cerebellar circuits. These deficits may reflect alterations in spinocerebellar tracts that innervate the cerebellum primarily through the inferior cerebellar peduncle (Cheng et al., 2010). Further studies examining spinocerebellar circuit anatomy and function are needed to better understand the integrity of these networks in ASD and their relation to gross motor abnormalities in patients.

Early Dysmaturation of Sensorimotor Systems in ASD

Multiple cerebellar circuits involved in sensorimotor control thus appear to be compromised in ASD. These circuits undergo rapid refinement during the early postnatal period that, if derailed, could have a significant impact both on sensorimotor behavior and other aspects of development (Ashwell and Zhang, 1992). The cerebellum undergoes rapid growth during the last trimester and early postnatal period. Mossy fibers form transient contacts with developing Purkinje cells during embryonic development, but they do not form their parasagittal zones with Purkinje cells until shortly after birth (Arsénio Nunes and Sotelo, 1985). Climbing fibers become organized into parasaggital stripes by late embryogenesis in the rat and mouse (Sotelo et al., 1984; Chedotal and Sotelo, 1992; Paradies et al., 1996). At a more macroscopic level, the cerebellum has been shown to undergo the greatest amount of volumetric increase among all studied brain regions during the first 30 days of life (Holland et al., 2014). Perinatal disruptions of neurodevelopment could selectively affect these processes that are occurring rapidly around birth.

To add to the vulnerability of the cerebellum, Purkinje cells are a relatively large (50–80 μm) class of neurons whose synapses with olivary climbing fibers form some of the most energy demanding connections in the brain (Sugimori and Llinás, 1990; O'hearn and Molliver, 1997; Welsh et al., 2002). As a result of the high level of excitatory amino acid synaptic connections and the response of the Purkinje cell that is mediated by voltage-gated and receptor-gated calcium channels, the Purkinje cell has an exceptionally high metabolic demand. This high metabolic demand combined with constant input from the inferior olive and large amounts of calcium stores and influx makes the cell particularly vulnerable to excessive rises in intracellular calcium that are associated with excitotoxicity and cell death (Vajda, 2002).

These factors may help explain the repeated findings of Purkinje cell pathology in ASD. Studies of other cerebellar cells provide insight into possible timing and mechanisms. Purkinje cells synapse with basket and stellate interneurons to support their survival. In the lone study to count the number of basket and stellate cells in brain tissue from individuals with ASD, no difference in the number of these interneurons were found suggesting that Purkinje cells were generated, migrated to their

proper location and then atrophied or died (Whitney et al., 2009). There also is no apparent loss of climbing fibers from the inferior olive (Whitney et al., 2008). Climbing fibers synapse with Purkinje cells shortly before birth but die off if there is Purkinje cell loss (Holmes and Stewart, 1908; Whitney et al., 2008). Findings that there is no loss of climbing fiber inputs suggest that Purkinje cell loss occurs prenatally, rather than as a regressive effect of later alterations in cortical feedback. Consistent with this hypothesis, Bauman and Kemper (1985) report an absence of gliosis. However, Bailey et al. (1998) reported gliosis in a subset of post-mortem tissue of individuals with ASD that could be associated with postnatal loss of Purkinje cells. Further research is needed to resolve this important discrepancy.

Sensorimotor abilities also undergo rapid maturation during the early postnatal period. These skills form the basis for multiple aspects of cognitive and language development, and their disruption could directly contribute to the social-communication deficits that define ASD (Lebarton and Iverson, 2013). Sensorimotor impairments have been repeatedly shown to be associated with social and language impairments in ASD (Takarae et al., 2004b; Haswell et al., 2009; Mosconi et al., 2009, 2015; Cook et al., 2013) and variable in terms of their severity over the course of development (Takarae et al., 2004b; Luna et al., 2007; Mosconi et al., 2015). Both retrospective videotape analyses and prospective studies of infant siblings of children with ASD have documented abnormal sensorimotor development within the first year of life affecting postural control, crawling and early walking, fine motor movements, prehension, and eye movements (Brian et al., 2008; Lebarton and Iverson, 2013; Ben-Sasson and Gill, 2014; Leonard et al., 2014; Ozonoff et al., 2014; Sacrey et al., 2015). Evidence that sensorimotor deficits may be present before the defining features of the disorder further indicates that their study in infancy may be highly informative for early diagnostic efforts aimed at guiding early interventions.

Preclinical Modeling of Cerebellar Involvement in ASD

In addition to being affected in idiopathic ASD, the cerebellum has been consistently implicated in several monogenetic syndromes associated with ASD (e.g., FXS, Phelan-McDermid Syndrome). And, its disruption appears to be selectively related to the severity of patients' ASD symptoms (Kaufmann et al., 2003; Eluvathingal et al., 2006; Aldinger et al., 2013). These findings suggest that preclinical genetic models may advance a more mechanistic understanding of the cerebellum's contributions to the pathogenesis of ASD. Preclinical models also provide a means to develop and ultimately test targeted therapeutics that will benefit sensorimotor and behavioral dysfunctions in ASD. To this end, multiple preclinical models have emerged that have shed light on the underlying pathophysiology of ASD. Here, we will focus on several models of known monogenetic disorders in which a disproportionate number of patients meet criteria for ASD, and chromosomal or gene abnormalities associated with high rates of ASD. Sensorimotor impairments have been reported for many of these models, and cognitive and behavioral dysfunctions consistent with and pathognomonic for cerebellar dysfunction also have been reported.

Fragile X Syndrome (FXS)

FXS is caused by expansion of CGG trinucleotide repeats in the Fragile X mental retardation 1 (FMR1) gene, which codes for the Fragile X Mental Retardation Protein 1 (FMRP1). Resulting methylation of the FMR1 promoter results in the absence of functional FMRP1 protein product. Individuals with FXS show developmental/intellectual disability and high rates of ASD and account for ∼1–2% of total ASD cases. Fmr1 knockout mice display hyperactivity, repetitive behaviors, impaired learning and memory, and variable social impairments (1994; Koekkoek et al., 2005; Spencer et al., 2005; McNaughton et al., 2008; Moy et al., 2009). Mutant mice also demonstrate cerebellar abnormalities with elongated spines and enhanced LTD (Koekkoek et al., 2005) consistent with enhanced plasticity in other brain regions (Bear et al., 2004). Fmr1 global knockout mice as well as Purkinje cell specific fmr1 knockouts demonstrate impairments in eyeblink conditioning, a form of associative learning requiring intact cerebellar function (Freeman and Steinmetz, 2011). Patients with FXS demonstrate similar deficits in eyeblink conditioning (Koekkoek et al., 2005) as well as cerebellar-associated motor dysfunctions (Zingerevich et al., 2009). Taken together with studies showing abnormal eyeblink conditioning in human studies of patients with idiopathic ASD (Sears et al., 1994; Oristaglio et al., 2013), these data support the presence of abnormal cerebellar function in patients with idiopathic ASD and ASD associated with Fragile X disorders.

Tuberous Sclerosis Complex (TSC)

TSC, like FXS, is a monogenetic disorder associated with intellectual and neurodevelopmental disability, seizures, and ASD (∼50%), contributing to 1–2% of ASD patients. This disorder results from mutations of a single copy of either TSC1 or TSC2, whose protein products heterodimerize and act to negatively regulate the mechanistic target of rapamycin (mTOR) protein, a critical regulator of protein translation (Kelleher and Bear, 2008; Thoreen et al., 2012). Patients with TSC show fine motor impairments (Jeste and Geschwind, 2014), and patients with mutations in the TSC2 gene have demonstrably smaller cerebella (Weisenfeld et al., 2013). Moreover, cerebellar lesions associated with ASD in TSC and the deep cerebellar nuclei have been found to be abnormal in patients with ASD and TSC (Weber et al., 2000; Eluvathingal et al., 2006), suggesting that cerebellar dysfunction may play a selective role in the pathogenesis of ASD in TSC (Asano et al., 2001).

To better understand whether cerebellar dysfunction was sufficient to cause ASD behaviors, Tsai et al generated a mouse model lacking tsc1 in cerebellar Purkinje neurons. These mutant mice demonstrated behaviors associated with ASD—social impairment, repetitive behavior, inflexible behavior, vocalization abnormalities—in addition to electrophysiologic abnormalities and cellular pathology similar to that seen in TSC patients (Tsai et al., 2012). In addition, abnormalities in delayed

eyeblink conditioning similar to those seen in patients with ASD and FXS are seen in these mice (Kloth et al., 2015). Early postnatal treatment of these mutant mice with the mTOR specific inhibitor rapamycin prevented the development of motor and ASD behaviors and the development of cerebellar pathology (Tsai et al., 2012). These findings were subsequently replicated in a mouse model where tsc2 was deleted in cerebellar Purkinje cells (Reith et al., 2013). Whether later treatment will be efficacious in treatment of these abnormalities remains an interesting avenue for further study.

Shank 3

SH3 and multiple ankyrin repeats 3 (Shank3)/proline rich synapse associated protein 2 (ProSap2) has been implicated to be the critical, pathologic gene responsible for Phelan-McDermid Syndrome in patients with 22q13 deletions/mutations. Phelan-McDermid Syndrome is characterized by elevated rates of neurodevelopmental disability, seizures, sleep disorders, and ASD (Phelan and McDermid, 2012; Soorya et al., 2013; Sarasua et al., 2014). Although mutations can be causal in Phelan-McDermid Syndrome, SHANK3 mutations have also been identified in several cases of idiopathic ASD. SHANK3 is a postsynaptic scaffolding protein that plays critical roles in excitatory synaptic transmission (Zoghbi and Bear, 2012) and is expressed widely throughout the brain, including the cerebellum (Peça et al., 2011). Rodent models with loss of Shank3 display social impairments, repetitive behaviors, abnormal vocalizations, and impaired learning (Bozdagi et al., 2010; Peça et al., 2011; Wang et al., 2011). Although the precise role for cerebellar Shank3 has not been studied, Shank3 mutant mice demonstrate significant abnormalities in cerebellar anatomy (Ellegood et al., 2014) and deficits in cerebellar function with impaired delayed eyeblink conditioning (Wang et al., 2014).

15q11-13 Duplication

15q11-13 duplication has been identified in up to 3% of cases of ASD, making it the most frequently identified chromosomal abnormality in ASD (Urraca et al., 2013). This region is genetically complex as maternal deletion of the region results in Angelman's Syndrome while paternal deletion results in Prader-Willi syndrome. Patients with 15q11-13 duplications have elevated rates of motor dysfunction (Urraca et al., 2013), while both deletion related syndromes (Angelman's and Prader-Willi) also are characterized by profound motor abnormalities (Buiting, 2010). Mouse models of paternal 15q11-13 duplication demonstrate autistic-like behaviors including social dysfunction, behavioral rigidity, and abnormal vocalizations (Nakatani et al., 2009). To investigate cerebellar contributions to these behaviors, Piochon et al. (2014) examined these mice and identified abnormalities in motor learning, reduced eye blink conditioning, impaired parallel fiber-Purkinje cell LTD, and impaired elimination of surplus climbing fiber inputs to Purkinje cells.

Neuroligin 3

Cerebellar dysfunction also has been implicated in models of nonsyndromic ASD. Neuroligin 3 (nl3) encodes a postsynaptic adhesion molecule involved in synapse assembly (Südhof, 2008). Mutations (both point mutations and deletions) have been identified in ASD patients (Jamain et al., 2003; Levy et al., 2011; Sanders et al., 2011). Nl3 mutant mice (either point mutation knockin or deletions) demonstrate autistic-like behaviors including motor coordination impairments, social impairments, repetitive behaviors, and abnormal vocalizations (Baudouin et al., 2012), while mice with a nl3 knockin mutation demonstrate changes in cerebellar anatomy on MRI (Steadman et al., 2014). Purkinje cell specific nl3 mutant mice demonstrate increased hyperactivity (Rothwell et al., 2014), while Purkinje cell specific expression of nl3 rescued motor coordination deficits in knockout mice, consistent with a critical role for cerebellar nl3 in the pathogenesis of ASD behaviors (Baudouin et al., 2012).

Engrailed 2

ENGRAILED 2 (En2) is a homeobox transcription factor that has been implicated in ASD through numerous genetic association studies (Gharani et al., 2004; Benayed et al., 2005). En2 is highly expressed in the cerebellum and abnormalities in En2 expression levels have been identified from postmortem ASD cerebellum (James et al., 2013; Choi et al., 2014). En2 transgenic mice demonstrate abnormal cerebellar development while En2 knockout mice display reduced cerebellar volumes, reduced Purkinje neuronal numbers, and abnormalities in cerebellar foliation (Millen et al., 1994; Ellegood et al., 2014). Knockout mice display motor and social impairments but demonstrate normal vocalizations and grooming behaviors (Brielmaier et al., 2012). They also demonstrate reductions in noradrenergic levels. When targeted with norepinephrine reuptake inhibitor therapy, amelioration of abnormal behaviors results, suggesting a potential avenue of targeted therapy (Brielmaier et al., 2014).

CADM1

Cell Adhesion molecule 1 (Cadm1) is a synaptic cell adhesion molecule that has been identified as a rare genetic cause of ASD (Zhiling et al., 2008). Cadm1 is highly expressed in the dendritic arbor of Purkinje neurons and Cadm1 knockout mice demonstrate reductions in cerebellar size (Fujita et al., 2012) and abnormal social behaviors, abnormal vocalizations, increased anxiety, and abnormal motor coordination (Takayanagi et al., 2010; Fujita et al., 2012).

RORα

Retinoic Acid receptor-related orphan receptor alpha (Rorα) has been implicated in sporadic cases of ASD. The naturally occurring staggerer mouse has a mutation in this gene, and as its name suggests, it displays profound motor dysfunction and ataxia (Sotelo and Changeux, 1974; Steinmayr et al., 1998). Loss of this gene results in subsequent cerebellar hypoplasia with marked loss of Purkinje neurons (∼80%) and a comparable loss of granule cells. In addition to motor deficits, these mice demonstrate abnormal learning and aberrant responses to novel stimuli (Goldowitz and Koch, 1986; Lalonde et al., 1996a,b), although these abnormalities are potentially adversely affected by the profound motor dysfunction seen in these animals.

Integrin 3

Hyperserotonemia has been identified in ∼30% of sporadic ASD cases, and Integrin 3β (ITGB3) has been implicated in genetic regulation of serotonin levels through interactions with serotonin transporters. Certain haplotypes of itgb3 are associated with ASD (Weiss et al., 2006; Napolioni et al., 2011) while a mutation in itgb3 has been identified in a patient with ASD (O'roak et al., 2012). Because of critical roles for itgb3 in platelet function, autistic behaviors in knockout models have not been fully evaluated, although cerebellar anatomy has been shown to be reduced in these mutant mice (Steadman et al., 2014). However, heterozygous itgb3 mutants display both abnormal social behaviors and increased repetitive behaviors (Carter et al., 2011), implicating potential roles for cerebellar ITGB3 in the pathogenesis of ASD.

Valproic Acid

Environmental exposures have also been linked to elevated ASD risk. One such model that has been widely examined is exposure to valproic acid (VPA). VPA is widely used as an antiepileptic medication and/or for its mood stabilization properties in the treatment of bipolar disorders. *In utero* exposure to VPA during the first trimester has been linked to increased risk of congenital malformations including neural tube defects but has also been linked to increased risk of ASD development (reviewed in Roullet et al., 2013). Numerous rodent models of VPA exposure have demonstrated impairments in motor, social, and communication behaviors in addition to increased repetitive behaviors (Roullet et al., 2013). Reductions in Purkinje cell numbers and cerebellar size have been demonstrated in these models (Ingram et al., 2000), and abnormal eye blink conditioning has been observed in mice with early VPA exposure (Stanton et al., 2007). Interestingly, motor activity has been reported to ameliorate VPA induced behavioral alterations while exposure to the antioxidant piperine has been reported to ameliorate behavior and pathology in the cerebellum (Pragnya et al., 2014). As markers of oxidative stress are noted to be increased in the cerebellum in postmortem studies of ASD brains (Sajdel-Sulkowska et al., 2009, 2011) and in genetic models of ASD (Tsai et al., 2012), these findings suggest the possibility of shared mechanisms in the pathogenesis of ASD.

Future Studies

Findings from histopathology, gene expression, *in vivo* imaging and sensorimotor studies each suggest a critical role for the cerebellum in the pathophysiology of ASD. Comparisons across studies suggest that different cerebellar circuits may be variably affected in different patients. Given the crucial problem of resolving biological heterogeneity in ASD, clarification of patterns of altered function in these different circuits may provide a much needed window into biological mechanisms affecting different patients or patient subgroups and their clinical implications. Studies aimed at determining the regional specificity of cerebellar pathology across different lobules and subregions are necessary for identifying whether cerebellar defects are more diffuse or specific to distinct circuits. Comprehensive assessments of multiple distinct sensorimotor abilities and their development over early childhood in ASD are needed. Further, integrated analyses across different levels including combined preclinical and clinical assessments are needed to better understand how genetic and molecular processes relate to cellular and brain system anomalies as well as clinical symptoms.

Rodent models of ASD provide an important means through which the contribution of cerebellar dysfunction to the pathogenesis of ASDs can be better understood. However, most studies thus far have been limited to describing evidence of cerebellar dysfunction in global models of ASD (Zingerevich et al., 2009; Baudouin et al., 2012; Roullet et al., 2013; Brielmaier et al., 2014; Ellegood et al., 2014; Piochon et al., 2014; Steadman et al., 2014). Only a few studies have specifically examined the effects of targeted disruption of cerebellar neurons on sensorimotor functions, cerebellar learning paradigms such as eye blink conditioning (Koekkoek et al., 2005; Kloth et al., 2015), ASD defining behaviors (Tsai et al., 2012; Reith et al., 2013), and other behaviors associated with neurodevelopmental disorders (Rothwell et al., 2014). As these models are further evaluated, the contributory role for the cerebellum in motor and non-motor behavioral dysfunction can be elucidated.

The neuronal circuitry by which the cerebellum regulates these diverse behaviors and aspects of development also remains to be further clarified. Studies demonstrate connections between the cerebellum and medial prefrontal cortices in rodents and primates, with ASD models demonstrating dysfunction in these cerebellar mediated circuits (Rogers et al., 2011, 2013; Bostan et al., 2013). Technology has been developed to pair genetic tools with neuromodulatory paradigms through chemical means (Sternson and Roth, 2014) or light based approaches (Steinberg et al., 2015) for use *in vitro* and for *in vivo* preclinical animal studies. These technologies provide a promising approach for teasing apart circuit based mechanisms underlying complex behaviors, such as those dysregulated in ASD. In combination with cerebellar targeting, these technologies offer intriguing potential to identify the pathogenic cerebellar circuits mediating behaviors that are dysregulated in neurodevelopmental disorders such as ASD, and they raise the possibility of targeted, cerebellar mediated therapeutic development.

Mouse models, as demonstrated, provide a powerful model system to further explore the contribution of cerebellar dysfunction to sensorimotor and cognitive/behavioral dysfunction in patients with ASD. With sensorimotor behavioral paradigms in combination with cerebellar learning modalities (eye blink conditioning) and cerebellar mediated reflexes (oculocephalic reflex), these models provide tools to better delineate cerebellar dysfunction in ASD. Considering that some of these paradigms—motor function, eye blink conditioning as examples—can be tested during early development or even during the neonatal period (Little et al., 1984; Fifer et al., 2010), these modalities could also emerge as biomarkers that could contribute to early ASD diagnosis.

Summary

Converging clinical and preclinical data identify an important role for the cerebellum in the pathogenesis of both monogenic syndromes associated with ASD and idiopathic ASD. These data identify anatomical and functional alterations of multiple distinct cerebellar circuits involving areas of neocortex and subcortical regions such as the basal ganglia. The cerebellum's rapid postnatal growth suggests that there may be critical periods of development during which it helps to scaffold the specialization of association cortices and other later developing brain systems (Rice and Barone, 2000). While gene expression studies show that ASD implicated networks are highly expressed in postnatal cerebellum, the timing of cerebellar defects across circuits has not yet been assessed. Narrowing the window during which these genetic and epigenetic events may disrupt cerebellar maturation will be critical for developing biomarkers and effective therapeutics for different forms of ASD. Evidence from preclinical model studies shows the possibility for selective rescue of cerebellar pathology in TSC (Tsai et al., 2012) and perhaps other forms of ASD. Increased attention to the role of cerebellar pathology in sensorimotor and other ASD-related behaviors may thus provide critical insights into pathogenic mechanisms as well as novel targeted molecular, cellular, and anatomic based therapeutics.

Author Contributions

All authors contributed to the conception of this work and revising it critically for important intellectual content. All authors provided final approval of the version to be published and agree to be accountable for all aspects of the work including ensuring that questions related to the accuracy or integrity of any part of the work be appropriately resolved. MM contributed to drafting, organizing, and critically evaluating all sections of this paper. ZW contributed to drafting sections relating to upper limb control and gait/posture. LS contributed to drafting the oculomotor section. PT contributed to drafting the section on preclinical modeling.

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REVIEW
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Cerebro-cerebellar circuits in autism spectrum disorder

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The cerebellum is one of the most consistent sites of abnormality in autism spectrum disorder (ASD) and cerebellar damage is associated with an increased risk of ASD symptoms, suggesting that cerebellar dysfunction may play a crucial role in the etiology of ASD. The cerebellum forms multiple closed-loop circuits with cerebral cortical regions that underpin movement, language, and social processing. Through these circuits, cerebellar dysfunction could impact the core ASD symptoms of social and communication deficits and repetitive and stereotyped behaviors. The emerging topography of sensorimotor, cognitive, and affective subregions in the cerebellum provides a new framework for interpreting the significance of regional cerebellar findings in ASD and their relationship to broader cerebro-cerebellar circuits. Further, recent research supports the idea that the integrity of cerebro-cerebellar loops might be important for early cortical development; disruptions in specific cerebro-cerebellar loops in ASD might impede the specialization of cortical regions involved in motor control, language, and social interaction, leading to impairments in these domains. Consistent with this concept, structural, and functional differences in sensorimotor regions of the cerebellum and sensorimotor cerebro-cerebellar circuits are associated with deficits in motor control and increased repetitive and stereotyped behaviors in ASD. Further, communication and social impairments are associated with atypical activation and structure in cerebro-cerebellar loops underpinning language and social cognition. Finally, there is converging evidence from structural, functional, and connectivity neuroimaging studies that cerebellar right Crus I/II abnormalities are related to more severe ASD impairments in all domains. We propose that cerebellar abnormalities may disrupt optimization of both structure and function in specific cerebro-cerebellar circuits in ASD.

Keywords: autism spectrum disorder, cerebellum, neuroimaging, diffusion tensor imaging, voxel based morphometry, resting state MRI, cerebo-cerebellar circuits, functional connectivity

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INTRODUCTION

Post-mortem, genetic, animal models, neuroimaging, and clinical evidence suggest that cerebellar dysfunction may play a crucial role in the etiology of autism spectrum disorder (ASD; for reviews, see Becker and Stoodley, 2013; Wang et al., 2014). The cerebellum is one of the most consistent sites of abnormality in autism (Allen, 2005; Fatemi et al., 2012), with differences reported from the cellular to the behavioral level. The majority of post-mortem studies of ASD report decreased Purkinje cell counts in the cerebellar cortex (Fatemi et al., 2002; Bauman and Kemper, 2005), and ASD-like symptoms can be induced by specifically targeting cerebellar Purkinje cells in animal models (Tsai et al., 2012). Cerebellar structural differences are associated with social and communication impairments as well as restricted interests and repetitive behaviors,

the hallmarks of the ASD diagnosis, in both human studies (Pierce and Courchesne, 2001; Rojas et al., 2006; Riva et al., 2013; D'Mello et al., 2015) and animal models of ASD (Ingram et al., 2000; Brielmaier et al., 2012; Tsai et al., 2012). The cerebellar cortex was consistently abnormal in an analysis of over 26 mouse models of ASD (Ellegood et al., 2015), and cerebellar atrophy is characteristic of one of the most widely used animal models of ASD, the valproic acid model (Ingram et al., 2000). At the genetic level, genes implicated in ASD (e.g., SHANK3, EN2, RORA) are often involved in cerebellar development (see Rogers et al., 2013 for review). This suggests that cerebellar development may be disrupted in ASD, which could have major knock-on effects on the structure and function of the multiple regions of the cerebral cortex with which the cerebellum forms reciprocal connections (see Wang et al., 2014; for reviews, see Strick et al., 2009; Stoodley and Schmahmann, 2010; Buckner et al., 2011).

The cerebellum is interconnected with distributed regions of the cerebral cortex, including regions involved in sensation (e.g., Snider and Stowell, 1944), movement (e.g., Snider and Eldred, 1951), attention (e.g., Kellermann et al., 2012), reward/motivation (e.g., Snider and Maiti, 1976), language (e.g., Schmahmann and Pandya, 1997; Kelly and Strick, 2003; Booth et al., 2007; Strick et al., 2009), social processing (e.g., Jissendi et al., 2008; Sokolov et al., 2012; Jack and Pelphrey, 2014), memory (e.g., Heath and Harper, 1974), and executive function (e.g., Middleton and Strick, 2000; Habas et al., 2009). This extensive connectivity provides an anatomical substrate by which cerebellar dysfunction could be involved in the large spectrum of symptoms that comprise the autism diagnosis (Rogers et al., 2013). We hypothesize that disruptions in *specific* cerebro-cerebellar loops in ASD might impede the functional and structural specialization of cortical regions involved in motor control, language, and social interaction, leading to developmental impairments in these domains. Here, after providing background information about cerebellar topography and cerebro-cerebellar circuits, we discuss the potential importance of the cerebellum in development, and review structural and functional neuroimaging studies describing regional cerebellar differences and disrupted cerebro-cerebellar circuits in ASD. We frame these findings in the context of the broader cerebro-cerebellar circuits involved in movement, language, and social cognition. We then address potential mechanisms by which cerebellar dysfunction could impact the core behavioral features of ASD. Finally, we suggest future directions for research.

CEREBELLAR TOPOGRAPHY AND CEREBRO-CEREBELLAR CIRCUITS

The emerging topography of sensorimotor, cognitive, and affective subregions in the cerebellum provides an important framework for interpreting the functional significance of cerebellar findings in ASD and their relationship with broader cerebro-cerebellar circuits. The cerebellum forms reciprocal, closed-loop circuits with much of the cerebral cortex as well as subcortical structures; because of this closed-loop organization and uniform circuitry, it is thought that the cerebellum contains repeating processing modules, the function of which is driven by the input the module receives (Schmahmann, 1991; Ito, 2006). Therefore, functional subregions of the cerebellum exist because different regions of the cerebellum form circuits with specific regions of the cerebral cortex. The anterior cerebellum is structurally and functionally connected to sensorimotor areas of the cerebral cortex, while the posterior cerebellum is structurally and functionally connected to "cognitive" regions, including prefrontal, and parietal association cortices (Strick et al., 2009; Stoodley and Schmahmann, 2010; Buckner et al., 2011; see **Figures 1**, **2**). The cerebellar deep nuclei—which receive projections from the cerebellar cortex and send output fibers from the cerebellum—also mirror this functional topography. In particular, the large dentate nuclei can be separated into dorsal and ventral regions that project to non-motor and motor regions of the cerebral cortex, respectively (Dum and Strick, 2003; Küper et al., 2011). This cerebellar functional topography is robust and is evident even at the individual level (Stoodley et al., 2010).

The specific cerebro-cerebellar circuits described above are involved in different aspects of behavior. In clinical studies, the location and lateralization of cerebellar damage can predict the resulting symptomology. Damage to the anterior cerebellum can result in motor symptoms such as ataxia (Schmahmann et al., 2009), while posterior lobe damage can lead to cognitive impairments and affective dysregulation (the Cerebellar Cognitive Affective Syndrome; Schmahmann and Sherman, 1998). Posterior vermal tumor resection has been associated with behavioral disturbances and affective dysregulation (Levisohn et al., 2000; Riva and Giorgi, 2000), reflecting anatomical connections between the vermis and limbic areas (Stoodley and Schmahmann, 2010; Buckner et al., 2011). Deficits following cerebellar damage can reflect the largely contralateral cerebro-cerebellar projections, as right cerebellar hemispheres interconnect with left cerebral language regions, and left cerebellar hemispheres form circuits with right cerebral cortical regions involved in spatial processing. Consistent with these projections, damage to the right cerebellar hemisphere in children has been associated with reduced verbal and literacy skills (Riva and Giorgi, 2000; Scott et al., 2001; Bolduc and Limperopoulos, 2009; Bolduc et al., 2012), while left cerebellar damage has been associated with impaired non-verbal or spatial skills (Riva and Giorgi, 2000; Scott et al., 2001). Lastly, lesions and neuromodulation of the cerebellum alter neural activity in regions of the cerebral cortex to which the cerebellum projects (e.g., Galea et al., 2011; Adamaszek et al., 2015), reflecting the functional impact of these long-range cerebro-cerebellar projections.

THE CEREBELLUM, THE DEVELOPING BRAIN, AND NEURODEVELOPMENTAL DISORDERS

Cerebellar structural and functional differences are found in several neurodevelopmental disorders, including attention deficit hyperactivity disorder (ADHD) and developmental dyslexia as

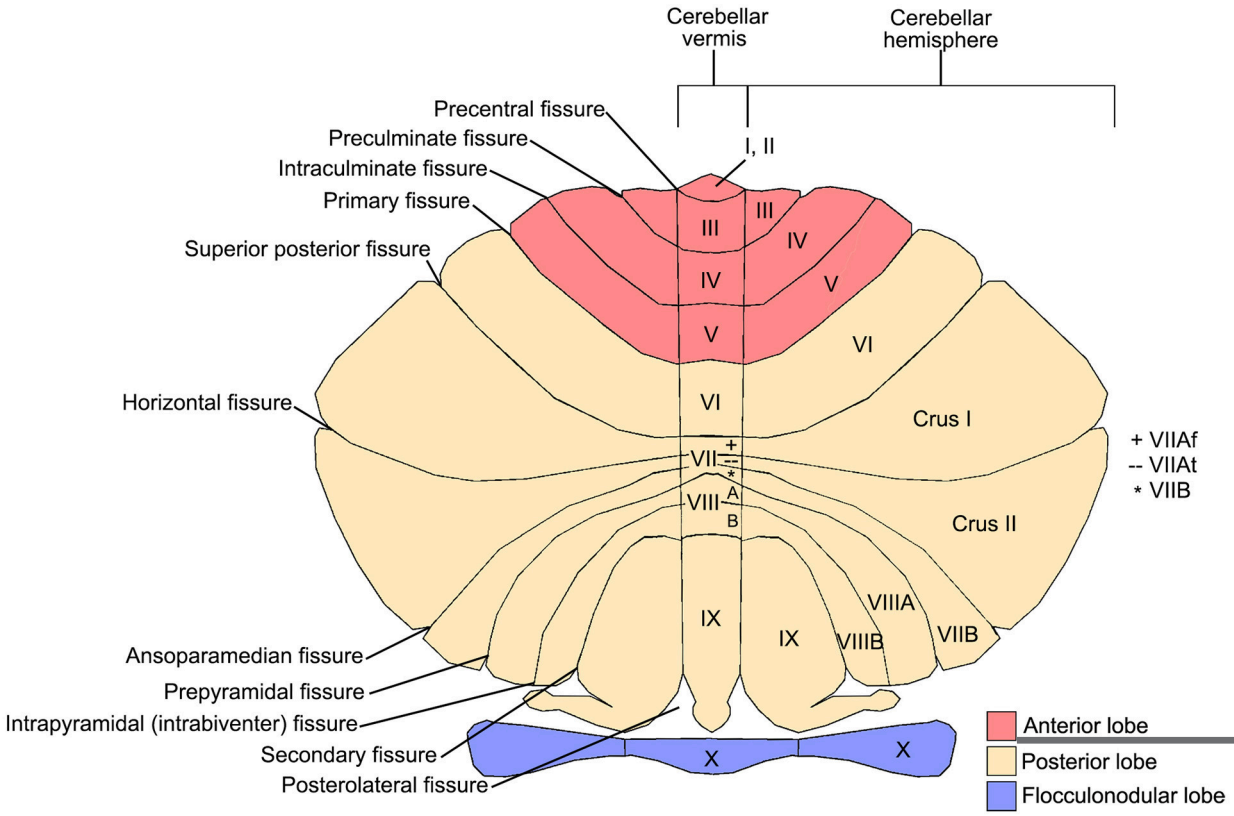

FIGURE 1 | Cerebellar anatomy showing major fissures, lobes, and lobules. The cerebellum is flattened to show the anterior lobe (red; lobules I-V), posterior lobe (cream; lobules VI-IX), and flocculonodular lobe (purple; lobule X). The 10 cerebellar lobules (labeled I-X) are labeled in both the vermis and hemispheres. Lobule VII is subdivided into Crus I, Crus II, and VIIB in the hemispheres, and VIIAf, VIIAt, and VIIB in the vermis. Lobule VIII is subdivided into VIIIA and VIIIB. Figure courtesy of Professor Jeremy Schmahmann, Massachusetts General Hospital, Boston.

well as ASD; it is important to note that different regions of the cerebellum show structural differences in each of these disorders, suggesting different cerebro-cerebellar circuits may be affected in ASD, ADHD, and dyslexia (see Stoodley, 2014). Other neurodevelopmental disorders, such as developmental coordination disorder (DCD), frequently co-occur with ADHD and dyslexia and are also hypothesized to be a product of cerebellar dysfunction (Zwicker et al., 2011; Biotteau et al., 2015). Why might cerebellar dysfunction be involved in so many developmental disorders, and what can we learn from the localization of cerebellar differences in each disorder? Relative to other regions of the brain, the cerebellum undergoes enormous growth between 24 and 40 weeks post-conception, increasing approximately 5-fold in volume and over 30-fold in surface area (see Volpe, 2009 for review). While this rapid cerebellar growth slows postnatally, neural differentiation and growth of axonal inputs and outputs continue throughout the first postnatal year (Volpe, 2009). This substantial prenatal growth, continued postnatally, might render the cerebellum especially vulnerable to developmental disruptions and damage. Consistent with this, premature infants for whom this rapid cerebellar development is interrupted are at increased risk of cerebellar hemorrhages and future neurodevelopmental disabilities (Volpe, 2009). As mentioned above, cerebellar damage is associated with a range of long-term motor, cognitive and affective outcomes, and cerebellar injury in childhood can often result in poorer outcomes than cerebellar damage in adulthood (Scott et al., 2001; Wang et al., 2014). This is evident in the assessment of acquired ASD symptoms: while damage to the cerebral cortex early in life does not lead to long-term ASD symptoms or diagnoses (Wang et al., 2014), early cerebellar injury results in an increased risk of internalizing behavioral problems, withdrawal from social contact, and affective and attentional deficits (e.g., Limperopoulos et al., 2007). Following cerebellar tumor resection, children are at an unusually elevated risk for cognitive and adaptive impairments (Beebe et al., 2005) and damage to the vermis can lead to long-term affective dysregulation (Levisohn et al., 2000). Malformations of the vermis are also associated with higher rates of affective and behavioral deficits, including ASD symptomology (Tavano et al., 2007).

More specifically, congenital cerebellar malformations and a variety of early cerebellar lesions have been directly associated with ASD diagnoses. In fact, Schmahmann included "autism spectrum" amongst the clinical characteristics of psychiatric outcomes associated with cerebellar damage or disease (Schmahmann et al., 2007). Damage to the cerebellum in infancy is one of the highest risk factors for developing ASD (estimated 40-fold increase; Limperopoulos et al., 2007), second

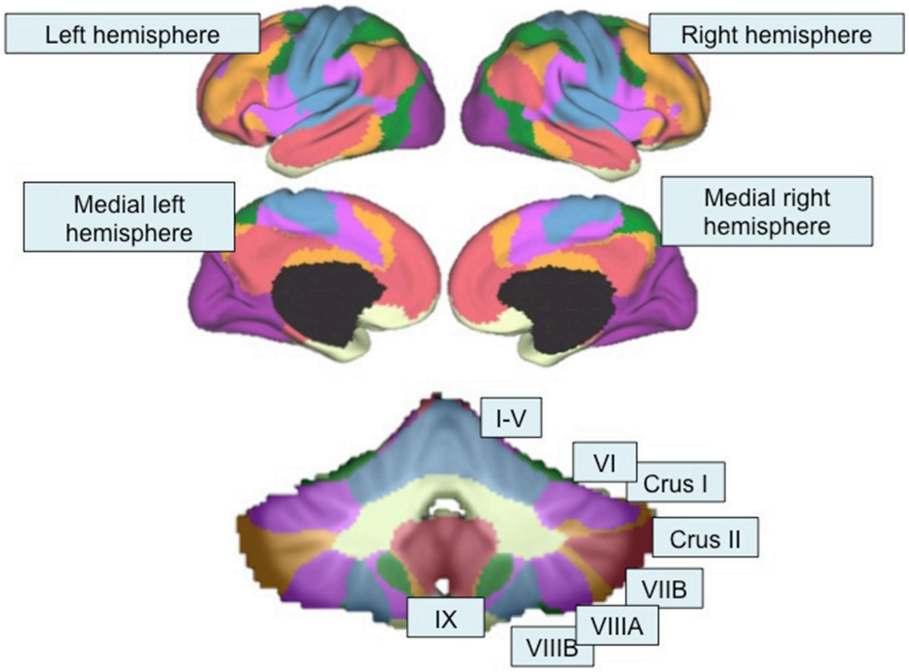

FIGURE 2 | Functional topography revealed by resting-state functional connectivity mapping. Top, Color-coded seven-network map of the cerebral cortex as revealed by resting-state functional connectivity (adapted with permission from Yeo et al., 2011). Bottom, resting-state functional connectivity network map of the cerebellum using the same seven-network solution (Buckner et al., 2011) from the Spatially Unbiased Infratentorial (SUIT) Atlas (Diedrichsen, 2006; Diedrichsen et al., 2009). Lobules are labeled according to the scheme shown in **Figure 1**. Purple, visual network; blue, somatomotor network; green, dorsal attention network; violet, ventral attention network; cream, limbic network; orange, fronto-parietal network; red, default mode network.

only to having an identical twin with autism, and conferring a larger risk than having a sibling with ASD (Wang et al., 2014). In children with tuberous sclerosis, tuber load in the cerebellum was a specific predictor of ASD (Weber et al., 2000). In one pediatric case, cerebellar damage led to stereotyped movements, gaze aversion, linguistic impairments, and a complete avoidance of physical contact, ultimately resulting in an ASD diagnosis (Riva and Giorgi, 2000).

These data from clinical disorders and acquired cerebellar damage suggest that disrupted cerebellar processing has long-term effects in developmental populations, including increases in ASD diagnoses. It has been proposed that early cerebellar damage impacts the development of cerebral cortical regions to which the cerebellum projects, via "developmental diaschisis" (Wang et al., 2014). Therefore, cerebellar developmental differences in ASD could disrupt not only cerebellar function, but also could negatively impact the structure and function of multiple regions of the cerebral cortex to which the cerebellum projects.

ARE SPECIFIC CEREBRO-CEREBELLAR PATHWAYS DISRUPTED IN ASD? EVIDENCE FROM STRUCTURAL AND FUNCTIONAL CONNECTIVITY STUDIES

Reduced number and microstructural integrity of cerebellar fibers might disrupt the outflow pathways from the cerebellum to supratentorial regions important for movement, language, cognition, and social interaction. White matter (WM) abnormalities in the cerebellum have been consistently reported in ASD using a variety of analysis methods, including voxel-based morphometry (McAlonan et al., 2008; Sahyoun et al., 2010). More specific analysis of fiber tracts within the cerebellum and its input and output pathways have utilized diffusion tensor imaging (DTI) and tractography methods. Measurements of fractional anisotropy (FA) represent diffusion of water molecules within an axon; higher levels of FA are typically related to increased microstructural integrity or fiber organization, while reduced myelination, inflammation along the axon, and decreased fiber density or coherence might result in decreased FA. Measures of mean diffusivity (MD) are related to the interstitial space between gray and white matter, and higher MD values might reflect reduced number of neural and glial cells or reduced packing of these cells (Beaulieu, 2002). While DTI findings in ASD are not always consistent, multiple studies report decreased FA and increased MD in the corpus callosum, cingulum, and WM within the temporal and frontal lobes (Travers et al., 2012). While fewer studies have examined the cerebellum, individuals with ASD display abnormalities in structural connectivity both within the cerebellum and in the projection fibers carrying information to and from the cerebellum.

Within the cerebellum, decreases in FA and increases in MD might result from reductions in Purkinje cell size and number

(Fatemi et al., 2002; Bauman and Kemper, 2005), as well as increased inflammation and microglial activation (Vargas et al., 2005), both of which are well-documented in ASD. Cerebellar WM is an especially potent discriminator of ASD diagnosis: One study in preschool children reported that increased cerebellar WM was the strongest discriminator of future ASD diagnosis, and including cerebellar WM in the model led to a correct diagnosis in 95.8% cases (Akshoomoff et al., 2004). Cerebellar WM differences may be directly related to genetic factors in ASD, as only twins concordant for the ASD phenotype had concordant cerebellar WM volumes, while twins discordant for the ASD phenotype did not have similar cerebellar WM volumes (Kates et al., 2004).

The output and input pathways of the cerebellum also show differences in FA and MD in ASD. FA and MD abnormalities in the middle cerebellar peduncle (MCP) and inferior cerebellar peduncle (ICP) might affect the relay of information to the cerebellum from the cerebral cortex and the spinal cord/inferior olives/vestibular nuclei, respectively; structural differences in the superior cerebellar peduncle (SCP), the major efferent WM tract from the cerebellum to the cerebral cortex, could reflect disruption in pathways exiting the cerebellum. In particular, 6 out of 7 studies reporting abnormalities in the cerebellar peduncles in ASD found differences in the MCP (Brito et al., 2009; Cheng et al., 2010; Shukla et al., 2010; Sivaswamy et al., 2010; Groen et al., 2011; Hanaie et al., 2013), with fewer reports of differences in the SCP (Catani et al., 2008; Brito et al., 2009; Sivaswamy et al., 2010). Most of these reported decreased FA and increased MD (Brito et al., 2009; Shukla et al., 2010; Groen et al., 2011; Hanaie et al., 2013). Less often, *increased* FA was reported in the MCP (Cheng et al., 2010; Sivaswamy et al., 2010).

Reversals in FA lateralization patterns in the cerebellar peduncles are also associated with ASD. One study found a reversed pattern of FA asymmetry in the MCP and the ICP: Typically-developing children displayed higher FA in the left MCP, while children with ASD displayed the opposite pattern, with higher FA in the right MCP. This is consistent with lateralization differences seen in structural and functional imaging studies, whereby individuals with ASD show abnormal rightward lateralization in cerebral cortex (e.g., Dawson et al., 1982; Escalante-Mead et al., 2003; Takeuchi et al., 2004; Flagg et al., 2005; Knaus et al., 2010; Lindell and Hudry, 2013; Seery et al., 2013), which may continue through the MCP into the cerebellum, and within the cerebellum itself. A similar pattern was noted in the ICP: children with ASD had lower FA in the right ICP while their typically-developing counterparts displayed higher FA in the right ICP relative to the left (Sivaswamy et al., 2010).

Structural abnormalities in both the MCP and SCP imply disruption in the entire cerebro-cerebellar loop in ASD, from the cerebral cortex to the cerebellar cortex and back again. Decreased integrity of cerebellar outflow pathways might result in loss of modulatory input from the cerebellum to cortical regions involved in motor behavior and social processing. Behavioral evidence supports this, as decreased FA in the right and left SCPs were related to both increased repetitive behaviors and social impairments in ASD, respectively (Catani et al., 2008; Hanaie et al., 2013).

More specific investigations have shown that the cerebellar projections to the thalamus (which would then project to the cerebral cortex) are abnormal in ASD. In young ASD children (under 5 years of age), reduced FA was found in connections between the dentate nucleus and thalamus. Reduced FA in projections from the right *ventral* dentate to the thalamus correlated with more severe communication impairments in ASD, while reduced FA in projections from the right *dorsal* dentate to the thalamus showed a trend-level correlation with daily living skills (Jeong et al., 2012). Correlations between reduced FA in right ventral dentate nucleus projections and impaired communication in ASD might reflect disruption in cerebro-cerebellar loops between cognitive regions of the cerebellum and contralateral supratentorial language regions via the thalamus. On the other hand, reduced FA in efferents originating in the dorsal dentate nucleus and passing via the contralateral thalamus to motor cortices might impair daily living skills in which motor behavior is particularly important.

These findings of altered structural integrity of cerebro-cerebellar loops in ASD converge with the results of functional connectivity studies. Functional connectivity (FC) provides a measure of the correlation between distinct brain regions based on low-frequency fluctuations in the blood-oxygen level dependent (BOLD) signal. Resting state FC (rsFC) is acquired in the absence of any task and can provide insight into the intrinsic organization of the brain, while task-based FC can provide important information regarding network integrity during a task and can be related to task performance. In general, FC findings in ASD suggest that cerebro-cerebellar networks are poorly assembled, with both decreased connectivity within established networks and increased out-of-network patterns of connectivity (Noonan et al., 2009; Khan et al., 2015). Consistent with atypical lateralization in the peduncles, lateralization of functional connectivity patterns is abnormal in ASD. Children with ASD have increased functional connectivity between right hemisphere cerebral cortical regions and right hemisphere cerebellar regions, violating typical patterns of contralateral cerebro-cerebellar connectivity (Noonan et al., 2009; Khan et al., 2015).

Recent functional connectivity analyses in ASD suggest that the cerebellum is abnormally connected with both motor and non-motor regions of the cerebral cortex. For example, while the typically-developing group showed FC between the right cerebellum and left cerebral cortical areas, ASD participants showed atypical, additional FC between the right cerebellum and the right-hemisphere homologs of those regions (Noonan et al., 2009). This "extra" functional connectivity between regions that are not typically correlated often occurs outside of topographical principles of cerebellar organization. For example, the expected cerebro-cerebellar connectivity between left lobule VI and the middle frontal gyrus was noted in both typically-developing and ASD groups, but only the ASD participants had *additional* atypical connectivity between the left middle frontal gyrus and the right anterior cerebellum (lobules IV/V,

which usually show connectivity with somatomotor networks) (Noonan et al., 2009). This recruitment of additional or "non-canonical" cerebellar regions is found in both studies examining cerebro-cerebellar FC in ASD (Noonan et al., 2009; Khan et al., 2015). Children and adolescents with ASD displayed increased rsFC between non-motor areas of the cerebellum (lobules VI and Crus I) and sensorimotor cerebral cortical regions, such as the premotor/primary motor cortices, primary somatosensory cortex, and the occipital lobe (Khan et al., 2015). This increase in non-canonical rsFC with posterolateral cerebellar regions in ASD is also evident in task-based fMRI: During simple motor tasks, individuals with ASD activate posterior cerebellar regions in addition to the anterior cerebellar regions typically recruited (Müller et al., 2003; Allen et al., 2004). These findings suggest that, during simple motor tasks, the domain specificity of cerebro-cerebellar connections might be abnormal in ASD, and may reflect the reduced integrity and abnormal organization of WM pathways entering and leaving the cerebellum.

This increased functional connectivity between unexpected, non-canonical regions in ASD is accompanied by decreased typical (or canonical) connectivity, particularly in cerebro-cerebellar networks related to language and social interaction (see **Figure 3**). Compared to their typically-developing counterparts, ASD children and adolescents display reduced rsFC between right Crus I/II and contralateral prefrontal cortex, posterior parietal cortex, and the inferior/middle temporal gyrus (Khan et al., 2015). Similarly, reductions in rsFC between right Crus I and the contralateral superior frontal gyrus, middle frontal gyrus, thalamus, anterior cingulate gyrus, and parietal areas were found in ASD adolescents (Verly et al., 2014). In this study, reduced rsFC was also found with SMA and precentral gyrus (Verly et al., 2014), which is not consistent with the other studies reporting increased non-canonical FC between right Crus I/II and motor regions of the cerebral cortex in ASD described above (Khan et al., 2015).

These findings suggest that increases in resting-state cerebro-cerebellar connectivity in ASD might be primarily driven by atypical functional connectivity, particularly between lobules VI and VII (Crus I and II) and motor cortices. These increases in non-canonical connectivity might occur at the expense of canonical rsFC between the posterior cerebellum (Crus I and II) and cerebral cortical regions involved in language and social cognition, as evidenced by consistent FC decreases in these specific pathways. Indeed, such connectivity differences are associated with more impaired behaviors in ASD. Reduced connectivity between right Crus I/II and prefrontal regions such as the dorsolateral and medial prefrontal cortex correlated with increasing ASD symptoms and severity (Jung et al., 2014; Verly et al., 2014). In exploratory analyses, cerebellar connectivity with left sensorimotor and association cortices correlated with Social Responsiveness Scale (SRS) scores in ASD (Khan et al., 2015). Therefore, together with the structural data described above, these findings suggest that alterations in cerebro-cerebellar functional connectivity are related to symptom severity in ASD.

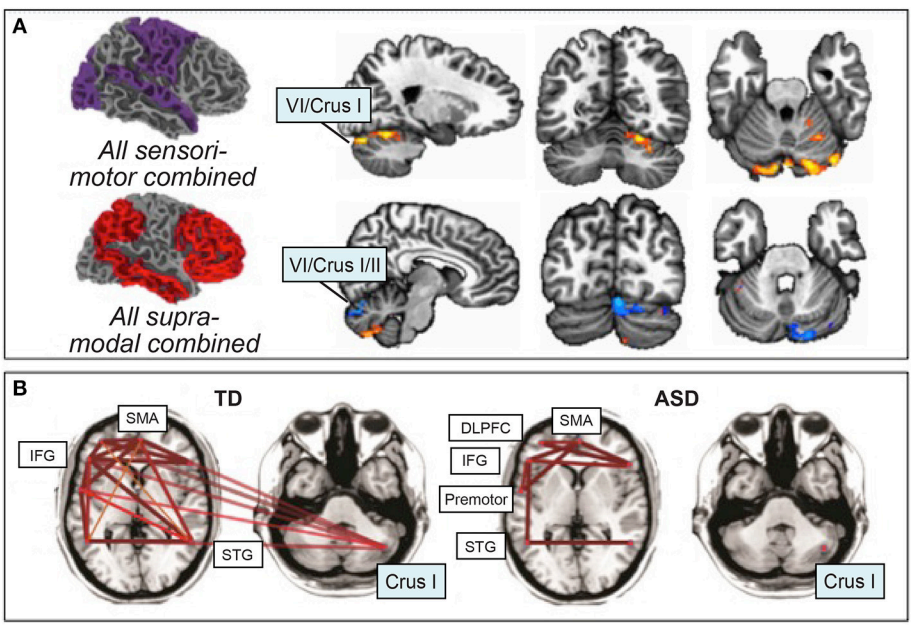

FIGURE 3 | Resting-state functional connectivity in ASD. (A) Atypical increased functional connectivity between sensorimotor regions of the cerebral cortex and cerebellar lobules VI and VII (orange), decreased functional connectivity between supra-modal association cortices and lobules VI and VII (blue). Orange, ASD greater rsFC than typically-developing; blue, ASD less rsFC than typically-developing. Figure adapted with permission from Khan et al. (2015). **(B)** Preserved functional connectivity in ASD between supratentorial language regions, in contrast with the lack of cerebro-cerebellar connectivity between right Crus I/II and left-hemisphere language regions. Figure adapted from Verly et al. (2014).

CEREBRO-CEREBELLAR CIRCUITS AND CORE ASD SYMPTOMS: SENSORIMOTOR, LANGUAGE/COMMUNICATION, AND SOCIAL INTERACTION

Cerebellar structural and functional neuroimaging findings in ASD conform to the principles of cerebellar functional topography and can be interpreted in the context of cerebro-cerebellar circuits. Below, we consider regional cerebellar findings from structural and functional imaging studies, as well as data emerging from investigations of cerebro-cerebellar circuits using structural and functional connectivity methods, in relation to sensorimotor, language, and social interaction deficits in ASD. Throughout, when data are available, we discuss how these findings relate to core ASD symptoms.

The Sensorimotor Cerebellum and Sensorimotor Cerebro-cerebellar Circuits in ASD

The anterior cerebellum (lobules I-V) forms reciprocal loops with sensorimotor regions of the cerebral cortex, including the primary motor cortex (Strick et al., 2009), supplementary motor area and premotor cortices (Strick et al., 2009), and the basal ganglia (Bostan and Strick, 2010). The cerebellum contains multiple homunculi, including a somatotopic representation of the body in the anterior lobe extending into lobule VI, and secondary representations in lobule VIII of the posterior lobe, which also interconnects with somatomotor networks (Snider, 1950; Grodd et al., 2001; Buckner et al., 2011).

Regional structural and functional findings in ASD can be correlated with performance on motor measures and interpreted in the context of these cerebro-cerebellar loops. Decreased gray matter (GM) in the anterior cerebellum (lobules IV and V) and lobule VIII have been found to correlate with increased severity of repetitive and stereotyped behaviors (Rojas et al., 2006; D'Mello et al., 2015). In typically-developing individuals, these regions of the cerebellum are strongly activated during simple motor tasks such as finger-tapping (Stoodley and Schmahmann, 2009; Stoodley et al., 2012). However, ASD individuals showed hypoactivation in the anterior cerebellum during motor tasks when compared to age-matched controls (Müller et al., 2003; Mostofsky et al., 2009; Murphy et al., 2014), even in the context of similar engagement of primary motor cortex in both groups (Mostofsky et al., 2009). Hypoactivation in the anterior cerebellum was also related to increased number of errors and slower reaction times relative to typically-developing counterparts (Müller et al., 2001, 2003; Murphy et al., 2014). These reductions in activation also extend to task-based functional connectivity: for example, during finger tapping, individuals with ASD had decreased FC between the anterior cerebellum and primary motor cortex, thalamus, and supplementary motor area (Mostofsky et al., 2009).

Other studies report *increased* activation in the anterior cerebellum during simple motor tasks in ASD (Allen and Courchesne, 2003; Allen et al., 2004). Increased anterior lobe activation in these studies was often accompanied by more diffuse cerebellar activation, which spread into the contralateral anterior lobe as well as posterior lobe regions not typically activated during motor tasks (Allen and Courchesne, 2003; Allen et al., 2004). However, increased activation was not associated with significant behavioral differences in motor performance between ASD and typically-developing groups (Allen and Courchesne, 2003; Allen et al., 2004). In typically-developing individuals, anterior lobe activation during finger-tapping is ipsilateral to the hand being moved and does not extend into Crus I/II or contralateral anterior lobe regions (Desmond et al., 1997; Stoodley et al., 2012). Activation in posterolateral regions of the cerebellum therefore might reflect the abnormal functional circuitry between the "non-motor" cerebellum and motor areas that has been reported in ASD (Khan et al., 2015). Alternatively, decreased anterior lobe activation in ASD might be related to behavioral impairments during motor tasks, while increased anterior lobe activation might be a compensatory mechanism, allowing ASD individuals to maintain typical levels of performance.

Posterior lobules of the cerebellum such as Crus I/II *are* activated in typically-developing individuals during more complex motor paradigms, particularly during motor imitation (Jack et al., 2011; Jack and Pelphrey, 2014), and motor imitation paradigms are associated with reduced activation in right Crus I in ASD (Jack and Morris, 2014). Some have suggested that impairments in imitation and praxis are core deficits in ASD and might contribute to social and communication impairments (Rogers and Pennington, 1991; Mostofsky et al., 2006). Supporting this, in typically-developing individuals, right Crus I/II typically interconnects with frontal and parietal association areas, including areas important for processing biological motion (e.g., superior temporal sulcus; Jack et al., 2011; Sokolov et al., 2012). Consistent with this, during a more complex motor imitation paradigm, adolescents with ASD had decreased connectivity between right Crus I and the superior temporal sulcus (STS) (Jack and Morris, 2014).

Differences in WM structure in motor regions of the cerebellum have also been reported in ASD. Consistent with motor symptoms being some of the earliest signs of ASD, young children with ASD had increased MD in the anterior cerebellum and lobule VIII (Walker et al., 2012). Further, decreased FA in bilateral lobule VIII has been correlated with increased repetitive behaviors (Cheung et al., 2009). As noted above, lobule VIII is activated by motor tasks and related to motor processing in typically-developing adults, and reduced GM in this region is associated with increased repetitive behaviors in ASD (Rojas et al., 2006; D'Mello et al., 2015). These behavioral correlates of WM abnormalities in ASD suggest that cerebellar structural differences have predictable behavioral consequences on stereotyped and repetitive behaviors.

Decreased GM in the posterior cerebellar vermis (vermal lobules VI-VII) and right Crus I have also been associated with increased repetitive behaviors and stereotyped interests (Pierce and Courchesne, 2001; D'Mello et al., 2015). While these posterior areas are typically considered part of cognitive control networks, it has been suggested that repetitive behaviors in ASD might reflect a loss of cognitive control over motor areas (e.g.,

Mosconi et al., 2009). There are anatomical links between Crus II/VIIB of the cerebellum and both associative (with input from prefrontal cortex) and sensorimotor (with input from premotor cortex and M1) regions of the basal ganglia, suggesting that this region of the cerebellum might be important for the integration of motor and non-motor information (Bostan and Strick, 2010). Consistent with this, in ASD basal ganglia dysfunction has been associated with increased repetitive and stereotyped motor behaviors (e.g., Hollander et al., 2005). Symptom severity in both Tourette syndrome/tic disorder (Stern et al., 2000; Bohlhalter et al., 2006; Lerner et al., 2007; Tobe et al., 2010) and obsessive-compulsive behaviors (Kim et al., 2001; Tobe et al., 2010; Hou et al., 2012), often likened to repetitive and stereotyped motor symptoms in ASD, have been associated with abnormal activation and structure in bilateral Crus I/II. Successful treatment for obsessive compulsive disorder was associated with increased activation in right Crus I (Nabeyama et al., 2008). It is possible that perseverative and repetitive behaviors might be due to loss of modulation of circuits between the posterior cerebellum and basal ganglia.

These results suggest a dissociation between cerebro-cerebellar circuits involved in different types of motor tasks in ASD. Simple motor tasks are associated with abnormal activation in the anterior cerebellum and differences in FC in cerebro-cerebellar somatomotor circuits, whereas reduced activation and FC with cerebro-cerebellar circuits involved in social cognition (right Crus I) are evident during complex motor tasks involving imitation. GM and WM structural differences in the anterior lobe and lobule VIII have been associated with repetitive and stereotyped behaviors in ASD.

The Linguistic Cerebellum and Cerebro-Cerebellar Language Circuits in ASD

In humans, lobule VII (subdivided into Crus I, Crus II, and VIIB), accounts for the largest proportion of cerebellar volume (Balsters et al., 2010). This considerable volumetric increase compared to phylogenetically older species mirrors the expansion of the frontal lobes, potentially conferring a cognitive advantage (Balsters et al., 2010). Viral-tract tracing studies report anatomical connections between right Crus I and II and BA 46, as well as other language regions of the cerebral cortex (Strick et al., 2009). In typically-developing individuals, right Crus I and II are activated during tasks of language processing, including verbal fluency, grammar, verbal working memory, and language learning tasks (Petersen et al., 1989; Fulbright et al., 1999; Papathanassiou et al., 2000; Mathiak et al., 2002, 2004; Chen and Desmond, 2005a; Booth et al., 2007; Stoodley and Schmahmann, 2010; Sens et al., 2011). The contralateral connections between the cerebellum and cerebral cortex are reflected in the right-lateralization of language-related tasks in the cerebellum, mirroring the left-lateralization of language in the cerebral cortex. Individuals with damage to the right posterior cerebellum can have deficits in both receptive language and expressive language (see Mariën et al., 2014 for review), suggesting that this region of the cerebellum subserves a variety of language functions.

Functional imaging studies in ASD report abnormal activation in these "language" regions of the cerebellum during a variety of language tasks (Harris et al., 2006; Wang et al., 2007; Redcay and Courchesne, 2008; Tesink et al., 2009; Groen et al., 2010). While in typically-developing individuals there was increased activation in right Crus I/II when hearing speech vs. non-speech sounds (Groen et al., 2010), children with ASD had reduced (Wang et al., 2007) or absent activation (Groen et al., 2010) in right Crus I/II in response to vocal stimuli. Reduced activation in right Crus I/II in ASD is often accompanied by hypoactivation in other language-processing regions, including the temporal lobes, medial prefrontal cortex, and Broca's area (Harris et al., 2006; Wang et al., 2007). These data suggest that activation in right Crus I/II and associated cerebro-cerebellar networks is related to basic receptive language processing, and abnormal activation here may be related to impaired communication in ASD.

More complex language processing is also associated with reduced cerebellar activation in ASD, particularly in right Crus I/II. Early PET studies suggested that individuals with ASD had decreased right dentate nucleus activation concomitant with decreased left BA 46 activation during both receptive and expressive language (Müller et al., 1998). During semantic processing (Harris et al., 2006) and processing of semantic anomalies (Tesink et al., 2009; Groen et al., 2010), typically-developing individuals activated right Crus I/II while individuals with ASD showed no statistically significant activation in this region. These data suggest that right Crus I/II might also play a role in semantic discrimination and error-processing in language tasks. Reduced activation here could contribute to the well-documented deficits in language discrimination and semantic processing in ASD (see Groen et al., 2008 for review). These paradigms further suggest that right Crus I/II is hypoactive at multiple stages of language processing in ASD—both initially during listening but also during later semantic processing.

Consistent with functional imaging studies indicating abnormal activation in the posterior cerebellum in ASD, structural differences in these regions are also related to language and fluency impairments in children with ASD. Reduced GM in right Crus I, vermis VI, vermis VIII, and lobule IX correlated with poorer communication skills as measured by standard autism scales (Riva et al., 2013; D'Mello et al., 2015), and reversed asymmetry was observed in lobule VIIIA in language-impaired children with ASD (Hodge et al., 2010). Further, neurochemical markers of reduced neuron density / viability in the right cerebellar hemisphere correlated with fluency deficits in ASD (Kleinhans et al., 2007).

Finally, appropriate recruitment of right Crus I and II might also be important for proper language acquisition and during language learning. In typically-developing infants, GM concentration in right lobule VIIB at 7 months of age predicted receptive language skills at 12 months of age (Deniz Can et al., 2013), and the cerebellum was one of two regions in the brain where GM predicted language skills later in childhood (Deniz Can et al., 2013). The degree of right lateralization in the cerebellum has been associated with stronger core language skills in children (Berl et al., 2014) and increased activation in this area predicted degree of language learning (Pliatsikas et al., 2014a). Studies of second-language acquisition in typically-developing

individuals report GM increases bilaterally in lobule VII, which were related to better performance on grammar tasks (Pliatsikas et al., 2014b) and improved fluency (Grogan et al., 2009). Cerebellar activation may also reflect the level of skill acquisition, from novice to expert: Activation in right lobules VI and VII were among the best classifiers of the results of intensive language training, distinguishing trained interpreters from controls (Hervais-Adelman et al., 2015). These findings suggest that the cerebellum may be a crucial neural determinant of language learning.

These data all support a role for the cerebellum (specifically, Crus I and II) in language development and learning. Loss of cerebellar modulatory input on language regions of the cerebral cortex could potentially result in sub-optimal specialization of language circuits, leading to difficulties automatizing language and communication. Consistent with this, lack of functional specialization of cerebral cortical language regions has been well-documented in ASD (e.g., Eyler et al., 2012), and lateralization of language is often abnormal in ASD, with language lateralized to right hemisphere homologs rather than typical left-hemisphere language regions (e.g., Dawson et al., 1982; Escalante-Mead et al., 2003; Takeuchi et al., 2004; Flagg et al., 2005; Knaus et al., 2010; Lindell and Hudry, 2013; Seery et al., 2013). MEG data suggests that while cerebral cortical language representation is originally bilateral in both typically-developing and ASD children, it shifts leftward in typically-developing individuals with age but shifts rightward in ASD (Flagg et al., 2005). The same pattern of abnormal lateralization is noted in the cerebellum. Two- to three-year old typically-developing children recruited right Crus I more strongly than left Crus I (Redcay and Courchesne, 2008), displaying typical contralateral patterns of language activation in the cerebellum. However, age-matched ASD toddlers recruited *left* VI more than right VI (Redcay and Courchesne, 2008). This improper cerebellar lateralization, occurring during a critical period in language development, might result in abnormal specialization of left supratentorial language regions for language.

On the other hand, increased leftward lateralization for language in the cerebellum might allow for compensatory rightward lateralization in the cerebral cortex in ASD (D'Mello et al., 2014). Right cerebral lateralization of language in ASD has been associated with earlier onset of language and better language skills (Joseph et al., 2014). A similar pattern has been noted in cerebellar GM patterns in ASD children with and without early language delay (D'Mello et al., 2014). Both ASD groups showed GM reductions in right Crus I/II, but language-delayed children with ASD also had decreased GM in left Crus I/II (D'Mello et al., 2014). In the face of reduced right Crus I GM, normal left Crus I volumes may enable children with ASD to shift language lateralization to right hemisphere language homologs and compensate for reduced functionality of left cortical language regions. Differences in both right and left Crus I/II might result in abnormal functional specialization of contralateral connected cerebral language homologs as well as right language homologs, leading to language delay (D'Mello et al., 2014).

In addition to well-documented GM reductions in right Crus I/II, ASD children display abnormal structural connectivity between right Crus I/II and the deep cerebellar nuclei. Using MRI tractography, one study found that children with ASD had reduced numbers of Purkinje cell fibers projecting from right Crus I/II of the cerebellar cortex to the right ventral dentate nucleus (Jeong et al., 2014), which then projects to non-motor associations areas of the cerebral cortex, including language regions. In addition, FA was reduced both in short intracerebellar fibers and between right Crus I/II of the cerebellar cortex and the dentate nucleus, which are thought to reflect parallel fiber and Purkinje cell axons, respectively (Catani et al., 2008; Jeong et al., 2014).

In summary, these findings suggest that regions of the cerebellum that interconnect with cerebral cortical language networks could be particularly important in receptive, expressive, and higher-level cognitive aspects of language, possibly due to deficient language learning. Recent resting-state connectivity data suggest that disrupted cerebro-cerebellar connectivity (e.g., Jones et al., 2010) is in marked contrast to intact functional connectivity *within* supratentorial language networks: While functional connectivity between cerebral cortical language areas was intact, language-impaired individuals with ASD displayed decreased rsFC between right Crus I/II and cerebral language regions (Broca's area and Wernicke's area, see **Figure 3**; Verly et al., 2014).

The "Social" and Affective Cerebellum and Associated Cerebro-cerebellar Circuits in ASD

Viral tract-tracing and human DTI studies link the posterior cerebellum (particularly Crus I/II, lobule IX, and the posterior vermis) with regions of the cerebral cortex involved in social processing and emotion, providing an anatomical substrate for cerebellar involvement in social cognition and affective regulation (Jissendi et al., 2008; Stoodley and Schmahmann, 2010; Buckner et al., 2011; Sokolov et al., 2012). In typically-developing individuals, cerebellar Crus I/II and lobule IX are functionally connected to the default mode and fronto-parietal networks, and largely overlap with regions of the cerebellum involved in language processing (Stoodley and Schmahmann, 2009; Buckner et al., 2011). These regions of the cerebellum are consistently activated during social paradigms, particularly during abstract mentalizing (Van Overwalle et al., 2014). Crus I/II is engaged during imitation, processing of biological motion, animacy attribution (Jack et al., 2011; Jack and Pelphrey, 2014), and emotional facial processing (Deeley et al., 2007); lobule IX has been found to be activated specifically when healthy individuals broke with social norms (Klucharev et al., 2009). These typical activation patterns suggest that Crus I/II might be important in supporting social processing functions while lobule IX might be involved in signaling social conflict. Both Crus I/II and lobule IX of the cerebellum are functionally connected to the temporoparietal junction, temporal poles, and prefrontal cortex, regions implicated in social cognition in typically-developing individuals (Mars et al., 2012) and which are consistently underactivated in ASD during socially awkward situations (Pantelis et al., 2015). Through these connections, the cerebellum might play a role in modulating supratentorial regions involved

in social processing and emotion. As discussed above, damage to the posterior cerebellum can result in sub-optimal regulation of mood and behavior, resulting in affective dysregulation, mood disruptions, and behavioral problems (Schmahmann and Sherman, 1998; Riva and Giorgi, 2000).

These activation patterns in typically-developing individuals are consistent with cerebellar regions where participants with ASD show reduced GM. Structurally, decreased GM in the anterior lobe, right Crus I/II, right lobule VIII, and left lobule IX in ASD have been correlated with increased symptom severity in social interaction (Rojas et al., 2006; D'Mello et al., 2015). Similarly, in DTI data, decreased FA in the anterior cerebellum was correlated with increased social impairment (Cheung et al., 2009). While we have categorized the anterior lobe as broadly motor, the medial portion shows functional connectivity with limbic networks (Buckner et al., 2011), and GM decreases in this region have been shown to correlate with increased social impairment in ASD (D'Mello et al., 2015).

Functional abnormalities in Crus I and II have been related to deficits in imitation and praxis, which are theorized to contribute to social and communication deficits in ASD (Rogers and Pennington, 1991). As mentioned above, during imitation individuals with ASD hypoactivate right Crus I/II and show decreased connectivity between right Crus I/II and supratentorial regions involved in social processing, such as the superior temporal sulcus and superior parietal lobe (Jack and Morris, 2014). Further, deficits in these circuits have been related to impairments on mentalizing tasks (Jack and Morris, 2014), and mentalizing / theory of mind deficits are commonly reported in ASD (e.g., Baron-Cohen, 2000). During mentalizing tasks, typically-developing individuals exhibited greater connectivity between the ventromedial prefrontal cortex and left IV/Crus I in self-mentalizing tasks when compared to mentalizing about others; this FC pattern was absent in ASD (Lombardo et al., 2010). Further, stronger FC between right Crus I and the superior temporal sulcus during mentalizing tasks was associated with better mentalizing abilities in ASD (Jack and Morris, 2014). On a related note, ASD individuals who are classified as highly alexythymic underactivated right VI/Crus I both during processing of pain to the self as well as during empathic pain tasks (Bird et al., 2010).

Crus I/II dysfunction might also contribute to the well-characterized deficits in face-processing in ASD. Activation in left Crus I/II was reported in individuals with ASD during stranger face-processing (Pierce et al., 2004) and during a face-memory task (Koshino et al., 2008), whereas typically-developing participants did not engage this region. During emotional face-processing of happy, sad, disgusted, and fearful faces, ASD individuals showed consistent hypoactivation in bilateral VI/Crus I/II of the cerebellum (Deeley et al., 2007). Unlike other regions of the brain, which were specifically hypoactive only for certain emotions or intensities, bilateral Crus I/II was consistently underactivated in ASD for all face stimuli (emotional faces and neutral faces) (Deeley et al., 2007). This is in marked contrast with the robust right Crus I/II activation in typically-developing individuals during processing and imitation of emotional facial expressions (Leslie et al., 2004; Schutter and van Honk, 2005; Dapretto et al., 2006; Schutter et al., 2009). Further, when attempting to detect irony in faces and prosody, ASD participants underactivated bilateral Crus I/II (Wang et al., 2007) and had fewer responses overall, potentially reflecting difficulty interpreting speaker intent (Wang et al., 2007). Combined with data implicating abnormal Crus I/II activation in language processing, irony, and prosody, abnormal activation in Crus I/II during face processing might further contribute to social impairments in ASD.

In terms of social interaction, children with autism showed abnormal age-related connectivity between the ventral striatum and bilateral lobules VI/Crus I. While typically-developing children showed decreasing rsFC between the cerebellum and ventral striatum with age, children with ASD show aberrant increases in cerebello-striatal connectivity with age (Padmanabhan et al., 2013). The ventral striatum is related to reward learning (Spanagel and Weiss, 1999; Haber, 2011) as well as affective processing (Haber, 2011), and rsFC abnormalities in these circuits could be related to deficits in social interaction in ASD. Consistent with this, some theories of autism suggest that individuals with ASD do not find social interaction rewarding, and are therefore unmotivated to engage in social interaction (e.g., Chevallier et al., 2012).

Connections between the cerebellar vermis and limbic regions of the cerebral cortex might also be relevant to ASD; structural and functional differences in these cerebro-cerebellar loops might be associated with difficulties in a range of affective processing tasks. One of the earliest reported neural differences in ASD was hypoplasia of the posterior cerebellar vermis (Courchesne et al., 1988, 1994a,b), and decreased volume in the posterior vermis inversely correlated with frontal lobe volumes in ASD (Carper and Courchesne, 2000). In typically-developing individuals, the posterior cerebellar vermis is functionally connected to the limbic network (Buckner et al., 2011) and is heavily implicated in affective regulation and emotion (see Schutter and van Honk, 2005; Stoodley and Schmahmann, 2009 for review). In children, damage to the vermis and vermal malformations are associated with affective dysregulation, behavioral deficits, and ASD symptoms (Levisohn et al., 2000; Tavano et al., 2007). Similarly, in ASD reduced GM volume in the anterior vermis and vermis VI correlated with more impaired social interaction scores (D'Mello et al., 2015). Functional MRI studies also report abnormal vermal activation in ASD: Processing of irony was related to decreased activation in medial lobule VIII (Wang et al., 2007), and processing of facial expression resulted in abnormal recruitment of the posterior cerebellar vermis in ASD participants (Critchley et al., 2000).

CONVERGING FINDINGS

Based on meta-analyses of structural and functional neuroimaging data, several regions of the cerebellum consistently emerge as abnormal in ASD. Out of 6 whole-brain structural MRI meta-analyses examining the current state of the ASD literature (Stanfield et al., 2008; Cauda et al., 2011; Via et al., 2011; Yu et al., 2011; Stoodley, 2014; DeRamus and Kana, 2015), all but one reported cerebellar differences in ASD (Via et al., 2011; this study

used a different approach than the other voxel-based analyses). The most commonly reported differences have been localized to right Crus I, lobule VIII, and lobule IX (Stanfield et al., 2008; Cauda et al., 2011; Yu et al., 2011; Stoodley, 2014; DeRamus and Kana, 2015; **Figure 4**). These regions, as discussed above, may be associated with specific aspects of the ASD phenotype (**Figure 5**). Functionally, a meta-analysis of fMRI findings in ASD further supports the relationship between disruption in specific cerebro-cerebellar circuits and task performance, with decreased activation in ASD during motor tasks in the anterior cerebellum, and differences in activation during auditory and language tasks bilaterally in VI and Crus I (Philip et al., 2012).

Abnormal findings in ASD are often right-lateralized, suggesting a specific dysfunction of the right cerebellum and its structural and functional connections with both contralateral and ipsilateral areas of the cerebral cortex (Noonan et al., 2009; Fitzgerald et al., 2015). Within the cerebellum, reduced FA between the right cerebellar cortex and right ventral dentate nucleus was found in over 70% of children with ASD (Jeong et al., 2014). Of note, decreased GM (Rojas et al., 2006; D'Mello et al., 2015) and abnormal activation in right Crus I/II has been related to motor, communication, and social symptoms in ASD, potentially speaking to the role of this region as a biomarker for the "core" ASD diagnosis. This region also shows abnormal structural and functional connectivity in ASD, both locally within the cerebellum (Paakki et al., 2010) and in long-range connections with motor and non-motor supratentorial regions (Noonan et al., 2009; Itahashi et al., 2014, 2015; Jung et al., 2014; Verly et al., 2014; Khan et al., 2015).

These converging findings emerge in the context of the well-documented heterogeneity in ASD, including inconsistencies in the direction of the GM differences in ASD (some studies

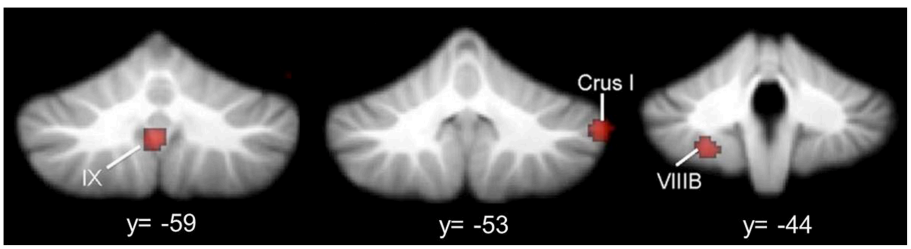

FIGURE 4 | Cerebellar gray matter reductions in autism. GM reductions in the cerebellum in ASD, based on a meta-analysis of voxel-based morphometry studies (Stoodley, 2014). Consistent GM reductions are evident in right Crus I, left VIIIB, and midline IX. Figure adapted from Stoodley (2014).

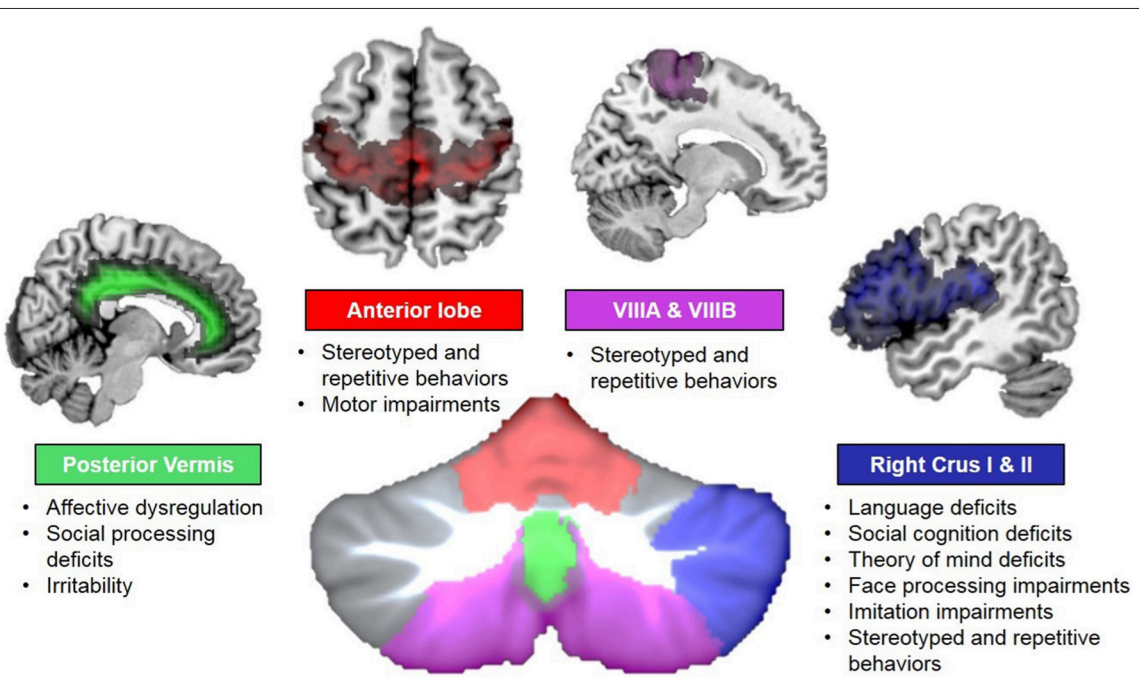

FIGURE 5 | Cerebro-cerebellar circuits in autism. Disruptions in specific cerebro-cerebellar circuits could result in different behavioral symptoms of ASD. Colors reflect connectivity of specific cerebellar regions: anterior lobe (red) and lobule VIII (violet) and somatomotor circuits; right Crus I and II (blue) and frontal language areas (among others); and posterior vermis (green) and limbic networks. Behavioral deficits associated with structural and functional disruptions in each circuit are noted.

report increases while others report decreases). Variations in age, IQ, and behavioral phenotypes of participants could contribute to such inconsistencies. Two recent meta-analyses suggest that certain regional cerebellar differences in ASD might be related to age and/or IQ of participants: DeRamus and Kana (2015) reported that decreased GM in vermal IX/VIIIB occurred in 6–16 year olds with ASD, but was not present in the 18–52 age group; Stanfield et al. (2008) also reported that decreased posterior vermal volumes in ASD become less apparent with increasing age, and, in older groups, differences here were less evident when groups were matched for IQ. Further, different behavioral phenotypes within ASD may also contribute to divergent structural findings in ASD. For example, we have found that ASD children with a history of early language delay show different GM patterns within the cerebellum, with decreased left Crus I/II volume specific to the children with early language delay (D'Mello et al., 2014).

WHAT IS THE SPECIFIC CONTRIBUTION OF CEREBELLAR PROCESSING DURING DEVELOPMENT?

Converging data suggest that the cerebellum may play an important role in the developing brain, and that dysfunction in specific cerebellar regions could lead to developmental disorders such as ASD. That said, it is clear that ASD results from dysfunction in multiple regions of the brain, and not only the cerebellum, which leads to the question: What is the specific contribution of the cerebellum to ASD?

In the motor domain, the cerebellum is involved in modulating and automatizing movement in order to optimize performance in a given context (Ito, 2002); transcranial magnetic stimulation of the cerebellum modulates activation patterns in the primary motor cortex (Galea et al., 2011), confirming that altering cerebellar activity has knock-on effects on the regions of the cerebral cortex to which it projects. Information sent from the cerebral cortex and spinal cord is used to create and train internal models of behavior, enabling optimization and prediction of future behavior (Ito, 2008). It is important to note that damage to the cerebellum does not result in complete loss of function (Schmahmann, 1991). For example, classic motor symptoms following cerebellar damage include not paralysis, but rather erroneous and poorly calibrated dysmetric movement. It has been suggested that the cerebellum plays a similar modulatory role in cognition and affect (see Ito, 2008). Akin to the motor symptoms following cerebellar damage, damage to the posterior cerebellum does not result in severely impaired cognition, but rather an inability to modulate and optimize cognitive performance (conceptualized as "dysmetria of thought," see Schmahmann, 1991). For example, posterior cerebellar damage can result in agrammatism or semantic fluency, but not complete loss of language (Schmahmann and Sherman, 1998).

The process of building and optimizing internal models is directly associated with the role of the cerebellum in implicit learning and skill acquisition. The cerebellum is thought to be maximally involved in initial motor skill learning, while other neural structures (including cortico-striatal pathways and primary motor cortex) are more involved in the retention of learned motor behaviors as a result of extended practice (Doyon et al., 2002; Galea et al., 2011). The same may be true in cognitive tasks, such as working memory: in a study of verbal working memory, right Crus I/II and the contralateral inferior frontal gyrus were maximally activated during the encoding portion of a letter-matching task, while lobule VIII and the posterior parietal cortex were activated during the maintenance phase; no cerebellar activation was associated with subsequent recall (Chen and Desmond, 2005b). A cerebellar role in implicit/procedural learning and skill acquisition is particularly compelling in the context of development and developmental disorders. Indeed, it has been proposed that while declarative memory and learning mechanisms are relatively intact in developmental disorders including dyslexia, developmental coordination disorder, and ASD, implicit skill acquisition is impaired (Biotteau et al., 2015; Ullman and Pullman, 2015). In our view, implicit learning of different types of information (e.g., literacy vs. motor skills vs. social skills) is supported by different cerebro-cerebellar circuits. This is consistent with the lack of overlap of cerebellar structural gray matter reductions between, for example, developmental dyslexia and autism (see Stoodley, 2014). Therefore, behavioral symptoms characterizing a given developmental disorder should reflect differences in structure and function of specific cerebellar regions (Stoodley, 2015); likewise, disorders sharing similar behavioral deficits may be associated with disruption in overlapping cerebro-cerebellar circuits. For example, Stuttering (a disturbance in motoric aspects of speech) is associated with over-activation in the cerebellar anterior lobe (see Stoodley and Schmahmann, 2015), whereas posterior regions of the cerebellum are associated with communication impairments in ASD. On the other hand, shared symptoms of compulsive/repetitive and stereotyped behaviors in obsessive-compulsive disorder and ASD are both associated with abnormalities in right Crus I/II (Kim et al., 2001; Tobe et al., 2010; Hou et al., 2012). The complex behavioral profile of ASD is reflected in the multiple cerebro-cerebellar circuits where structural and functional differences are found, encompassing cerebellar regions involved in movement, language, social cognition, and affective regulation. Disrupted implicit learning specifically affecting the circuits described above could impact the acquisition of motor, communication, and social skills during early development in ASD, leading to long-term deficits in these domains.

Is the Cerebellum Involved in "Optimization of Function" during Development?

As mentioned above, the creation of internal models to optimize both cognitive and motor behaviors may be crucial for skill acquisition during typical development. Consistent with this idea, it has been proposed that the integrity of cerebro-cerebellar loops might be especially important earlier, rather than later, during the course of development (Wang et al., 2014), as early cerebellar damage is related to worse outcomes than cerebellar damage in adulthood. For example, during a pivotal period in language development, toddlers aged 1-2 years showed *greater* activation in the anterior vermis as well as bilateral lobule VI of

the cerebellum than did older 3 year olds when listening to speech (Redcay et al., 2008). Evidence such as this suggests that cerebellar involvement might be age-dependent—more important earlier in life when cortical networks are first being established, and less important later in life when motor and cognitive behaviors have been appropriately set up in distributed cortical networks. For example, cerebellar processing might support language development by helping to organize cortical regions involved in language, which come on-line later in development and are reliant on appropriate input. In fact, activation in bilateral lobule VI, primarily seen in younger children, showed a negative relationship with expressive language scores, suggesting that decreased activation in this region as language skills develop might reflect a more mature language profile (Redcay et al., 2008).

Given the role of the cerebellum in modulating cerebral cortical activity, cerebro-cerebellar loops might inform early functional specialization of cortical regions. One study examining primary motor cortex in children with ASD found abnormal functional organization of M1 subregions, suggesting a lack and/or delay of functional specialization in this region (Nebel et al., 2014). Abnormal connectivity between the cerebellum and cerebral motor regions might result in sub-optimal automatization and modulation of motor behaviors, and might also be related to delayed acquisition of gestures important for social interaction and communication (Mostofsky et al., 2009). Similarly, abnormal connectivity between the cerebellum and cerebral cortical regions involved in language (Verly et al., 2014) could lead to atypical organization of language networks in ASD (Eyler et al., 2012; Verly et al., 2014), and be associated with delayed language acquisition in ASD. Finally, regions of the cerebellum showing abnormal structure and functional activation in ASD form circuits with cerebral cortices underpinning social cognition (e.g., superior temporal sulcus). It is possible, therefore, that early cerebellar dysfunction can result in sub-optimal specialization of functional networks related to core ASD symptoms of social and communication deficits and repetitive and stereotyped behaviors. Increased repetitive or stereotyped behaviors, atonal or agrammatical language, and impairments in social interaction all reflect not loss of function, but loss of *optimal* function.

Is the Cerebellum Involved in "Optimization of Structure"?

In addition to this proposed role for the cerebellum in optimization of function during the course of development, the cerebellum might also be involved in the optimization of structure. Optimization of structure relies on functional activation: myelination and pruning in the developing brain are known to be activity-dependent, shaping the specialization of structural networks. Longitudinal development of the cerebellum mirrors that of the cerebral cortex, with phylogenetically newer regions, such as the posterolateral cerebellum, reaching peak maturity later in development (Tiemeier et al., 2010). It is possible that these reciprocally-connected regions are developing in concert, such that cerebellar dysfunction has knock-on effects on cerebral cortical development. Consistent with the idea that the integrity of cerebro-cerebellar loops might be especially important for early cerebral cortical development (Wang et al., 2014), damage to the cerebellum early in life can affect the growth and structure of the cerebral cortical regions to which it projects. Infants sustaining cerebellar hemorrhages after birth later had reduced gray matter volume in the contralateral cerebral hemisphere (Limperopoulos et al., 2010, 2012), accompanied by long-term behavioral deficits in movement, language, and general cognition (Limperopoulos et al., 2007). In ASD, developmental differences in cerebellar structure may lead to improper processing of information that is then sent to the cerebral cortex, potentially impacting the activity-dependent structural specialization of the regions of the cerebral cortex to which these cerebellar regions project. Crucially, there is a specificity to the regional findings within the cerebellum in ASD, suggesting that impairments in specific cerebro-cerebellar loops might result in suboptimal structural development in cerebral regions involved in motor, language, and social function, resulting in long-term behavioral deficits.

Caveats and Limitations

While there is robust evidence of cerebellar structural and functional differences in ASD, multiple regions of the brain show abnormalities in this complex disorder. While in our description of abnormal cerebro-cerebellar circuits in ASD we have focused on the cerebellum as the potential "starting point," it is possible that the differences in cerebellar structure and function result from an initial developmental abnormality elsewhere in the brain. While genetic, animal, clinical, and post-mortem studies suggest that cerebellar differences arise very early in pre-natal development in ASD, and that cerebellar abnormalities alone are sufficient to produce ASD symptoms, it is possible that poor cerebellar information processing is a result of impoverished information reaching the cerebellum. Future studies, described below, should help to clarify if ASD can truly be considered a "disorder of the cerebellum" (Rogers et al., 2013).

CONCLUSIONS AND FUTURE DIRECTIONS

Anatomical, neuroimaging, and animal work suggest that the cerebellum is one of the most common sites of abnormality in ASD (Fatemi et al., 2012), and cerebro-cerebellar circuits provide a critical anatomical substrate by which cerebellar dysfunction impacts core ASD symptoms. Crucially, damage to the cerebellum can directly lead to an ASD diagnosis in a way that damage to other regions commonly implicated in ASD cannot, including the prefrontal cortex, basal ganglia, and parietal cortex (Riva and Giorgi, 2000; Limperopoulos et al., 2007; Wang et al., 2014). The localization of gray matter and white matter differences in the cerebellum in ASD suggest disruption of specific cerebro-cerebellar circuits involved in movement, language, social cognition, and affective regulation (**Figure 5**). We suggest that developmental abnormalities in the cerebellum could exert long-term effects via lack of appropriate modulation of the cerebral cortex, impacting the optimization of both structure and function.

Based on these data, future studies should not exclude the cerebellum in analyses of structural and functional differences in ASD. Further, to better characterize cerebellar abnormalities in ASD, neuroimaging investigations should aim to localize cerebellar differences to specific subregions. In addition, the location of these abnormalities must be considered in the context of broader cerebro-cerebellar circuits, in order to better understand the relationship between these differences and specific ASD symptoms. Recent technological advances in high resolution imaging of the cerebellum (e.g., Dell'Acqua et al., 2013) might provide improved understanding of the microstructural organization of cerebro-cerebellar circuits in ASD. In animal studies, disruption of specific cerebellar regions at particular time points could inform our understanding of the developmental relationships between the cerebellum and the cerebral cortex, and further characterize ASD-like behaviors following cerebellar damage. Similarly, human clinical lesion studies throughout the lifespan and longitudinal study designs are necessary to establish the developmental effects of cerebellar damage on optimization of structure and function in the cerebral cortex. Finally, the investigation of the role of the cerebellum in ASD should include tasks that tap not only cerebellar motor function, but also the broader role of the cerebellum in language and social interaction, consistent with our modern understanding of cerebellar function.

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REVIEW
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Autism spectrum disorders and neuropathology of the cerebellum

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The cerebellum contains the largest number of neurons and synapses of any structure in the central nervous system. The concept that the cerebellum is solely involved in fine motor function has become outdated; substantial evidence has accumulated linking the cerebellum with higher cognitive functions including language. Cerebellar deficits have been implicated in autism for more than two decades. The computational power of the cerebellum is essential for many, if not most of the processes that are perturbed in autism including language and communication, social interactions, stereotyped behavior, motor activity and motor coordination, and higher cognitive functions. The link between autism and cerebellar dysfunction should not be surprising to those who study its cellular, physiological, and functional properties. Postmortem studies have revealed neuropathological abnormalities in cerebellar cellular architecture while studies on mouse lines with cell loss or mutations in single genes restricted to cerebellar Purkinje cells have also strongly implicated this brain structure in contributing to the autistic phenotype. This connection has been further substantiated by studies investigating brain damage in humans restricted to the cerebellum. In this review, we summarize advances in research on idiopathic autism and three genetic forms of autism that highlight the key roles that the cerebellum plays in this spectrum of neurodevelopmental disorders.

Keywords: apraxia of speech, cerebellar cognitive affective syndrome, cerebellar vermis, deep cerebellar nuclei, fragile X syndrome, inferior olivary complex, Purkinje cell, tuberous sclerosis

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AUTISM SPECTRUM DISORDERS: CLINICAL FEATURES, DIAGNOSTIC CRITERIA, CLASSIFICATION, AND ETIOLOGY

Autism spectrum disorder (ASD, also known as autism spectrum condition), is a behaviorally defined neurodevelopmental disorder that is estimated to affect 1 in 88 children and is diagnosed approximately four times more frequently in males than in females (Halladay et al., 2015). Two international psychiatric classification systems are used for making clinical diagnoses: the Diagnostic and Statistical Manual of Mental Disorders (DSM) and the International Classification of Diseases. The diagnostic criteria for ASD are outlined in the widely used DSM where key revisions were put in place when DSM-IV was replaced by DSM-5 in 2013. The DSM-IV diagnostic criteria for Autistic Disorder defined three domains of behavior—social deficits, communication deficits, and repetitive/stereotypic behaviors. Under DSM-5 only two behavioral domains, social communication deficits and repetitive/stereotypic behaviors are stipulated (see Lai et al., 2013; Fung and Hardan, 2014 for reviews on this topic). Additional salient changes in DSM-5 include the elimination of both Asperger's Syndrome and Pervasive Developmental Disorder as diagnostic categories, while a new category, Social Communication Disorder, has been added. Regarding the

latter, persons displaying impaired social communication but not engaging in repetitive behaviors (or restricted interests) could now be diagnosed with Social Communication Disorder (Fung and Hardan, 2014).

The level of cognitive impairment in ASD varies over a wide range. In a study conducted on 156 ASD patients between ages of 10 and 14, 55% had intellectual disability (IQ < 70), 17% had below average intelligence (IQ 70–84), 25% had average intelligence (IQ 85–114), and 3% had above average intelligence (IQ > 115) (Charman, 2011). However, it is important to note that standard intelligence tests of IQ are not optimal for assessing much of the autism population, due in part to difficulties in language and communication (Baum et al., 2014). In addition to cognitive impairment and the core symptoms, there are several additional conditions associated with ASD that are correlated with each other and vary greatly in their severity. These include seizures, motor impairments, altered sleep, and increased anxiety (Matson et al., 2007; Maski et al., 2011). Many persons with ASD also experience heightened or reduced sensitivity to sensory stimuli such as sound and temperature.

Approximately 90% of autism cases are classified as idiopathic while about 10% are caused by known gene mutations (Betancur, 2011; Zafeiriou et al., 2013). The genetic forms of ASD are widely studied in humans and animal models in part because knowledge obtained may prove valuable for enhancing our understanding of idiopathic autism. Examples of ASDs caused by gene mutations include fragile X syndrome (FXS) and tuberous sclerosis (TSC) which have mutations in the FMR1 and TSC1/2 genes, respectively. Rett syndrome was previously classified under autism spectrum disorders but it has not been retained under the ASD umbrella in DSM 5. However, clinically, Rett syndrome patients frequently show autism spectrum characteristics and as such, Rett can serve as a model system for ASD. Although FXS and TSC are two of the most common genetic causes of autism, individual genes linked to ASD are each responsible for only a minor proportion of autism cases; other genetic forms of ASD account for even fewer cases of autism, typically 1% or less. However, the extent of epigenetic effects and regulatory changes also needs to be considered and remains under investigation. Estimates of the incidence of FXS and TSC in the overall ASD population vary considerably. In an analysis of patient records from almost 15,000 persons with ASD, Kohane et al. (2012) reported an incidence of 0.5% for FXS and 0.8% for TSC. Others have reported higher incidences of FXS ranging up to about 5% of the ASD population (Budimirovic and Kaufmann, 2011). It has been estimated that about 30% of patients with FXS and about 50–60% with TSC present with ASD core symptoms (Kong et al., 2014). However, in the case of FXS, Budimirovic and Kaufmann (2011) have stated that "the high frequency of relatively mild autistic features, and differences in ascertainment strategies and supportive diagnostic methods have led to reported rates of autism within the FXS population that range widely from 15 to 60% for prevalence of ASD in males with FXS." Therefore, relatively common but mild autistic characteristics can complicate the diagnosis of ASD in FXS.

Although much effort has centered on the identification of genes linked to ASD, there is also a growing trend toward more emphasis on investigating possible environmental causes. Examples include altered immune responses (Mead and Ashwood, 2015), maternal immune activation where a strong immune stimulus or infection during pregnancy is associated with an increased probability of giving birth to a child with ASD (Atladóttir et al., 2010a,b; Brown, 2012; Knuesel et al., 2014), and environmental contaminants (Kalkbrenner et al., 2014; Nevison, 2014; Posar and Visconti, 2014). However, these links so far are not well-established and will require more intense investigation to validate.

STRUCTURE AND ANATOMY OF THE CEREBELLUM

Recent work has suggested that the mammalian cerebellum has undergone substantial enlargement throughout the evolution of apes including humans, and that its size has increased much more than predicted based on analogous enlargement of the cerebral cortex. As a result, humans and other apes deviated significantly from the general evolutionary trend for neocortex and cerebellum to change in tandem, leading to a significantly larger cerebella relative to neocortex size than other primates (Weaver, 2005). In fact, it has even been suggested that "given the role of the cerebellum in sensory-motor control and in learning complex action sequences, cerebellar specialization is likely to have underpinned the evolution of humans' advanced technological capacities, which in turn may have been a preadaptation for language" (Barton and Venditti, 2014). The large expansion of the cerebellar hemispheres has been particularly prominent in the posterior lobe of the cerebellum, a substructure that is crucial for rapid processing of cognitive and language skills (Broussard, 2014).

The cerebellum comprises only about 10% of the brain's volume yet contains over half of all the neurons in the brain (Herculano-Houzel, 2010). In humans, the cerebellar cortex expanded with the cerebral cortex, especially in the hemispheric region (Balsters et al., 2010). As illustrated in **Figure 1**, the cerebellar cortex has one of the simplest configurations in the human brain. It is comprised of three layers: inner granular layer (GL), Purkinje cell layer (PCL), and outer molecular layer (ML). The underlying white matter contains the deep cerebellar nuclei, the main output region of the cerebellum. In development, billions of excitatory granule cells (GCs) have descended from the pia surface via the external granule layer to take residence in the GL, leaving "T-shaped" axons (i.e., parallel fibers, PF) that run parallel to the long axis of the cerebellar folia like telephone wires, synapsing on the elaborate dendritic arbors of GABAergic Purkinje cells (PCs) and continuing across the ML for long distances (e.g., Altman and Bayer, 1978). Each PC receives up to 100,000 PF synapses, and PC axons mainly travel to one of four pairs of deep cerebellar nuclei (fastigial, emboliform, globose, and dentate nuclei), but also give off collaterals to Lugaro cells. Lugaro cells, lie just beneath the PCL and contain long horizontal dendrites that can contact up to 10–15 PCs, hypothesized to monitor the environment around PCs (Lainé and Axelrad, 1998). Lugaro cell axons contact cerebellar interneurons in the ML and

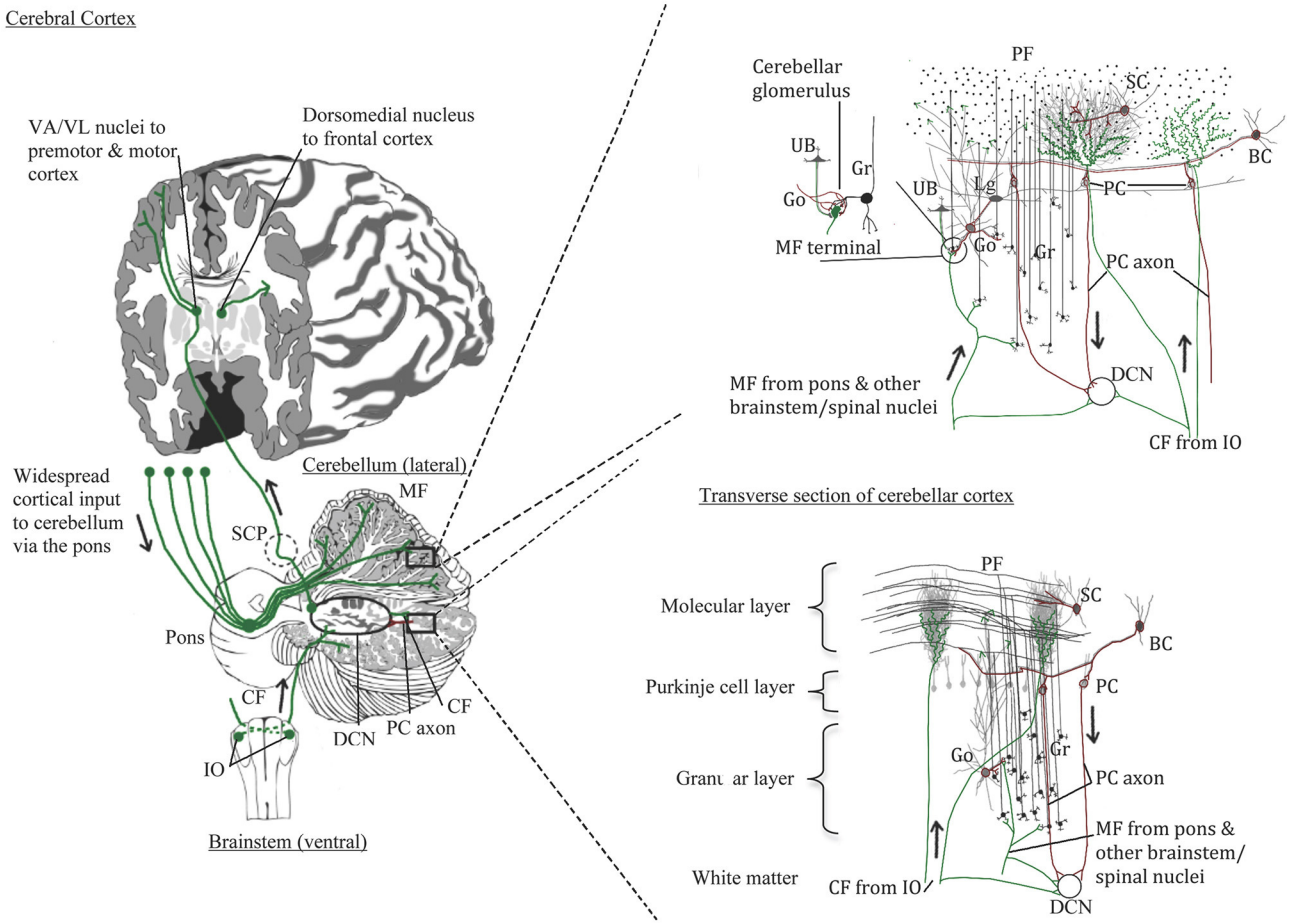

FIGURE 1 | Normal cerebellar circuitry. Main inputs to the cerebellum are from the inferior olivary nuclei via the inferior cerebellar peduncle providing climbing fibers (CF) to Purkinje cells (PC) and, from various spinal cord and brainstem nuclei including the pons that relays cerebral cortical input through the middle cerebellar peduncle to granule cells (Gr). Both climbing and mossy fibers (MF) send collaterals to cells in the deep cerebellar nuclei (DCN). Granule cells in turn send their parallel fibers to PC dendrites in the molecular layer. The PCs innervated by climbing fibers send their axons to the same DCN cells that climbing fibers innervate. Excitatory DCN cells project to the thalamus and cerebral cortex; inhibitory DCN cells project back to the inferior olive completing the loops. Other cerebellar neurons include the Golgi cells (Go) that send inhibitory input to the mossy fiber glomeruli and has dendrites extending into the lower molecular layer where it contacts parallel fibers; Lugaro cells (Lg) that contact multiple PCs via its horizontal dendrites thus sensing the local environment and also contacts Go cells; and unipolar brush cells (UB) found mostly in cerebellar areas with vestibular functions and sends its axon to the Gr cell glomeruli, similar to Go cells.

large inhibitory Golgi cells in the GL (Lainé and Axelrad, 1998). The intrinsic GABAergic interneurons in the ML include basket cells in the lower third of the ML that form perisomatic nests around PC somata and, stellate cells that reside in the outer two thirds of the ML and innervate PC dendrites. The large inhibitory Golgi cells in the GL, characterized physiologically by Eccles et al. (1967) and first called large stellate cells by Cajal, synapse on GC glomeruli and are part of an inhibitory feedback loop. Golgi cells also synapse on excitatory local circuit neurons (Mugnaini et al., 2011), the unipolar brush cells. Unipolar brush cells, initially described by Altman and Bayer (1978), have a round nucleus and a short tuft of dendrioles with large synaptic junctions with filapodia, which are thought to be involved in cell signaling. In the cerebellar cortex, unipolar brush cells are most abundant in regions involved in vestibular functions such as the vermis, flocculus, and paraflocculus (Diño et al., 2001; Englund et al., 2006). Granule cell axons (PFs) also form synaptic contacts on basket and Golgi cells, the latter of which, via its inhibition on GCs, can counteract the excitatory inputs onto PCs (Brodal, 1969).

A major input to the cerebellar cortex is excitatory (glutamate, aspartate) olivocerebellar climbing fibers (CFs) that cross at the level of the inferior olive, travel through the inferior cerebellar peduncle and the cerebellar white matter, and "climb" onto the primary, secondary and tertiary branches of PC arbors. In development, CFs innervate the PC soma but as the PC dendrites arborize, these connections are replaced by basket cell perisomatic "baskets." Each PC receives only one CF and

in the human CFs can innervate up to 10 PCs along its ascent (Chan-Palay, 1977). CFs give off collaterals to the deep cerebellar neurons on the same neurons that receives its PC innervation. Climbing fibers are thus topographically organized in parasagittal innervation patterns, but are less organized in patches laterally (Groenewegen et al., 1979). Inferior olivary neurons are electrotonically coupled and fire synchronously in groups thus activating clusters of PCs. Climbing fibers, which carry information from spinal cord and select brainstem nuclei as well as sensory and motor cortices generate complex spike excitatory postsynaptic potentials that are important in regulating the timing of PC activity, especially for motor function contributing to the "error correction system" of cerebellar activity (Eccles, 1967). More recently, cerebellar function in cognitive tasks has been recognized, and CFs may play an important role in contributing to a wide range of high order sensory associative cognitive behaviors.

Cerebellar mossy fibers produce simple spikes and arise from a variety of pre-cerebellar nuclei. Vestibulocerebellar fibers originate from vestibular ganglia and vestibular nuclei; spinocerebellar fibers arise from the dorsal spinocerebellar tract via the Clark's column (nucleus dorsalis); additional afferents arise in the anterior (ventral) spinocerebellar tract, cuneocerebellar tract (from the external cuneate nucleus), trigeminocerebellar fibers, lateral reticular fibers, and others. A massive projection arises from the cerebral cortex and travels through the pons (the cerebro-ponto-cerebellar tract) via the middle cerebellar peduncle. The topography of the cerebro-ponto-cerebellar projection has been well-established from tract tracing studies in primates (Schmahmann and Pandya, 1989, 1991, 1995, 1997a,b). Mossy fibers send collaterals to the respective deep cerebellar nuclei with the main axon terminating on GC dendrite glomeruli along with axon terminals from inhibitory Golgi cells and in some regions, unipolar brush cells. Thus, there is abundant sensori-motor input to the cerebellum from widespread regions of the cerebral cortex and the topographical ordering in the pons is relayed throughout region-specific areas of the cerebellum. For example, frontal projections via select parts of the pons project to the lateral hemisphere (Schmahmann and Pandya, 1995), mostly to the Crus I and II regions which have been implicated in high order cognitive functions (Schmahmann, 2010).

In terms of efferent projections, PC axons distribute topographically to the four deep cerebellar nuclei which in turn project through the crossed superior cerebellar peduncle mainly to the ventral anterior and ventral lateral nuclei of the thalamus and on to premotor and motor cortex. Other deep cerebellar nuclei projections include the red nucleus which gives rise to the rubrospinal tract, the vestibular nuclei (vestibulospinal tract), and other brainstem nuclei. Chan-Palay (1977) described GABAergic projection neurons in the deep cerebellar nuclei in rats to the inferior olivary complex that are different from intrinsic smaller GABAergic interneurons in the deep cerebellar nuclei (also articulated in Llinas et al., 2004). Some PC axons project directly to vestibular nuclei bypassing the deep cerebellar nuclei. Strick and colleagues used trans-synaptic transport via tracer injections in the frontal cortex and found strong labeling in Crus I and II regions via the mediodorsal thalamic nucleus (Strick et al., 2009). These studies demonstrated that the cerebellar hemisphere not only receives frontal lobe inputs, but also has a means to send projections back to these high-order association areas of the frontal lobe.

LINKS BETWEEN ASD AND THE CEREBELLUM—INFORMATION GLEANED FROM ASSESSING DAMAGE TO THE HUMAN CEREBELLUM

What aspects of ASD could be most affected by cerebellar pathology? There have been several excellent reviews on this topic in recent years (e.g., Fatemi et al., 2012; Reeber et al., 2013; Rogers et al., 2013; Wang et al., 2014; Ebrahimi-Fakhari and Sahin, 2015); our aim here is to update and extend the discussion on this key issue in ASD research. Although the cerebellum has previously been associated primarily with motor functions, it is increasingly accepted that it is also involved in cognitive functions, due in part to its interconnections with other brain regions including (via the thalamus) the cerebral cortex (Fatemi et al., 2012; Broussard, 2014). Because most of the known pathological brain alterations in ASD are not restricted solely to the cerebellum but extend to other brain regions (see below), an examination of cases of cerebellar congenital abnormalities, injury (e.g., trauma, stroke), and disease (e.g., tumors) is instructive. Collectively, the cognitive and affective symptoms have suggested that lesions in specific site(s) in the adult cerebellum produce effects throughout connected networks in the brain, simulating functional deficits from thalamic and/or cerebral cortical lesions that lie upstream within the circuits. Schmahmann postulated that the cerebellum is an important regulator of the speed, consistency and appropriateness of cognitive processes, and that patients with cerebellar damage were unable to control their thought processes (Schmahmann, 2004). This led Ito (2008) to develop his "internal-model hypothesis for mental activities" that forms an analogy between the cerebellums' role of regulating the control of motor function with its modulation of mental activities. The model involves the concerted activity of large scale encoded neural circuits including the prefrontal cortex, temporoparietal cortex, and the cerebellar hemispheres, each of which participate in some aspect of the temporally sequenced network activity.

Although the descriptions of the effects of cerebellar lesions are extremely variable, useful information gleaned from descriptions of human lesions of the cerebellum indicates that both the location of the lesion and the age at which the damage occurred are critical factors in determining the outcome and any potential recovery (Strick et al., 2009; Manto, 2010). As delineated further below, in addition to fine motor error correction, the cerebellum also plays a critical role in visuospatial perception, auditory processing, verbal memory, sequencing, executive functions, and language (Bolduc et al., 2011, 2012; Broussard, 2014). To illustrate one example, auditory and written language comprehension necessitates attention to the message of interest—and suppression of interference from distracting sources. In a

scan of multiple brain regions of typically developing individuals using structural and fMRI imaging, Filippi et al. (2011) showed that the only brain region of significance was the right posterior paravermis which showed a strong correlation between gray matter density and the control of verbal interference.

Autism is intimately associated with impaired communication. One manifestation of this is the difficulty with speech. Although DSM-5 does not incorporate specific criteria for deficits in speech and verbal communication, poorly integrated verbal and non-verbal communication is an example under the criteria for non-verbal communicative deficits. Additionally, individuals who meet criteria for autism spectrum disorder can also be further described with the specifier "with or without accompanying language impairment" (Fung and Hardan, 2014). Pertinent to this issue are recent findings that demonstrate an association between children with ASD and childhood apraxia of speech. Childhood apraxia of speech is defined as a neurological childhood speech sound disorder in which the precision and consistency of movements underlying speech are impaired in the absence of neuromuscular deficits. Children with apraxia of speech cannot produce the sounds necessary for speech due to deficits in motor planning, organization, and production. The muscles required for speech production are fully functional, but the ability to coordinate and plan motor movements to produce fluid intelligible speech is impaired. It is important to note that analysis of apraxia of speech can be difficult to disentangle from the clinical phenotype of ASD. Nevertheless, in a recent study, Tierney et al. (2015) examined young children with autism over the course of 3 years; 64% of children initially diagnosed with autism were found to also have apraxia of speech, and 37% of children initially diagnosed with apraxia also had autism (Tierney et al., 2015). The subjects with apraxia had "difficulty coordinating the use of their tongue, lips, mouth, and jaw and with accurately producing speech sounds, so that each time they say the same word, it comes out differently, and even their parents have difficulty understanding them." This study also showed that the commonly used Checklist for Autism Spectrum Disorder accurately diagnoses autism in children with apraxia.

In the context of this review, one critical question is, how important is the cerebellum in verbal communication? The cerebellum is often, perhaps arbitrarily, perceived as being responsible for automatic movements and reflexes but not higher functions such as speech. However, that distinction may be meaningless. In fact speech requires a great deal of coordination that is largely automatic. As noted by Broussard (2014) "some apraxias such as gait apraxia and limb-kinetic apraxia are routinely associated with cerebellar damage but speech apraxia can also result from cerebellar damage." Early documentation of this was described in a one-of-a-kind study of World War I soldiers with gunshot injuries restricted to the cerebellum where (Holmes, 1917) reported that speech was impaired in many of the casualties he examined. In another classic paper Schmahmann and Sherman (1998), reported on 20 cases where neurological disease was confined to the cerebellum. This group coined the term "cerebellar cognitive affective syndrome" to describe this patient population. Affected individuals exhibited impaired executive functions such as planning, set shifting, abstract reasoning, working memory, and difficulties with spatial cognition. Prominent features of this syndrome are language deficits and impaired verbal fluency (Schmahmann and Sherman, 1998). Some of these patients also presented with changes in their personalities including passivity, flattening, or blunting of emotion, decreased emotional expression and disinhibited or child-like behavior, often due to lesions in the vermis (see below and also Schmahmann, 2000, 2004, 2010).

The findings of Schmahmann and Sherman (1998) have been validated in a study by Tedesco et al. (2011) who retrospectively analysed patient records from the Ataxia Lab of Santa Lucia Foundation in Italy. Of the 223 medical charts reviewed, 156 were included in the study which focused on the role of the cerebellum in cognition and the relevance of lesion topography in defining the cognitive domains that were affected. Vascular topography and the involvement of deep cerebellar nuclei were established as the chief factors that determined the cognitive profile. Language, executive function, visuospatial abilities and sequences were the most adversely affected functions irrespective of whether the damage was focal or degenerative. Differences in the patterns appeared when the location of the focal lesion was considered. The vascular territory of the superior cerebellar artery primarily involves the anterior lobe, whereas that of the posterior inferior cerebellar artery mostly involves the posterior lobe including the inferior aspects of the cerebellar hemispheres and vermis, and also the dentate nucleus. Strokes in the superior cerebellar artery or posterior inferior cerebellar artery usually present with gait problems and/or ataxia depending on which aspects of the arteries are blocked (e.g., ataxia of stance and gait are a sign of medial superior cerebellar artery and lateral posterior inferior cerebellar artery infarction; Timmann et al., 2009). Strokes in the anterior inferior cerebellar artery which supplies the flocculus, and inferior and anterior aspects of the cerebellum and middle cerebellar peduncles, are rare and usually affect the brainstem. Therefore, assessment of patients with stroke lesions in the superior cerebellar artery or posterior inferior cerebellar artery territory allowed evaluation of anterior vs. posterior cerebellar lesions. Intriguingly, subjects with posterior inferior cerebellar artery strokes performed worse than patients with superior cerebellar artery on all cognitive domains, and in fact, statistically significant differences were noted with regard to verbal memory, language, visuospatial and executive functions (see **Figure 2**).

Another general feature that has emerged from recent studies is that injury or malformations of the vermis result in disabilities that resemble those encountered in ASD. In the context of mouse models of ASD, support for an essential role of the vermis is illustrated by $GABA_A$ receptor β3 (GABRB3) subunit knockout mice, which display autistic behaviors and selective vermal hypoplasia (DeLorey et al., 2008). The $GABRB3^{-/-}$ mouse has significantly decreased sagittal surface area of lobules II-VII measured semi-quantitatively and exhibits a reduced level of interaction with unfamiliar mice compared to controls. Specifically, $GABRB3^{-/-}$ mice show reduced social engagement

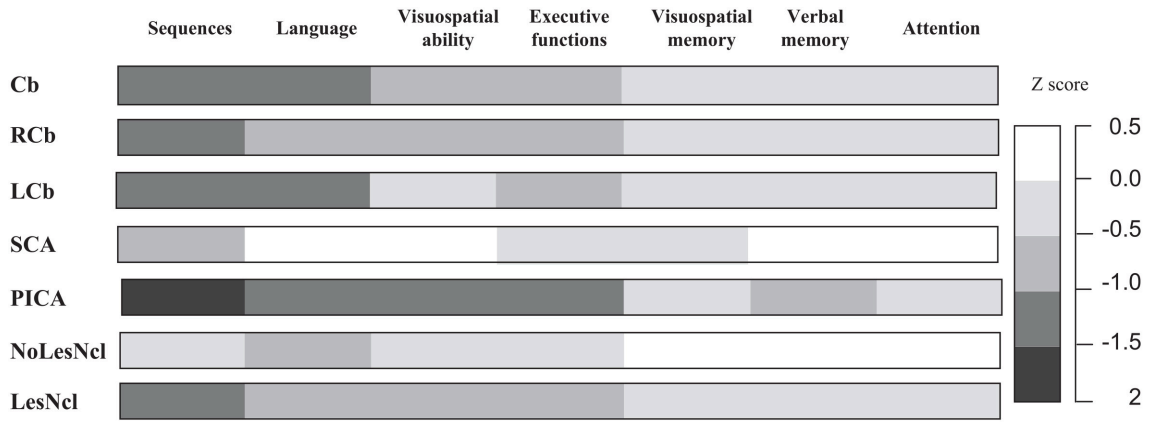

FIGURE 2 | Cognitive patterns of subjects with focal cerebellar lesions grouped according to lesion topography. To compare performances between tests, raw scores were converted to *Z*-scores (*z* score = subject raw score minus population mean)/population standard deviation. For each cognitive function, a single *Z*-score was obtained by calculating the mean *Z*-scores of the tests, grouped according to that function. *Z* scores of −1 or lower were considered to indicate pathology. Cb, cerebellar damage; LCb, left cerebellar damage; LesNcl, lesions of the deep cerebellar nuclei; NoLesNcl, no lesions of the deep cerebellar nuclei; PICA, posterior inferior cerebellar artery; RCb, right cerebellar damage; SCA, superior cerebellar artery. Adapted with permission from Tedesco et al. (2011).

in both sociability (interaction time with novel mouse) and social novelty testing (adding a second novel mouse, i.e., unfamiliar mouse to a chamber on the opposite side as the first now familiar mouse). In addition, the GABRB3$^{-/-}$ mouse in the DeLorey et al. (2008) study, exhibited hyperactivity and spent more time in the open portion of a circle than with the novel object in an exploratory behavior task and a stereotypical circling pattern. Although these authors mention that the vermal aplasia could contribute to the reported behavioral deficits in the GABRB3$^{-/-}$ mice, a previous study by Pierce and Courchesne (2001) did significantly correlate the degree of hypoplasia of vermal lobules VI-VII in children with ASD with reduced visuospatial exploration and with stereotyped behavior. In the context of human ASD, genetic studies have suggested that the GABRB3 gene may be a susceptibility locus (see Chen et al., 2014 and references therein) and duplication of a segment of chromosome 15q ("duplication 15 syndrome"), which contains several genes including the GABRB3 gene, occurs in approximately 2% of autism cases (Scoles et al., 2011). Moreover, postmortem samples from a small number of subjects have indicated reduced GABRB3 transcripts (Scoles et al., 2011) and protein (Fatemi and Folsom, 2011).

The vermis is phylogenetically the most ancient structure of the cerebellum (the paleocerebellum) and develops and becomes fully foliated by 4 months gestation in humans, while development of the large cerebellar hemispheres (neocerebellum) lags behind that of the vermis by 1–2 months. Studies conducted on children that have undergone surgery for cerebellar tumors affecting the vermis have also been informative. Typical problems include cognitive impairment and flattened affect manifesting as increased irritability, impulsiveness, disinhibition, poor attention and behavioral modulation (Levisohn et al., 2000; Riva and Giorgi, 2000). In an important study by Tavano et al. (2007), the clinical picture of 27 patients with congenital malformations restricted to the cerebellum was described in detail. Seventy-four percent of these patients presented with some degree of mental retardation. Notably, patients with cerebellar vermal agenesis or diffuse cerebellar hypoplasia presented with core ASD symptoms including language deficits, social interaction impairments, and some repetitive and stereotyped behaviors. In contrast, in cases where the lesions were restricted to the cerebellar hemispheres, the disabilities were less severe. Interestingly, motor deficits in patients with lesions in the vermis were less severe than patients with lesions in the cerebellar hemispheres. Overall, abnormalities affecting the vermis translated into the least favorable long-term outcomes and the most severe impairments, including pervasive developmental disorder, mental retardation, impaired social and communicative behavior, and social withdrawal.

In adults, damage to the cerebellar vermis may be relatively rare because some of these patients likely do not survive. However, a condition known as Dandy-Walker malformation primarily affects the vermis. The pathology consists of cerebellar vermal hypoplasia with upward vermis rotation and often elevation of the torcula, an enlarged fourth ventricle which extends posteriorly as a retrocerebellar cyst, and hydrocephalus which is present in 50–80% of subjects. This condition often presents with macrocephaly in the neonatal period, and infants may come to medical attention because of hydrocephalus, developmental delay, or ataxia (Parisi and Dobyns, 2003). Apnea and seizures are seen in a significant proportion of children and about one quarter of patients with this condition die. No studies have specifically investigated the incidence of ASD in Dandy-Walker malformations. However, developmental delay and mental retardation are common but highly variable in Dandy-Walker; as noted by Parisi and Dobyns (2003), the distribution of intelligence scores appears to be bimodal, suggesting that there may be two distinct groups: those with normal cognition (47%), and those with severe impairment (IQ $<$ 55), which represented 35% of the cohort.

CEREBELLAR PATHOLOGY IN ASD—HUMAN POSTMORTEM AND IMAGING STUDIES

Idiopathic Autism

Early neuropathological studies examining eight serially sectioned autism brains revealed a reduction in PCs most pronounced in the lateral hemispheric region coupled with a pallor of Nissl staining in the granular cell layer in the same region (Bauman and Kemper, 1985; Kemper and Bauman, 1993). The reduction in PC density was later reported by a number of investigators (e.g., Bailey et al., 1998; Whitney et al., 2008; Skefos et al., 2014) and in total, about 75% of reported autism cases that involved quantitative studies had a reduction in PCs (see Schumann and Nordahl, 2011 for review). In contrast, the medial vermis displayed a less severe reduction in PCs (Bauman and Kemper, 1985 and others). Fatemi et al. (2002a) reported a 24% decrease in the size of remaining PCs in the cerebellum in autism cases compared to controls.

An open question is, with the reduction in PCs in the autism cerebellum, how is the distribution of CFs affected? An insight into this question was found in studies of spontaneous cerebellar mutants that despite having decreases in PCs, the CFs distributed in a topographically normal pattern (Blatt and Eisenman, 1988, 1989, 1993). Electrophysiological studies in the adult homozygous weaver mouse revealed that CFs hyperinnervated the PCs in the vermis, with each PC receiving up to four CF inputs (Crepel and Mariani, 1976; Puro and Woodward, 1977; Mariani, 1982) instead of the normal monoinnervation first described anatomically by Ramon y Cajal in the late nineteenth century. It remains to be determined whether the autism brain has a similar PC multiple innervation pattern due to decreased PCs. This may contribute to sustain inferior olivary neurons, which are unaffected in number in the medulla in postmortem autism cases (Blatt, 2012).

We hypothesize that due to the absence of some PCs, those that border remaining clusters are likely to be multi-innervated by excitatory olivocerebellar CFs (illustrated in **Figure 3**). The remaining PCs in the cerebellar hemisphere in postmortem cases contain decreased GAD67 mRNA (Yip et al., 2007) suggesting abnormal functional connectivity with the deep cerebellar nuclei. In the autism cerebellum, there was low Nissl staining in serial sections through the deep cerebellar nuclei in Bauman and Kemper's (1985), cases suggesting a possible deficiency in cell number and/or density in this key output structure. In that same study and confirmed by Blatt (2012), the inferior olivary complex contained ectopic linearly-oriented neurons in the principal

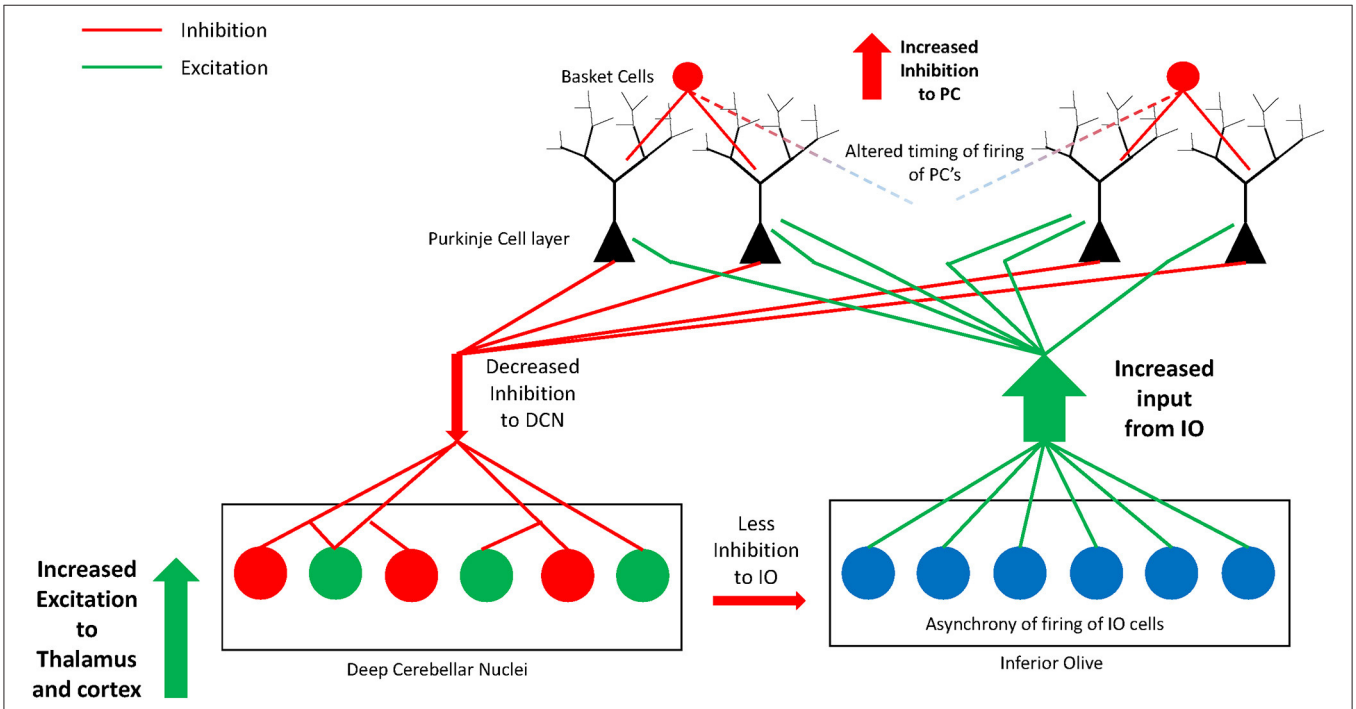

FIGURE 3 | Illustration of the hypothesis of increased excitatory:inhibitory circuitry within the cerebellum in autism. Missing Purkinje cells may disrupt olivocerebellar circuitry in individuals with autism. Some of the remaining Purkinje cells are likely multi-innervated by climbing fibers as previously shown in animal models of spontaneous cerebellar mutations. Remaining Purkinje cells have decreased GAD67 mRNA and protein that could translate to decreased GABA inhibition to excitatory cells in the deep cerebellar nuclei. The deep cerebellar nuclear cells in turn send increased excitatory input to thalamus and cerebral cortex thus affecting higher circuitry within the network. The circuitry may be further perturbed by an abnormal nucleo-olivary projection from the deep nuclei to the inferior olive. GABAergic projection cells in the deep nuclei (dentate nucleus) have decreased GAD65 mRNA and project to the inferior olive. This can result in asynchrony of firing of groups of inferior olivary neurons whose altered climbing fiber complex spike activity would cause abnormal timing of firing in select subgroups of Purkinje cells. Thus, alterations in cerebellar circuitry in autism likely contribute to motor and non-motor aspects of the phenotype by affecting higher-order association cortices within the networks.

olive on the lateral edge of the ribbon, that may disrupt the electrotonically coupled synchrony of firing of principal olivary neurons and affect the timing of PC activity (Welsh, 2002; Welsh et al., 2005). There were also age-related changes in the size of inferior olivary neurons with larger cells in children with autism and smaller neurons in adults (Bauman and Kemper, 1985). Interestingly, the principal olive has a robust projection to the lateral cerebellar hemisphere, the cerebellar region that contains the greatest decrease in PCs (Bauman and Kemper, 1985; Whitney et al., 2008; Skefos et al., 2014), and is the recipient of cortical association inputs especially from the frontal lobe via the pons (Schmahmann and Pandya, 1995, 1997a; see Ramnani, 2012 for review). Neurochemical studies in the Crus II region revealed decreased glutamic acid decarboxylase 67 (GAD67) mRNA in the remaining PCs in young adults with ASD (Yip et al., 2007), and subsequent studies showed likely compensatory increases in GAD67 mRNA in cerebellar interneurons in the molecular layer (Yip et al., 2008).

Fatemi et al. (2002b) demonstrated an overall reduction in GAD in the cerebellum using specimens from different cerebellar regions in autistic brains. Yip et al. (2009) added a study analyzing the GABAergic neurons within the dentate nuclei and found significantly decreased GAD65 mRNA levels in the adult autism group in a larger sized subpopulation of GABAergic neurons, described by Chan-Palay (1977) as projecting to inferior olivary neurons in rodents and confirmed by Llinas et al. (2004). In contrast, the smaller sized GABAergic interneurons contained normal GAD65 mRNA levels (Yip et al., 2009). If the anatomy is similar in primates, that is, if these larger GABA-containing neurons of the dentate do project to the inferior olive, then it could potentially have a profound effect on the synchrony of firing of olivary neurons and influence the timing of the remaining PCs in the hemisphere (see **Figure 3**). These events could help reaffirm one of the more interesting hypotheses of disturbed neural circuitry in autism resulting in an excitatory:inhibitory imbalance (Blatt et al., 2001; Hussman, 2001; Rubenstein and Merzenich, 2003; Pacey et al., 2009; Blatt, 2012) affecting motor and non-motor functional activity.

Additional observations on postmortem autism brains include flocculonodular dysplasia and hypoplastic lobes, which may indicate cell migration abnormalities to defined areas (Wegiel et al., 2010). Cytoarchitectural laminar abnormalities are not common in autism but have been reported sporadically in the anterior cingulate (Simms et al., 2009), posterior cingulate (Oblak et al., 2011) and, the fusiform gyrus (van Kooten et al., 2008). Reports of neuroglia at pathologic sites in the autism brain were reported by Bailey et al. (1998) who found increased glial fibrillary acidic protein (GFAP) in Bergmann glia in the cerebellum in three cases of autism and increased glial proliferation in one case. Vargas et al. (2005) also reported increased Bergmann glia reactivity in the PC layer and increased microglia reactivity in the granular layer and accompanying white matter in 9 of 10 patients. These investigators also demonstrated activated microglia in the vicinity of degenerating PCs and granule cells (Vargas et al., 2005) but it is unclear whether epilepsy and/or anti-epileptic therapies contributed to the cell loss and subsequent increased glial activity.

Antibodies to Cerebellar Antigens

Serum autoantibodies directed against human brain were identified via ELISA and Western immunoblot in children with ASD and their non-autistic siblings as denser bands of 73 kDa in the cerebellum and cingulate cortex and, as 100 kDa bands in the caudate, putamen, and prefrontal cortex compared to controls (Singer et al., 2006). Serum antibodies from mothers of children with autism have the capability to cross the placenta and possibly alter brain development (Zimmerman et al., 2007). The Van de Water group followed with a study extracting human cerebellar protein with a 52 kDa band present in about 20% of children with autism (Wills et al., 2009). This same cerebellar extract was examined via immunohistochemical staining of antibodies in Macaque fascicularis monkey cerebellum. Intense staining was found in Golgi cells and, in some cases, basket cells in the deep molecular layer. Furthermore, these antibodies also immunostained Golgi cells in 21% of children with ASD (7/34) compared to controls (0/23). No relationship was found between antibodies for the 52 kDa protein or with immunohistochemical staining, when compared against the behavioral outcome which was based on subjects with a diagnosis of regressive vs. early onset autism (Wills et al., 2009). Although unclear, autoantibodies may bind to and affect the physiological role of these key inhibitory neurons in the cerebellum with downstream pathological effects. In a later study, GABAergic interneurons were found to be co-localized with calcium binding proteins including calbindin, parvalbumin, and calretinin and were immunoreactive in additional brain regions (Wills et al., 2011). In another study, 45 and 62 kDa bands were isolated from Western blot of autoantibodies toward target cerebellar proteins (Goines et al., 2011). The presence of the 45 kDa protein autoantibodies were associated with the diagnosis of autism whereas the 62 kDa protein autoantibodies were associated with ASD diagnosis (Goines et al., 2011). However, children with autism, ASD and typically developing children that contain reactivity to these autoantibodies, all displayed altered basic cognitive and adaptive behavioral traits (Goines et al., 2011). Plasma samples from children with or without subtypes of ASD were immunostained against monkey cerebellum and other brain regions including dentate gyrus, were significantly correlated with behavioral and emotional problems utilizing the Child Behavior checklist (Rossi et al., 2011). A comprehensive study examining the behavioral profiles in Italian children with ASD and their unaffected siblings was reported by Piras et al. (2014). In that study, the 45 and 62 kDa cerebellar specific antibodies correlated with cognitive impairment and motor stereotypies, respectively, in the ASD group and both correlated with increased head circumference. Also, the presence of maternal anti-brain autoantibodies of 37, 39, and 73 kDa in children with ASD correlated with cognitive behavioral abnormalities including verbal and non-verbal language development, sleep/wake cycle alterations and developmental delay (Piras et al., 2014). The authors also found a correlation between the mothers of autistic children having the 39 or 73 kDa autoantibodies directed against the fetal brain, with the presence of the 62 kDa autoantibodies in the ASD child. Therefore, this is an exciting area of research that may represent an important subset of ASD children with neurologic

profiles based on immunological factors including maternal contribution.

Neuroimaging Studies

A discussion of neuroimaging studies in ASD should be prefaced with the suggestion that there may be a disconnection between neuropathological reports from postmortem cases with moderate to severe forms of idiopathic autism, vs. imaging studies that are primarily conducted in higher functioning individuals. Very few high functioning or Asperger's autism postmortem cases have been obtained from the brain banks that would match up well with the higher functioning cohorts typically utilized in imaging studies. It therefore remains unclear whether alterations in brain activity within specific patients or cohorts are due to observed postmortem changes in histology of the same brain area(s).

Early neuroimaging studies of the cerebellum in ASD reported a reduction in the size of cerebellar regions including the vermis (Hashimoto et al., 1995; Kaufmann et al., 2003). Another study confirmed that the size of the vermis is indeed reduced in ASD but noted that the reduction in vermal volume did not correlate with symptom severity or with verbal, non-verbal or full scale IQ (Webb et al., 2009). In a pathology report, Kemper and Bauman (1998) found much variability of the size of different lobules in the vermis with widening of the spaces of the folia. These neuropathologic observations may explain the contrasting imaging results in the vermis in autism cases. For example, Courchesne et al. (1988) reported a significantly smaller mean area of vermal lobules VI and VII but not lobules I-V and reported hypoplasia (16% smaller) or hyperplasia (34% larger) of vermal lobules VI and VII across ages in a subgroup of autism patients (Courchesne et al., 1994). A smaller cerebellar vermis in autism cases was also reported by others (e.g., Kaufmann et al., 2003; Scott et al., 2009). In contrast, Piven et al. (1997) did not replicate these findings but did find that the total cerebellar volume was increased in ASD. However, the increase in cerebellar volume was found to be in general, and proportional to total brain volume.

A meta-analysis of structural MRI studies in the literature by Stanfield et al. (2008) reported the general findings of increased total brain volume and volume of the cerebral hemispheres, cerebellum and caudate, whereas in contrast, the corpus callosum volume was reduced. These authors also concluded that there was an age and IQ effect on the volume of vermal lobules VI-VII and for age in the amygdala. In contrast, a well-designed structural MRI study was conducted by Scott et al. (2009) that included low functioning individuals with ASD, high functioning individuals with autism, an Asperger's group, and a typically developing control group amongst 62 male subjects 7.5–18.5 years old. The authors concluded that the midsagittal area of the vermis or of subgroups of vermal lobules in any of the autism groups was not significantly different from typically developing children and neither age nor IQ predicted the size of the vermis in the autism groups (Scott et al., 2009). Other early observations included an increase in cerebellar white matter volume, and a reduction in the gray/white matter ratio (Courchesne and Pierce, 2005). In a study of 103 subjects, Cheung et al. (2009) using diffusion tensor imaging, reported a correlation of repetitive behaviors via ADI-C diagnostic algorithm scores with reduced cerebellar white matter fractional anisotropy indices. In another study, significant differences in white matter volume in the autism group included the corticoponto-cerebellar tract, arcuate fasciculus, uncinated fasciculus and corticospinal tract (Ecker et al., 2012).

An MRI study by Bolduc et al. (2012) on young children with developmental cerebellar pathologies found that the lateral cerebellar hemisphere is important in cognitive skills and language. These authors also found that there is a stronger association between language skills and the right cerebellar hemisphere, previously demonstrated in tumor resection studies (Levisohn et al., 2000; Riva and Giorgi, 2000). Using voxel-based morphometry, Ecker et al. (2012) found a negative correlation between cerebellar gray matter volume vs. group (ASD vs. controls). Similar findings were found in the posterior cingulate, fusiform gyrus (posterior), and amygdala. In contrast, using partial least squares analysis, in many regions in the autism brain, these authors concluded that there is positive correlation with gray matter volume in the following areas: the cerebellar tonsil, dorsomedial prefrontal cortex, fusiform gyrus (anterior) parahippocampal gyrus, superior temporal gyrus, mid-and anterior-cingulate, inferior frontal gyrus, caudate, and insular cortex.

SYNDROMIC FORMS OF AUTISM—POSTMORTEM, IMAGING, AND MOUSE STUDIES

Fragile X Syndrome

FXS is the most common form of X-linked intellectual learning disability affecting about 1 in 2500 individuals, and is associated with a cytogenic marker in the distal region of the long arm of the X chromosome. The disorder is caused by an unstable trinucleotide repeat of cytosine guanine guanine (CGG) near the 5′ promotor region of the FMR1 gene. Normal individuals have 5–55 CGG repeats whereas in FXS, repeat lengths of greater than 200 causes gene hypermethlyation and loss of the FMR1 gene product, Fragile X mental retardation protein (FMRP). FXS is associated with abnormal dendritic development and maturation of synaptic connectivity that contributes to the cognitive deficits (see Bagni et al., 2012; Gallagher and Hallahan, 2012 for reviews). Approximately 20% of individuals with FXS have epilepsy (Berry-Kravis, 2002).

FXS is associated with abnormalities in cortical dendritic spines; they are longer, thinner, and lack the mushroom-shaped spines that are characteristic of mature dendrites (Hinton et al., 1991; Irwin et al., 2000, 2001, 2002). As seen in idiopathic autism, cerebellar PC loss and cell displacement have been reported in a human postmortem study of FXS (Greco et al., 2011). Cerebellar pathology has also been noted in adult onset pre-mutation carriers of fragile X where repeat lengths are in the intermediate range (55–199). These carriers are susceptible to fragile X-associated tremor ataxia syndrome (FXTAS), a neurodegenerative condition that typically occurs in late middle age (Hagerman and Hagerman, 2015). Neuropathology in this syndrome is associated with patchy loss of axons in the brain,

spongiosis of the middle cerebellar peduncles, and loss of PCs (Greco et al., 2002, 2006, see Gallagher and Hallahan, 2012, for review).

There are some data indicating that that the posterior-superior vermis is significantly larger in boys with FXS and autism than in boys with FXS without ASD; these changes appear to be specific to the individuals with ASD (i.e., autistic disorder) since boys with FXS and social anxiety show increased anterior, but not posterior vermis size (Kaufmann et al., 2003). Moreover, Gothelf et al. (2008) reported positive correlations between size of the posterior vermis (and the caudate) and several subscales of the Autism Behavior Checklist in persons with FXS. Curiously, the increased posterior-superior cerebellar vermis size affects the same region in FXS (i.e., lobules VI–VII) that is smaller in individuals with idiopathic ASD (Budimirovic and Kaufmann, 2011).

The Fragile X Knockout Mouse

The Fmr1 knockout (KO) mouse is generally considered a good animal model of human FXS and has been intensively studied on the molecular, cellular, anatomical, and behavioral levels (Hampson et al., 2012). Results from studies on the Fmr1 mouse were instrumental in facilitating translation of metabotropic glutamate receptor 5 antagonists and a $GABA_B$ receptor agonist into human clinical trials, although those conducted to date have all failed due to lack of efficacy and/or meeting endpoints. Nevertheless, this mutant mouse continues to provide useful information on the neurobiology of FXS, and will likely continue to be used for preclinical drug studies.

In the context of neuroanatomical aberrations, a recent study classified the Fmr1 mouse in relation to other mouse models of ASD. Most studies investigating mutant ASD mice have examined only a single mouse model. However, Ellegood et al. (2015) pursued a more holistic approach whereby the clustering of autism was studied based on anatomical phenotyping (using various modes of high resolution MRI) of 27 different ASD mouse lines. The results indicated that ASD mouse lines separated into three major clusters; a cerebellar set, a cortico-thalamic-striatal set, and a set related to limbic structures (Ellegood et al., 2015). The strongest connections in the clustering of models were between En2, Nrxn1α, and Fmr1. This cluster (Group 1), which also included Shank 3 mice, displayed increases in large white matter structures including the corpus callosum, fimbria, and fornix, as well as increases in the frontal and parieto-temporal lobe, and decreases in the cerebellar cortex. It is intriguing that the Fmr1 mouse appears to be anatomically related to the En2 knockout mouse as the En2 gene is a homeobox gene that is essential for the proper development of the cerebellum (Cheng et al., 2010; Choi et al., 2014). Previous analyses on the Fmr1 knockout mouse conducted by this group provided insight into how the cerebellum is affected by the loss of FMRP. Ellegood et al. (2010) used high resolution MRI imaging to show that of 62 brain regions examined, the cerebellum stood out as one of the few regions showing significant volume changes compared to wild-type controls. Specifically, it was demonstrated that the deep cerebellar nuclei, which as noted above, transmit the sole output of the cerebellar cortex to the thalamus and cerebral cortex, were smaller in volume in the Fmr1 mice. Moreover, immunocytochemical analyses indicated a loss of neurons and an increase in astrocytes in the deep cerebellar nuclei (Ellegood et al., 2010). Pacey et al. (2013) conducted a detailed study of postnatal development of the Fmr1 mouse cerebellum and showed reduced volume in the first few weeks after birth (as determined by diffusion tensor imaging), and a delay in myelination which was likely due to a reduced number of oligodendrocyte precursor cells. A delay in myelination may in turn affect the trajectory of neuronal maturation which could have permanent consequences affecting the adult CNS (Pacey et al., 2013).

Rett Syndrome

Rett syndrome, first discovered in the late 1970s/early 1980s occurs in 1/10,000–1/23,000 girls with 90% of cases involving a mutation in the X-linked methyl-CpG binding protein 2 gene (MECP2). Rett syndrome is a progressive neurological condition that presents early with autistic features, but then progresses to severe neurological and cognitive dysfunction. Rett primarily affects females where beginning at 12–18 months of age they regress, losing speech, and motor skills and suffer from additional problems including mental retardation, respiratory impairment, and seizures (Armstrong, 1992, 2005). Rett differs from ASD in that patients with Rett syndrome have a reduced lifespan, although greater than 70% live beyond age 45 (Tarquinio et al., 2015). The leading cause of premature death is cardiorespiratory failure. Mutations in MECP2 can result in cessation and regression of development in early childhood, ataxia, stereotypies as well as severe cognitive and spastic motor abnormalities (Ropers and Hamel, 2005). Patients have reduced brain weight (not accounted for by age-related atrophy) directed more toward the cerebral hemispheres than the cerebellum (Armstrong, 2005). The Rett syndrome brain is characterized by widespread reduced dendritic branching in many areas in the frontal and motor cortices, hippocampus, thalamus, basal ganglia and amygdala (Bauman et al., 1995). Also, decreased size of cortical minicolumns in select frontal and superior temporal areas was reported in five Rett patients with mean age of 14.4 years (Casanova et al., 2003).

In contrast, the cerebellar literature for Rett syndrome is very sparse. One account of five patients by Oldfors et al. (1990) reports that all five had smaller brains compared to controls by 25% with the cerebellum decreased proportionately in size. One patient had a patchy loss of PCs with several folia having a complete loss and, the ML had cell loss and gliosis. Another patient had moderate generalized loss of PCs and thinner folia with gliosis. The other patients had milder effects but also focal loss of PCs, minor gliosis in the cerebellar cortical layers and a few of the patients were observed to have a loss of myelin in the white matter (Oldfors et al., 1990). In addition to mutation of the MeCP2 in Rett syndrome, MECP2 gene duplication is also associated with severe neurological consequences including mental retardation, hypotonia, recurrent infections, and interestingly, cerebellar degeneration (Reardon et al., 2010).

Mouse studies specifically focusing on the cerebellum in Rett syndrome are also relatively sparse. Steadman et al. (2014)

used MRI imaging to compare three genetic ASD mouse models: Neuroligin-3 R451C knock-in, integrin β3 homozygous knockout, and MECP2 308-truncation mice, and focused on assessing morphological differences specific to the cerebellum. All three ASD lines showed significant volume changes in the cerebellum. Of note is the observation that the posterior vermis was morphologically altered in the two mouse lines with reported repetitive behaviors (MeCP2 and integrin β3 mice). An unexpected finding by Steadman et al. (2014) was that MECP2 mutant mice had cerebellar volume changes that increased in scope depending on the genotype; compared to age and sex matched wild-type mice MeCP2 heterozygous males had reduced cerebellar volumes while homozygous females had increased cerebellar volumes.

Tuberous Sclerosis Complex (TSC)

In a recent analysis of the field (Sundberg and Sahin, 2015) indicated that approximately 50% of patients with TSC present with ASD and that preclinical and clinical investigations conducted to date indicate the cerebellum has a profound regulatory role during development of social communication and repetitive behaviors in TSC. TSC is an autosomal dominant condition that affects cerebral cortical development (the lesions are called tubers), and in a smaller cohort, the development of the cerebellum, where reduced volume has been reported (Weisenfeld et al., 2013). The pathogenesis may lead to developmental delays, epilepsy, and autism. Vaughn et al. (2013) retrospectively reviewed MRI images from 145 children with TSC with a mean age of 7.6 years old. These investigators found cerebellar tubers present in 35 of 145 patients (24%) while 6 of 35 patients had cerebellar atrophy. This incidence of cerebellar tubers was in agreement with Ertan et al. (2010) who reported 8 out 27 patients (30%) had tubers while only 1/27 showed atrophy. Tubers in some patients changed while others remained static; in those that changed, the pathogenesis may have been due TSC2 gene mutations resulting in disordered migration and/or accelerated astroglial apoptosis (Chu-Shore et al., 2009). Previously, Jurkiewicz et al. (2006) selected 12 patients with TSC (median age of 10.6 years old) and 8 of 12 had a mutation in the TSC2 gene, with 50% displaying cerebellar atrophy. Further analysis of the cerebellum in TSC was reported in an MRI and PET imaging study of 78 patients with TSC; 27% had cerebellar pathology that was positively correlated with the severity of autistic symptoms (Eluvathingal et al., 2006). In this study, within-group analyses of the cerebellar lesion group revealed that children with right-sided cerebellar lesions had higher social isolation and communicative and developmental disturbance compared with children with left-sided cerebellar lesions. The side of the cerebellar lesion was not related to adaptive behavior functioning. These findings provide additional empiric support for a role the cerebellum in autistic symptomatology. It was concluded that further investigation of the potential role of the right cerebellum in autism, particularly with regard to the dentatothalamofrontal circuit, is warranted.

Several exceptionally informative lines of mutant Tsc mice have been generated and studied in detail. FXS and TSC are both caused by mutations in genes that regulate protein synthesis in neurons and it has been hypothesized that excessive protein synthesis is one core patho-physiological mechanism of intellectual disability and autism. Using electrophysiological and biochemical assays of neuronal protein synthesis in the hippocampus of Tsc2 ± and Fmr1 knockout mice, Auerbach et al. (2011) demonstrated that synaptic dysfunction caused by these mutations actually falls at opposite ends of a physiological spectrum. Synaptic, biochemical and cognitive defects in these mutants are corrected by treatments that modulate metabotropic glutamate receptor 5 in opposite directions, and deficits in the mutants disappear when the mice are bred to carry both mutations. It was concluded that normal synaptic plasticity and cognition occur within an optimal range of metabotropic glutamate-receptor-mediated protein synthesis, and deviations in either direction can lead to shared behavioral impairments. In terms of treatment, these findings support the concept that antagonists of mGluR5 (including negative allosteric modulators) could be used in FXS, while mGluR5 agonists (or positive allosteric modulators) might be useful for treating TSC.

Reith et al. (2011) studied a mutant mouse line in which the Tsc2 gene was selectively deleted from PCs starting at postnatal day 6. A crossed line was generated (Way et al., 2009) that produced mice with one functional Tsc2 allele (Tsc2f/-;Cre); this line was examined because these mice model a TSC patient with one non-functioning TSC2 allele. Somatic cell loss of the remaining Tsc2floxed allele occurs primarily in PCs. The loss of Tsc2 caused a progressive increase in PC cell size and subsequent death from apoptosis. PC loss was predominantly cell type specific and associated with motor deficits. Immunohistochemical analysis showed that both endoplasmic reticulum and oxidative stress were increased in Tsc2-null PCs. The cell death and ER stress phenotypes were rescued by treatment with the mTORC1 inhibitor rapamycin. To assess whether the murine PC loss had a correlate to the human TSC, postmortem cerebellum samples from TSC patients were examined and found to have PC loss in half of the samples. These findings support a role for the TSC complex in PC survival by regulating endoplasmic reticulum and oxidative stress and reveal a salient feature of TSC neuropathology.

In a subsequent study, (Reith et al., 2013) conducted a series of behavioral tests to determine if Tsc2flox/-;Cre mice displayed autistic-like deficits. Tsc2f/-;Cre mice demonstrated increased repetitive behavior as assessed with marble burying activity. Using the three chambered apparatus to assess social behavior, Tsc2f/-;Cre mice showed behavioral deficits by exhibiting no preference between a stranger mouse and an inanimate object, or between a novel and a familiar mouse. The observations indicate that selective loss of Tsc2 in PCs in a Tsc2-haploinsufficient background leads to autistic-like behavioral deficits.

Another mouse model of TSC has been described where the selective loss of Tsc1 gene expression only in PCs also resulted in autistic-like behaviors including abnormal social interaction, repetitive behavior and vocalizations (Tsai et al., 2012). In addition, PC loss was apparent in Tsc1 −/− mice at 2 months of age but not in +/− mice at 2 or 4 months of age. Most startling were the observations that (a) both Tsc1 −/− and +/− mice showed significant reductions in PC excitability (including

reduced PC firing rates), and (b) that not only homozygous Tsc −/− mice, but also heterozygous Tsc1 +/− mice showed autistic like behaviors *in the absence or prior to any PC loss*. Importantly, the findings of Tsai et al. (2012) in Tsc1 +/− mice indicate that an autistic behavioral phenotype can be induced not only by the loss of PC, but also solely by impaired PC excitability.

In summary, mice with loss of Tsc1 or Tsc2 restricted to PCs both resulted in PC degeneration (in Tsc1 −/− mice and in Tsc2 +/− mice) and haploinsufficiency of either Tsc1 or Tsc2 is necessary and sufficient to induce an autistic phenotype in mice. These observations also overlap and are consistent with results from chimeric Lurcher mice which exhibit partial depletion of PCs during development and have been reported to display ASD characteristics such as motor hyperactivity, increased repetitive behavior, and deficits on a reversal learning task (Dickson et al., 2010; Martin et al., 2010). Collectively, these findings strongly indicate that PC loss and/or dysfunction may be an important link between TSC and ASD, and suggest the possibility that this phenomenon could contribute to other forms of ASD.

CONCLUSIONS AND OPEN QUESTIONS

We present the following major points gleaned from this review:

- Studies investigating congenital and acquired damage limited to the cerebellum generally support the proposition that many of the symptoms of ASD have a cerebellar component. Some studies appear to indicate that the vermis and lateral hemisphere might be considered regions of special interest.
- The cerebellum is intimately linked to many if not most of the behavioral symptoms seen in ASD. These include disruption or impairments in motor activity and coordination, repetitive behaviors, sensory perception, cognitive ability, and language and speech generation and comprehension. The involvement of the cerebellum in this array of different symptoms is likely a result of the absolute necessity of its powerful and rapid computational power in processing sensory, motor, and cognitive information in the brain.
- Overall, human brain imaging studies that included the cerebellum have reported highly variable results in ASD, especially in the vermis which also varies in size and volume in typically developing individuals. Imaging studies on mutant ASD mouse lines have suggested that a subset have significant alterations of cerebellar size and/or structure (e.g., fragile X, En2, Nrxn1α, and Shank 3 mice).
- Human postmortem analyses are still limited by relatively small numbers of individual brains examined. Nevertheless, collectively these studies indicate a loss of PCs in a high percentage of cases with idiopathic autism. This finding is additionally supported by a more limited data set from published cases of FXS and TSC. The fact that PCs are the sole output of the cerebellar cortex, and that their axons project to the deep cerebellar nuclei which in turn project directly and indirectly to multiple forebrain regions, places this unique cell type in a strategic position in information processing and overall brain function.
- Neurochemical studies in postmortem autism cerebella have revealed alterations in GAD65/67 in PCs, basket cells and in the dentate nuclei suggesting an imbalance of excitatory:inhibitory circuitry within the lateral hemisphere Crus II region. Further, cytoarchitectural abnormalities in the inferior olivary complex and in its inhibitory modulation from the dentate may lead to asynchrony of firing of inferior olivary neurons altering excitatory input to the remaining PCs. PCs bordering areas of absent PCs are hypothesized to be multi-innervated, thus affecting cerebellar output to high order association cortices.
- Results from human imaging and neuropathological studies of TSC strongly support an important contribution of cerebellar pathology to the overall clinical picture including the autism spectrum characteristics associated with the disorder. Additionally, results from Tsc mice with mutations in Tsc1 and Tsc2 genes restricted only to PCs have been particularly enlightening. These studies complement human neuropathological findings demonstrating a reduction in PCs and provide strong support for an essential role of the cerebellum in ASD.
- The demonstration by Tsai et al. (2012) from the analysis of mice with PC-restricted mutations in Tsc1 showing that autistic symptoms occur in the presence of altered PC excitability, but prior to PC loss, is particularly intriguing. This finding suggests that impaired PC excitability might be sufficient to induce ASD symptoms, and that cases where PC loss can be demonstrated may represent a later stage pathological state.
- Future human and animal studies of ASD should incorporate, and perhaps give priority to, assessing cerebellar parameters at the molecular, cellular, anatomical, physiological, and behavioral levels.

The following questions arise delineating potential priorities for future studies to address:

- Could reduced numbers of PCs, or even intact but dysfunctional PCs, be a feature common to many cases or forms of ASD? If so, this would represent a clear focus for future efforts in ASD research. In addition to the recent novel findings from mutant Tsc mice, the issue of the status of PC excitability and other physiological properties of PCs should be explored in other models of syndromic ASDs.
- In light of PC loss in the autism cerebellum, how is the distribution of CFs affected? It remains to be determined whether the autism brain displays a PC multiple innervation pattern due to decreased PCs. This may contribute to sustain inferior olivary neurons, which are unaffected in number in the medulla in postmortem autism cases.
- What is the relationship between the cerebellum and the basal ganglia in autism? Both structures are now known to participate in motor and non-motor functions, have indirect connectivity via the subthalamic nucleus, and, have reciprocal connectivity either directly or indirectly with similar thalamic nuclei and cortical targets. Could the two brain regions both participate in the alteration of fine motor control and high order cognitive behaviors in autism?

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REVIEW
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Moving from capstones toward cornerstones: successes and challenges in applying systems biology to identify mechanisms of autism spectrum disorders

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The substantial progress in the last few years toward uncovering genetic causes and risk factors for autism spectrum disorders (ASDs) has opened new experimental avenues for identifying the underlying neurobiological mechanism of the condition. The bounty of genetic findings has led to a variety of data-driven exploratory analyses aimed at deriving new insights about the shared features of these genes. These approaches leverage data from a variety of different sources such as co-expression in transcriptomic studies, protein–protein interaction networks, gene ontologies (GOs) annotations, or multi-level combinations of all of these. Here, we review the recurrent themes emerging from these analyses and highlight some of the challenges going forward. Themes include findings that ASD associated genes discovered by a variety of methods have been shown to contain disproportionate amounts of neurite outgrowth/cytoskeletal, synaptic, and more recently Wnt-related and chromatin modifying genes. Expression studies have highlighted a disproportionate expression of ASD gene sets during mid fetal cortical development, particularly for rare variants, with multiple analyses highlighting the striatum and cortical projection and interneurons as well. While these explorations have highlighted potentially interesting relationships among these ASD-related genes, there are challenges in how to best transition these insights into empirically testable hypotheses. Nonetheless, defining shared molecular or cellular pathology downstream of the diverse genes associated with ASDs could provide the cornerstones needed to build toward broadly applicable therapeutic approaches.

Keywords: autism, ASD, WGCNA, systems biology, network analysis, review, CSEA

Introduction

Autism spectrum disorder (ASD) is a pervasive developmental disorder, affecting around one of every 100 children. ASD is characterized by profound deficits in communication and social interaction as well as restricted interests and resistance to change. ASD clearly has a strong genetic component, with a 60–90% concordance between monozygotic twins. However, the disorder shows

BOX 1 | | Definitions of key terms.

Network: A graphical representation of entities and their relationships. Entities are represented as nodes and relationships between pairs of entities, as defined by some experimental measure, are represented as weighted or unweighted edges (lines) between the corresponding node pairs. Experimental measures might include correlated expression, weighted evidence of protein–protein interaction, or number or presence of curated connections from the literature. Many of the capstone analyses used network-based tools.

Module: A subgraph of a network that contains nodes that are more highly interconnected to each other than to other nodes in the network. Various formal definitions exist, but we use this general intuitive definition for the purposes of this review. Modules are typically identified using clustering or graph partitioning algorithms.

Gene set: A group of genes that share a particular feature. Straightforward statistics exist for determining if two gene sets overlap more than expected by chance. Some examples include:

1. A candidate gene set (for example, all genes implicated in ASD in a particular study).
2. A set of genes that all belong to the same co-expression module.
3. A set of genes sharing known features as exemplified by gene ontologies categorization. For example, a set of genes sharing a molecular function (e.g., all kinases) or presumed biological process (e.g., members of the Krebb cycle metabolic pathway).
4. A set of genes expressed in a given cell type during a specific developmental period.

Pathway: A series of events that link molecules and leads to a final product or change in the cell or organism. There are varied uses of this word in the literature.

1. A **metabolic pathway** might be a series of enzymes that progressively alter a metabolite (for example, the Krebb cycle).
2. A **signaling pathway** is a series of molecules, usually proteins, that transmit biological information, primarily using chemical modifications to activate or inhibit signaling activity of downstream targets.
3. A **genetic pathway** is a set of genes that contribute to a common final phenotype *in a related manner*, as determined by epistatic analyses. Note that additive effects on phenotype are not sufficient to place two genes into the same pathway. To be firmly placed in the same genetic pathway, gene products must be shown to be complementary, dominant, or suppressors of one another.

For the purposes of this review, metabolic and signaling pathways are referred to and treated simply as gene sets. The term 'pathway' will be used to refer exclusively to genetic pathways. Note that discovery of genetic pathways historically has led to the elucidation of a corresponding specific type of pathway (e.g., a signaling pathway such as Wnt signaling), though initial definition of a genetic pathway requires no knowledge of molecular function, only measurement of an effect on a phenotype.

Circuit: Generally, a course along which chemical and electrical signals travel. While a cellular circuit has some analogy to a 'molecular circuit' or signaling pathway, here we are distinguishing between these two levels of analysis. For the purposes of this review, circuits only refer to series of interconnected neural cells that mediate a particular behavior.

remarkable heterogeneity in the genetic risk factors. Common variant analyses have identified few reproducible associations across studies, and meta-analyses suggest that what common variants do exist likely have small individual effects (odds ratios less than 1.2) and act in a highly polygenic manner (Anney et al., 2012; Klei et al., 2012; Gaugler et al., 2014). Thus, the recent focus has been on rare variants, including copy number variations (CNVs), and exome sequence analyses (Pinto et al., 2010; Sanders et al., 2011, 2012; Chahrour et al., 2012; Malhotra and Sebat, 2012; Neale et al., 2012; O'Roak et al., 2012b; Yu et al., 2013; De Rubeis et al., 2014; Iossifov et al., 2014). These studies collectively have identified a clear role for rare and private deleterious coding mutations, both *de novo* and inherited. However, though of larger effect size, the rarity of these individual events limits statistical power. For example, while *de novo* loss-of-function mutations may collectively account for around 10% of ASD cases, any given gene might be seen to be mutated only in 2 or 3 cases out of the thousands now sequenced (Sanders et al., 2011; De Rubeis et al., 2014). Nonetheless, since 2012 a number of *de novo*, apparent loss-of-function mutations have been described that are found primarily in individuals with ASD, and a growing number of the same genes have been mutated frequently enough to indicate clear association. Ongoing efforts are poised to discover many more. Current estimates indicate there will be several hundred genes implicated by this approach when sufficient sample size is obtained (Krumm et al., 2014), in addition to the >100 genetic syndromes which already show some shared genetics or comorbidity with ASD (Betancur, 2011; Yu et al., 2013). With the number of new ASD variants being discovered the research bottleneck now is the identification of the neurobiological mechanisms by which they act. Since the genetic heterogeneity is so substantial, it is hoped that the identification of common neurobiological mechanism(s) across these diverse genetic causes may suggest some common routes to treatments.

The relatively recent advent of computational science has produced tools that enable opportunities to unveil truths that are not reachable using only theoretical or experimental approaches alone (Reed et al., 2005). Consequently, many recent scientific advancements have materialized thanks to two alternating and complementary modes of reasoning (Kell and Oliver, 2004). Discovery-driven approaches focus on *inductive* reasoning; they examine wide sources of data and attempt to define hypotheses from the emergent patterns that describe cause and effect relationships. In contrast, hypothesis-driven approaches leverage *deductive* reasoning to identify the logical consequences of a specific theory or hypothesis; consequences that can then be tested in an experimentally rigorous manner. The dawn of the genomic era, with the ability to measure the expression of thousands of genes, protein–protein interactions, epigenetic marks, etc., has produced fertile grounds for discovery-driven analyses, and many groups are leveraging these data resources in joint analyses with human genetics data for ASD to provide novel insights into any shared

characteristics of the genes and potential mechanisms of this disorder. Here, we review these studies with a particular focus on what bioinformatic approaches may have indicated about the molecular or cellular mechanisms of ASD. Then, we also highlight some of the successes and the challenges facing these approaches, along with a limited number of recommendations toward possible solutions. The overall aim of this review is to spur robust, critical, and creative thinking to advance the field.

Evolution of Discovery-Driven Applications for ASD-Related Genes

Studies of ASD genetics have evolved substantially over the last 15 years. As it was realized that common variants of large effects would be truly rare, it became evident that large sample sizes would be necessary to power both common and rare variant analyses. To amass these samples, large gene discovery projects required the coordinated efforts of hundreds of researchers with specialized expertise (clinicians, biologists, statisticians, programmers, etc.). The end results of these studies were essentially tables: tables of SNPs showing tentative association, linkage, or transmission disequilibrium (Ma et al., 2009; Wang et al., 2009; Weiss et al., 2009), or tables of CNVs (Sebat et al., 2007; Marshall et al., 2008; Bucan et al., 2009; Glessner et al., 2009; Pinto et al., 2010; Levy et al., 2011; Sanders et al., 2012), or *de novo* and recessive single nucleotide variants (SNVs; Gilman et al., 2011; Chahrour et al., 2012; O'Roak et al., 2012b; Sanders et al., 2012; Yu et al., 2013; De Rubeis et al., 2014; Iossifov et al., 2014) occurring, with some statistical confidence, in individuals with ASD and other forms of developmental delay. These tables, collectively, have provided the foundational resource to begin understanding the human biology of ASD.

The results in these tables are arguably significant enough that a study is complete when they are generated. But they are difficult to reduce to a single statement for a title, or to summarize in an abstract, and perhaps aesthetically unpleasing as a final figure. Thus, the emergence of a 'capstone analysis.' Early on, if only a single candidate region or two arose from a study, such an analysis might be as assessing association between a SNP and gene expression (e.g., *CDH9*) or between cases and controls for gene expression (e.g., *SEMA5A*), which were the capstone figures of two early common variant GWAS studies (Wang et al., 2009; Weiss et al., 2009). But as the tables became longer, the capstone analysis was often focused on summarizing the likely candidate genes on the table as a whole, i.e., to provide a systematic gestalt of these genes. Examples included leveraging the GOs resource to identify disproportionately represented categorical terms [e.g., Cytoskeletal elements or Rho GTPases (Pinto et al., 2010), known to regulate neurite outgrowth (Hall and Lalli, 2010)], or an attempt to organize all the resulting genes into some kind of network (**Box 1**) using other data resources. In more recent years, these capstones have expanded in scope and in effort (Gai et al., 2012; De Rubeis et al., 2014; Pinto et al., 2014), sometimes sufficiently to become companion and *post hoc* analytical manuscripts focused on finding common themes to the discovered genes, and presumably the disorder (Gilman et al., 2011; Ben-David and Shifman, 2012; Parikshak et al., 2013; Willsey et al., 2013; Krumm et al., 2014; Xu et al., 2014; Chang et al., 2015; Hormozdiari et al., 2015). In a review by Willsey, the earlier works have been characterized as initially using 'static' data resources to contextualize the findings, but eventually turning to more 'dynamic' resources such as gene expression across brain regions or cell types in the CNS (Willsey and State, 2015). As gene expression inherently includes an aspect of brain region and developmental time, they could be equally described as moving from trying to find a shared molecular pathology for these genes, to trying to find a shared regional or cellular pathology. Below, we review capstones from both types of analyses and highlight recurrent themes that may be emerging across groups.

A Shared Molecular Pathology for ASD-Related Genes?

Given a set of genes, a variety of mature tools exists for identifying disproportionately shared molecular functions for these genes, mostly based on researcher-curated collections of gene functions (e.g., GO), or empirically determined sets of protein–protein interactions, derived from literature mining or high throughput screens in simplified model systems (e.g., yeast 2-hybrid; Ashburner et al., 2000; Lage et al., 2007; Rossin et al., 2011; Szklarczyk et al., 2015). These approaches have highlighted a variety of enriched molecular functions amongst ASD related gene sets (**Table 1**). However, the utility of the results from these approaches have two limitations; they are dependent on manually curated annotations, and they do not lead directly to falsifiable hypotheses.

First, while these GO-based tools are indescribably preferable to the alternative (attempting to manually curate the literature for dozens or 100s of genes simultaneously), it is clear that they also suffer from a derivative of one of the classic barriers to unadulterated inductive reasoning described by Francis Bacon – a sort of collective version of his 'idols of the cave.' The term classically refers to how an individual's interpretations of data are colored by their prior knowledge and experiences (Bacon, 1620). Likewise, GO terms are assigned based on the collective experiences of researchers, as reflected in the literature, and thus they can only be readily leveraged for well-annotated genes. In addition, even known genes may have unidentified pleiotropic molecular functions. For example, FMRP, the RNA binding protein disrupted in Fragile X syndrome, has recently been shown to also physically regulate presynaptic voltage gate potassium channels through protein–protein interactions (Deng et al., 2013), independent of any RNA binding activity. It may likewise be found that genes currently annotated as chromatin modifiers (e.g., *CHD8)* or histone deacetylase (e.g., *HDAC5*), may have as yet unknown roles in directly modifying cytoskeletal elements regulating neurite morphogenesis. Simply put: analyses based on curated knowledge cannot account for currently unknown functions.

Second, it is not always clear how the insights from these molecular gene set analyses might be actionable for identifying mechanistic hypotheses for ASD or developing new therapeutics. Results such as an enrichment of genes in the GO gene set 0045216 (intercellular junction assembly and maintenance),

TABLE 1 | Summary of findings regarding enriched functional categories for ASD genes.

Reference	MAGI	NETBAG+	WGCNA	CSEA	DADA	Other	DAWN	ASD postmortem	CNV	SNP	Exomes (SNV)	SFARI/AutDB	TADA	GO	bacTRAP	Cahoy	Interpro	PPI	KEGG	GeneATLAS	FMRP	BrainSpan	Brain-MAP	TRANSFAC	Encode	Brainspan lamina	Molecular pathways implicated	Notes
Pinto et al., 2010									●					●													Neural development GTPase/RAS signaling Cytoskeleton projections (neurites) motility	
Gilman et al., 2011		●							●		●			●			●	●	●		●						Kinase activity GTPase signaling Cell adhesion Neurite morphogenesis cytoskeleton/synaptogenesis	
Chahrour et al., 2012											●			●													GTPase signaling Transcriptional regulation/chromatin Protein modifying activity/ubiquitination	
O'Roak et al., 2012a					●				●		●			●				●									Wnt signaling Chromatin modifying activity Ubiquitination	
Parikshak et al., 2013			●					●	●	●	●	●		●				●			●	●		●	●	●	Transcription regulation (exomes) Post-synaptic signaling (SFARI, SNPs)	M2,M3 M13,M15,M16
De Rubeis et al., 2014							●				●		●	●			●										Channels (calcium)and synaptic transmission Transcription regulation/chromatin	
Krumm et al., 2014									●		●			●				●			●						Chromatin/transcription WNT signaling Synaptic function	
Pinto et al., 2014		●							●		●			●				●			●	●					MAPK signaling Migration, proliferation Chromatin, transcription regulation Synaptic components Cell projections/neurite	
Chang et al., 2015		●							●		●			●	●		●	●	●		●	●					Chromatin modification regulation Post-synaptic density Neuronal signaling/cytoskeleton Channel activity	
Hormozdiari et al., 2015	●										●			●				●	●	●	●	●					Chromatin, NCOR, SWI/SNF related Notch pathway Wnt pathway Channel activity (calcium)	Module1 Module1 Module1 Module2

Red columns: primary computational approach used. Green columns: data set used to derive ASD gene list. Blue columns: resources leveraged. In parentheses – details added by authors if different data sources indicated different enrichement of different gene sets.

which contains 159 genes, provides limited insight on which direction to pursue. In addition, the specificity of the 159 genes in the entire GO gene set to ASD or a particular question (e.g., drug targets, causative genes, and temporal expression of ASD genes) is unknown. It has been long shown in model systems that genes that perform functions in the same genetic pathway or encode for proteins in the same protein complexes lead to similar phenotypes when disrupted, but it is not clear how closely linked a particular genetic pathway (**Box 1**) is with a given GO gene set. Thus, it would be ambitious to assume that disrupting any of the 159 genes associated with this GO gene set will lead to ASD. This is also because the 159 genes could be expressed in markedly different locations in the brain and the behavioral manifestations of such molecular disruptions will be highly dependent on the specific neural circuits that utilize each of these proteins.

In contrast, there are clear successes – that have led to purposeful experiments and meaningful treatments – arising from identifying the relevant neural circuit for a particular disorder. Note the rich variety of treatments arising from the knowledge that Parkinsonism is due to loss of dopaminergic cells of the Substantia Nigra. Long before any genes were identified that contributed to the development of this disorder, knowledge of the afflicted circuit (**Box 1**) led to the identification of viable treatment strategies. If the dysfunction of particular circuits in the brain manifests as explicit behavioral abnormalities (e.g., specific symptoms), then it is reasonable to assume that the shared symptomatology across distinct genetic causes of ASD implies some convergent neural circuit disruption downstream of these distinct genetic pathways. Encouragingly, if the diverse set of rare causative genetic mutations in ASD does share a common cellular or circuit mechanism, then we do not need to devise treatments for each specific rare mutation. Rather, treatments focused on correcting the common cellular dysfunction could be applied to individuals who have a variety of underlying causes, analogous to the common treatments used regardless of which genetic factor or environmental exposure was the underlying cause for a case of Parkinson's disease. Thus, identifying common cellular circuits mediating the behavioral disruptions seen across a variety of distinct ASD genetic etiologies is essential for designing practical treatments for this disorder.

A Shared Cellular Pathology for ASD-Related Genes?

To address the two limitations outlined above and to attempt to identify some shared neurobiological circuit disrupted across distinct genetic causes, we and others have focused on complementary analyses leveraging gene expression data resources. As gene expression is readily measured even for unannotated genes, it is unbiased and does not suffer from the 'idols of the cave.' And, as gene expression varies substantially across cell types or circuits, it may be possible to implicate particular circuits by expression alone. At an extreme, a disease gene selectively expressed in a single cell type in the brain (e.g., the narcolepsy-related peptide Hypocretin found only in a population of cells in the hypothalamus; Peyron et al., 2000), clearly implicates that cell type as a vulnerable population in the disorder and any related circuits as targets for treatment. While such all-or-none expression of genes in a single cell type is rare, the logic of this 'selective expression' hypothesis may be somewhat extensible to a more moderate statistical enrichment of expression as well: disproportionately enriched expression of a large number of disease genes in a particular cell type or tissue could indicate a relevant anatomical intermediary of a disorder. Indeed, we have now shown that retinopathy-causing genes are disproportionately expressed in rods and cones (Xu et al., 2014). Likewise, SNPs associated with autoimmune diseases by GWAS tend to be eQTLs for genes expressed in the blood where immune cells are prevalent (Ardlie et al., 2015). And knowledge of anatomical intermediaries leads to testable hypotheses: individual cell types can be disrupted in model organisms quite readily using Cre/Lox, optogenetics, and related approaches, and behavioral consequences examined.

Before summarizing the results of the analyses leveraging expression data, it is worth noting that while gene expression data have the advantage that they are relatively unbiased for specific genes, several caveats remain. First, determination of expression levels can be affected by variations in sample collection and preparation, technician experience, equipment calibration, and choices of pre-processing algorithms, statistical tests, thresholds, microarray/RNAseq platforms, and other aspects of study design (Gudjonsson et al., 2010; Suárez-Fariñas et al., 2010). However, stringent consistency throughout the study and prudent design choices can help to ensure reasonable accuracy with regard to *relative* differences between expression levels, and these relative differences are adequate for most subsequent analyses. Second, covariates such as differences in gender, age, cause of death, time to preservation of the sample, and batch effects are sources of potential bias that are typically corrected using standard methods, such as ANCOVA (Huitema, 2005). While commonly overlooked, in order to ensure spurious relationships do not slip past these corrections, it is important that covariate information is double-checked following subsequent analyses. For example, if a co-expression module of a couple dozen genes is identified, the individuals bearing most, or all, of the expression pattern should be extracted and the degree of correlations with covariates should be determined. Third, variations in ancestry or overlooked sample relatedness can present unexpected sources of bias. An effective, albeit not always practical, way to identify either of these potential pitfalls is to collect genotype data and analyze them using packages such as Structure (Pritchard et al., 2000) and PLINK (Purcell et al., 2007). If this additional data collection is impractical, thorough screening of study participants can help alleviate these possible sources of bias. Finally, inadequate sample size can lead to serious issues as described later in this review.

If these issues are addressed, then two approaches can be taken to leverage gene expression data. The approach we took in our particular analyses were 'top down.' We defined sets of genes with enriched expression in different tissues based upon available body-wide RNAseq data resources (GTEX: Ardlie et al., 2015), in different cell types based on cell specific profiling technologies from mouse data (bacTRAP: Doyle et al., 2008), and profiles of human brain regions across development (Brainspan: Kang et al., 2011). We then examined the overlap of these lists with candidate disease genes, in a manner very analogous to

the tools overlapping candidate gene sets with GO. However, there are weaknesses to this approach. Our use of mouse data assumes conservation of gene expression in particular cell types across mammals – a reasonable, but clearly not perfect assumption (Zeng et al., 2012). And our approach also does not explicitly leverage the correlation structure of gene expression across tissues. Likewise, human brain-region and tissue-wide data sets lose the cellular level resolution that may be most useful for identifying targets for treatments. Both data resources are limited of course to the samples that were collected, and other cell types, tissues, or perhaps key developmental windows might be absent from a particular analysis. Human data in particular have focused heavily on cortex, potentially under-representing other regions that may be of importance (e.g., hypothalamus or brainstem). Thus, these analyses are moving *toward* being potentially usable as cornerstones for developing hypotheses of the cellular mechanisms underlying ASD, and will hopefully provide additional insights as more data become available.

A complementary set of 'bottom up' data-driven studies address some of these concerns. Several groups used a variety of clustering analyses to first organize the ASD related genes into networks, often leveraging their correlated expression across human brain development to group them into co-expression modules using WGCNA (Ben-David and Shifman, 2012; Parikshak et al., 2013), or philosophically similar approaches using additional data resources (Willsey et al., 2013; Chang et al., 2015). Resulting modules can be used for GO analyses or examined for enriched expression in particular developmental windows, brain regions, or cell types. It is worth noting here that it has long been recognized that one of the primary drivers of correlated gene expression across different brain regions is the consistent changes in proportions of different cell types (e.g., neurons and glia) across regions (Geschwind, 2000). Thus it is likely that many co-expression modules might correspond to genes enriched in a particular cell type. Our cell-type specific expression analysis (CSEA) approach (Doyle et al., 2008; Xu et al., 2014) or other datasets (Cahoy et al., 2008; Zhang et al., 2014) can be used to rapidly identify this. Regardless, in the above analyses, either co-expression or somewhat more inclusive human genetics criteria has been used to expand these ASD-related gene sets into larger modules. This allows for more genes to be included in these analyses, facilitating better network insights, though it is currently unclear if there is a particular cost in terms of a potentially inflated false positive rate associated with this expansion of gene sets.

However, in spite of the moderate differences in the precise ASD-related gene sets, differences in leveraged data resources, differences in the use of 'top down' or 'bottom up' methods and statistical approaches, some themes seem to be emerging regarding where ASD-related genes show enriched expression (**Table 2**). *First*, amongst the rare mutations that were highlighted in the recent exome studies, several groups have reported disproportionate expression in the mid fetal developing cortex and/or striatum (Parikshak et al., 2013; Willsey et al., 2013; Xu et al., 2014). Though there is some disagreement on the exact lamina that might be implicated (frankly, relatively few gene expression differences define distinct cortical lamina (Doyle et al., 2008; Dougherty et al., 2010; Xu et al., 2014) relative to the robust expression differences between cell types in other brain regions such as the cerebellum), many of these genes show relatively high expression in forebrain development. This is consistent with the long known roles in telencephalic development for at least two of the recently implicated genes (*TBR1* and *RELN*; Caviness and Sidman, 1973; Hevner et al., 2001), and suggest that mutations profoundly affecting forebrain development may have ASD as one (of perhaps many) deleterious consequences. This is consistent with the replicated finding that individuals with *de novo* loss-of-function mutations have lower IQ than other individuals with ASD (Samocha et al., 2014). *Second*, genes downregulated in human ASD postmortem transcriptomic studies (Voineagu et al., 2011; Gupta et al., 2014), and ASD candidate genes compiled prior to exome studies (Basu et al., 2009) seem to map most strongly to cortical interneurons, as well as a striatal cell type: medium spiny neurons (Xu et al., 2014). These findings suggest that perhaps there might be some shared abnormalities in cortical and striatal circuits across distinct genetic causes of ASD. In contrast, for example, none of the analyses have implicated cell types of the cerebellum, suggesting these are perhaps less commonly involved in ASD.

While overall both 'top down' and 'bottom up' discovery-driven approaches have highlighted potential circuits of interest in ASD, it is also clear that this disorder is not like retinopathies, which have a dominant signal in one or two cell types (minimum p-values $< 10e^{-20}$). The significant, yet relatively modest statistical signal in ASD studies (minimum p-values around $10e^{-3}$ for medium spiny neurons or cortical interneurons) indicate there may be substantial heterogeneity in cellular mechanisms for the disorder, just as there is extensive heterogeneity in genetic mechanisms. Further, as many of these methods start from largely similar ASD-related gene lists, and leverage a small number of overlapping data resources, they do not truly represent independent replications. Thus, in the final section, we outline some of the challenges facing application of these approaches to ASD and present some examples of solutions and recommendations. The recommendations are not exhaustive and it is likely other elegant solutions exist as well. The challenges can be organized into three groups. *First*, how do we best identify and rule out alternative explanations that may also account for the relationships between these genes? How do we define the null hypothesis? And what are likely sources of false negatives? *Second,* how do we assess the reproducibility of a discovery-driven network analysis result? What constitutes a replication of one of these findings? *Finally*, how do we convert discovery-driven network-based insights into empirically testable hypotheses, and from there into informed treatments?

Challenges Posed by Systems Biology Approaches using ASD-Related Genes

Challenge 1: Selecting the Correct Interpretation of a Network Analysis Result

Networks are graphical descriptions of the relationships between the embedded entities. They provide the ability to display more numerous relationships than could be efficiently conveyed with

TABLE 2 | Summary of findings regarding cell types, brain regions, or developmental windows with enriched expression of ASD genes.

Reference	MAGI	NETBAG+	WGCNA	CSEA	DADA	Other	DAWN	ASD postmortem	CNV	SNP	Exomes (SNV)	SFARI/AutDB	TADA	GO	bacTRAP	Cahoy	Interpro	PPI	KEGG	GeneATLAS	FMRP	BrainSpan	Brain-MAP	TRANSFAC	Encode	Brainspan lamina	Cells, regions, or developmental windows implicated	Notes
Ben-David and Shifman, 2012			●					●	●	●		●				●						●	●				Most highly expressed in cortical and striatal/hippocampal brain regions (SNPs, SFARI genes) Enriched in a module harboring interneuron genes (ASD postmortem) Salmon: peak expression in infancy, light green: constant, gray: increasing postnatally	Light green, salmon Gray
Parikshak et al., 2013			●					●	●	●	●	●		●				●			●	●		●	●	●	Peak expression during fetal brain development Cortical neurons of various layers (postmortem ASD, SFARI, SNPs)	M2,M3
Willsey et al., 2013						●					●		●									●				●	Peak expression during fetal brain development (exomes) Highest expression in layers 5/6 glutametergic projection neurons	
Xu et al., 2014				●				●			●	●			●							●					Increased expression of astrocyte and immune cell genes (postmortem ASD) Decreased expression of neuronal, especially interneuronal genes (postmortem ASD) Enriched in strial medium spiney neurons and cortical interneurons (SFARI) Enriched expression in midfetal striatum and cortex (exomes)	
Chang et al., 2015		●							●		●			●	●		●	●	●		●	●					ASD-network genes tended to be highly expressed in the brain Chromatin-ASD genes were expressed during early brain development PSD-ASD genes were expressed more postnatally ASD-network genes have biased expression in cortical, striatal neurons and granule cells of cerebellum	
Hormozdiari et al., 2015	●										●			●				●	●	●	●	●					Peak expression during fetal brain development Peak expression during postnatal brain development	Module1 Module2

Red columns: primary computational approach used. Green columns: data set used to derive ASD gene list. Blue columns: resources leveraged. In parentheses – details added by authors if different ASD gene lists indicated different regions.

Key for both tables.

Approaches

1. CSEA: cell specific expression analysis. A method to define sets of genes with enriched expression in particular samples – i.e., to create datasets that can serve as a "Gene Ontologies" for expression of genes in specific cell types. Applied initially to bacTRAP data.
2. DADA: disease aware disease-gene association: A method for prioritizing candidate genes within protein–protein interacting (or other networks) accounting for connectedness to other disease genes.
3. DAWN: detecting association within networks. A method leveraging gene expression data and genetic association scores to generate networks of related genes and prioritize additional candidate autism genes.
4. MAGI: merging affected genes into integrated networks. A method leveraging PPI and expression data to identify modules enriched in ASD genes. Can be used to prioritize additional candidate genes.
5. NETBAG: network based analysis of genetic associations. A method of constructing networks of related genes based on a variety of data sources including GO, KEGG, and PPI data.
6. WGCNA: weighted gene coexpression network analysis. A method of defining modules of genes with correlated expression patterns across samples.

ASD geneset sources

1. ASD postmortem: Microarray data comparing cortex and cerebellum of ASD patients and controls (Voineagu et al., 2011);
2. CNV: copy number variant studies;
3. SNP: single nucleotide polymorphism (common variant) studies;
4. Exomes/SNVs: single nucleotide variants (rare and *de novo*) studies;
5. SFARI/AutDB: a curated database of autism genes (initially AutDB), now hosted by Simons Foundation (Basu et al., 2009).

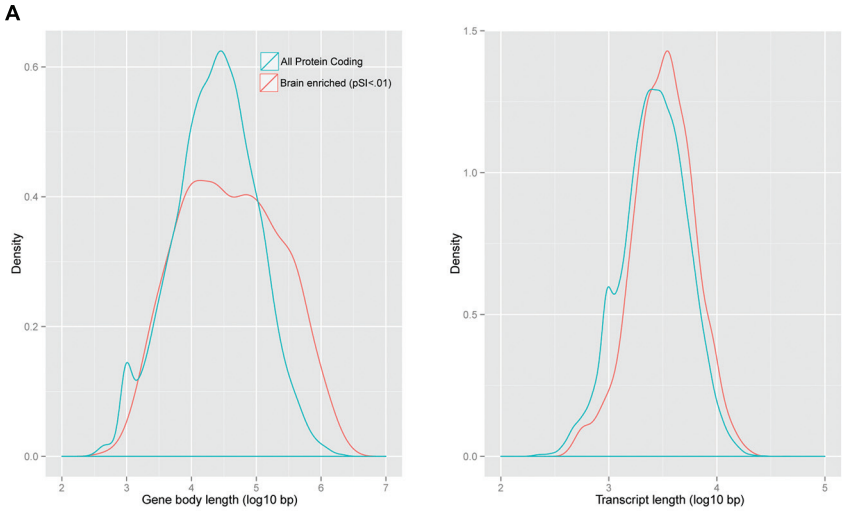
Resources leveraged

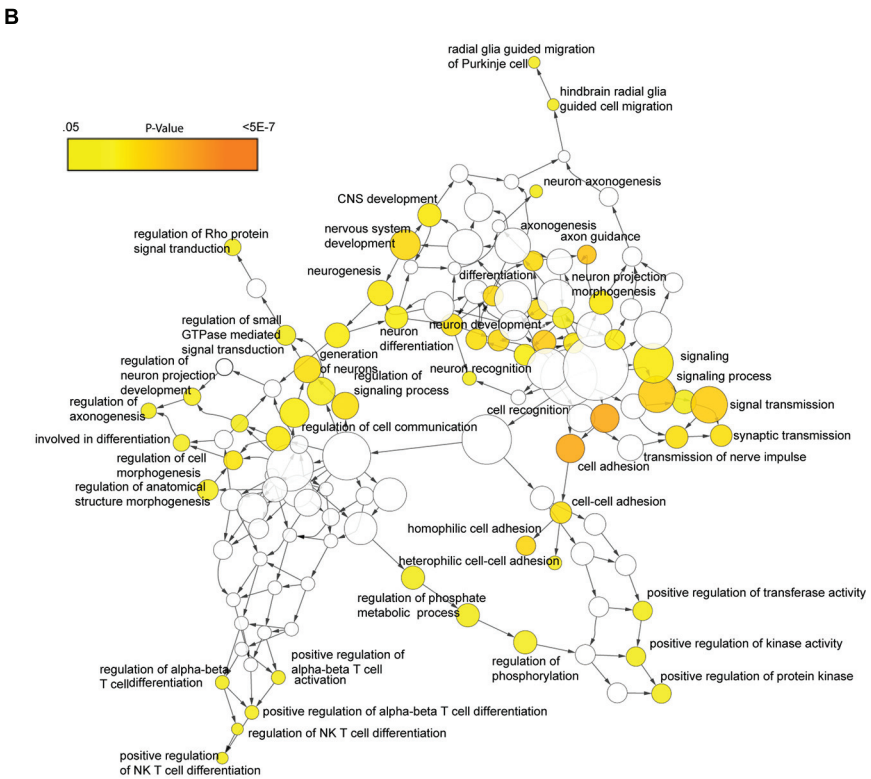
1. GOs: gene ontologies. Curated lists of functional annotations for all genes based on a defined hierarchical vocabulary. Accessed through a variety of portals across studies.
2. KEGG: Kyoto encyclopedia of genes and genomes. A curated collection of metabolic and signaling pathways, and associated genes.
3. Interpro: database of annotating the domains found in proteins.
4. PPI: protein–protein interaction data. Various sources across the studies (e.g., StringDB, Szklarczyk et al., 2015).
5. TADA: transmission and *de novo* association. A method for weighting genes by their likely contribution to ASD based on multiple sources of information (He et al., 2013). Here, used to refer to gene lists deriving from that method. Can also be used to prioritize genes, and is integrated with DAWN.
6. bacTRAP: gene expression profiles of ~25 genetically defined cell populations in adult mice (Doyle et al., 2008).
7. Cahoy: gene expression profiles of the four major classes of cells in developing mouse brain (Cahoy et al., 2008).
8. GeneAtlas: a microarray study of dozens of distinct tissues in mouse and human (Su et al., 2004).
9. FMRP: the set of transcripts detected as binding the RNA binding protein FMRP in mouse brain (Darnell et al., 2011).
10. Brainspan: a collection of human brain transcriptomic data across multiple developmental time points and regions (Kang et al., 2011). Accessed through different portals in different studies.
11. Lamina: a collection of transcriptomic data using laser capture microdissection to harvest RNA from specific layers of developing human and mouse cortex (Miller et al., 2014).
12. BrainMap: a collection of human brain transcriptomic data from a single time point but across multiple regions from two individuals generate by Allen Institute as part of the Human Brain Atlas Microarray Survey.
13. Transfac: transcription factor database. A collection of sequence motifs known to bind specific transcription factors.
14. ENCODE: encyclopedia of DNA elements. A collection of many different types of data focused on identifying regions of genome covered by specific sets of epigenetic marks found on DNA in a range of tissues and cultured cell types.

words. However, a mind presented with such a large amount of data will rapidly organize it by drawing on examples from our own experience as researchers (idols of the cave yet again). Cortical development researchers might tend to migrate toward the cytoskeletal and Rho-GTPase genes, while physiologists may be most stimulated by the channel genes. One who has worked for many years with the transcriptional profiles of different cell classes in the brain, when looking at a network (or gene set), might have a bias to interpret it in terms of the cell types these genes are expressed in (i.e., one could view an 'immune' module in transcriptomic data as reflecting changes in the proportion of microglia in the tissue, rather than immune genes being upregulated in neurons). Thus, all investigators must be careful to recognize their individual biases for what they are and shield analytical approaches as best possible from them. In addition, there can be biases in the discovery methods and resources themselves that might create statistically significant results for scientifically insignificant reasons (**Figures 1** and **2**). Therefore, we also need to define as carefully as possible our null hypotheses and be attentive to circularity and alternative explanations.

Recommendation 1: define the null and rule out alternative explanations

Not all genes are equally likely to be implicated in genetic studies. A simple example is that longer genes will tend to, by nature of their size, overlap with more markers present on SNP microarrays, provide more bases that could have a *de novo* SNV and are more likely to be disrupted by a random CNV mutation. And of course, mutations are not randomly distributed: different regions of the genome, or even particular nucleotide contexts, have different rates of mutation (Krawczak et al., 1998; Michaelson et al., 2012; Samocha et al., 2014). Furthermore, transcript length, and potentially gene body size,





GO	Name	P-Value	Corrected (B-H)
7411	axon guidance	2.28E-07	1.50E-04
23060	signal transmission	1.32E-06	4.36E-04
7156	homophilc cell adhesions	3.03E-06	8.56E-04
48667	cell morphogenesis involved in neuron differentiation	4.44E-06	1.02E-03
7399	nervous system development	4.66E-06	1.02E-03
48812	neuron projection morphogenesis	7.34E-06	1.32E-03
7409	axonogenesis	8.50E-06	1.40E-03
19226	transmission of the nerve impulse	1.17E-05	1.79E-03
7268	synaptic transmission	1.33E-05	1.88E-03

FIGURE 1 | Genes with enriched expression in the brain are long. (A) Genes with enriched expression in the brain are longer both as mature transcripts (right) and in terms of gene body length (left), when compared to protein coding genes as a whole. **(B)** Random GWAS results show enrichment in a network of brain-related GO terms: using a uniform distribution between 0 and 1, random *p*-values were assigned to SNPs in the genome, and SNPs were mapped to genes using ANNOVAR. The SNP with the lowest *p*-value in a gene was used to determine the 500 most significant genes that were then used for a GO analysis and displayed as a network using BINGO. Dozens of categories related to CNS function were significant (examples shown in table at bottom).

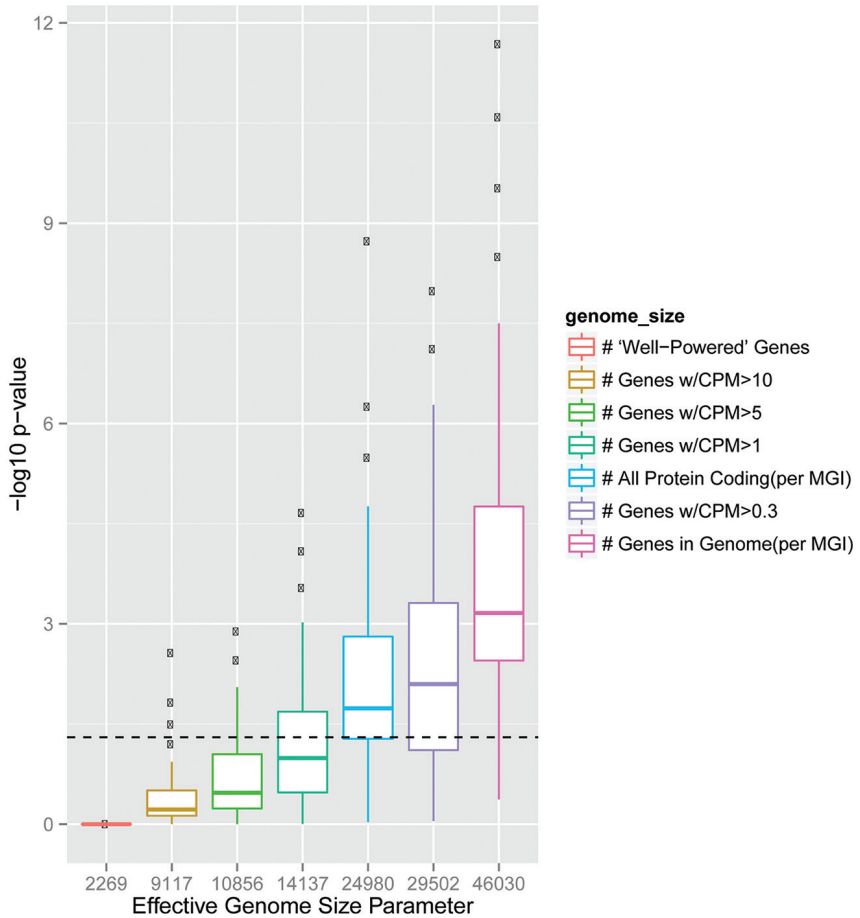


FIGURE 2 | Choice of effective genome size in GO analysis of transcriptomic data can substantially influence statistical results. *Boxplots show distribution of −Log10 p-values for Fisher's Exact Test overlapping the GO:007268, Synaptic transmission, with 100 different sampled lists at each effective genome size indicated. Dashed line is p = 0.05* Power to detect differential expression in RNAseq is influenced by total count number for a particular gene, and thus longer or higher expressed transcripts in a tissue are more likely to be found as significantly different (Bullard et al., 2010; Young et al., 2010). To determine if this could create spurious GO results from a brain transcriptomic experiment, we randomly sampled genes from the 10% most robustly detected genes in a brain RNAseq experiment (Ouwenga and Dougherty, 2015) to mimic results a 100 hypothetical differential expression experiments. If it was assumed these genes were randomly drawn without bias from the whole genome ($n = 46030$ genes) this consistently resulted in a statistically significant, but scientifically meaningless, enrichment in the GO category 0007268 (Synaptic Transmission). More conservative estimates which only include genes at least lowly expressed in the brain (CPM > 0.3 or CPM > 1) still frequently yield spurious overlap (*blue, purple*). Because of this, using an effective genome or 'background gene set' based on the transcripts which are well-powered for differential expression is recommended.

bear some relationship to biological function. Notably, genes expressed in the nervous system tend to be longer in both regards (**Figure 1A**). For example, randomly sampling SNPs from the genome and mapping them to overlapping genes will result in an enrichment for brain-related GO categories (**Figure 1B**). Likewise in transcriptomic studies, the appropriate background 'genome' needs to be carefully defined (**Figure 2**). Transcripts clustering in modules or being differentially regulated in a particular tissue, by necessity must first be expressed in that tissue. Thus, the effective genome and genome size for statistical analysis of overlap should be restricted to those genes whose transcripts could have plausibly been identified in the analysis. As an example, both length and expression can come into play when considering overlaps with the known Fmrp-interacting RNAs (Darnell et al., 2011), as for potentially methodological reasons these tend to include long transcripts that are highly expressed in the brain. Therefore, the overlap of these RNAs with gene sets derived from either human genetic studies or potentially transcriptomic studies may reflect these primary features of the transcripts rather than a central role for Fmrp in the particular experiment (Ouwenga and Dougherty, 2015).

Overall, correcting for gene body length, transcript length, and brain expression level are challenging. For example, simply down-weighting GWAS results for those genes that are tagged by more SNPs, under the assumption that every gene in the genome is equally likely to contribute to disease, would be too conservative – long genes could legitimately be more vulnerable to mutation/polymorphism because of their length. And, an evolutionary argument could be made that genes requiring more

careful regulation have evolved to be longer – permitting the presence of more potential regulatory sites (e.g., enhancers in the genome, or protein binding motifs in the RNA) to finely tune final protein levels. Indeed, genes that do not appear to tolerate heterozygous mutations in humans (Samocha et al., 2014), tend to be longer than the average gene. Thus, there is a risk that fully removing the influence of gene or transcript length in some analyses might be too conservative. Nonetheless, these are issues that should be explicitly addressed in analyses and chosen parameters should either be well-justified, or systematically varied to demonstrate robustness.

Therefore, an appropriate null for discovery-driven analyses of ASD-related genes should take these primary sequence features into account. A common approach is to conduct comparison analyses using sampled control sets of genes that share length, connectivity, or mutability with the ASD related genes (Willsey et al., 2013; Krumm et al., 2014; Chang et al., 2015). An additional control commonly used are genes actually detected as mutated (SNVs or CNVs) from control populations such as unaffected siblings, population databases of variation, or an unrelated disease (Pinto et al., 2010, 2014; Gilman et al., 2011; Chahrour et al., 2012; Parikshak et al., 2013; Krumm et al., 2014; Samocha et al., 2014; Chang et al., 2015; Hormozdiari et al., 2015). This controls for both the known biases highlighted above and any currently unrecognized biases in the ASD gene discovery methods.

Challenge 2: Independent Replication of a Network Analysis Result

One of the tenants of the scientific method is reproducibility. Experiments should be able to be reproduced by other labs and result in substantially identical findings. Furthermore, following the deductive tradition, tests of the same hypothesis using different methods should produce convergent results if the model is correct and the methods are robust. While discovery-driven analyses are typically insight- or hypothesis-generating rather than hypothesis-testing endeavors, reproducibility and replication are criteria that are still applicable.

Recommendation 2: parameter choices and code sharing

Just as in biological studies, where minor changes in the composition of a buffer can sometimes substantially alter biochemical findings, minor alterations in parameters in bioinformatic analyses can result in substantial differences in the results. For example, the choice of the effective genome size can dramatically influence p-values in analyses overlapping two gene sets with a Fisher's Exact Test (**Figure 2**), and parameter choices in aligners have at times created misleading results such as an overestimation of RNA editing (Schrider et al., 2011). Frequently there may not be a strong *a priori* reason for choosing a particular parameter setting, which might lead to a 'parameter placebo:' an accidental or subconscious tuning of the parameter to produce the most striking results. To avoid this, key parameters can be varied systemically with the results presented in such a way that allows the reader to judge for themselves the robustness. For example, we were interested in overlapping sets of genes with 'enriched' expression in a particular cell type with ASD-related gene sets. We could rank genes from most enriched to least, but justifying a precise threshold was challenging. Uniquely expressed in these cells? In them, but in a few other populations as well (i.e., moderately enriched)? As there was no clear answer, we designed the analysis to systematically vary the parameter and present the results at multiple thresholds, with the most intuitive confidence given to overlaps that occurred significantly across some or all thresholds (Xu et al., 2014).

Thus the researcher's choice for how parameters are set (or the range of values tested) needs to be well-justified. Code for conducting the entire analysis should be made available on request, or perhaps even hosted in its entirety on a public forum. However, to enable this, the discovery of a bug in code that has been made available should be treated as an opportunity to raise the quality of the scientific analyses as a whole, rather than as an opportunity to cast stones at a competing lab.

Recommendation 3: replication replication replication. . .

It is a common experience at the bench – the first replicate of an experimental series that matches predictions perfectly or that hints at exciting new biology. And then the second replicate that does not match the first, and then third, fourth, fifth, until it becomes apparent that the first experiment was the outlier, whether due to some technical mishap or simple winner's curse. At the bench one has the (dubious) luxury of being able to repeat an experiment as many times as cost and time constraints allow to convince ourselves of the reproducibility of an outcome. However, in systems biology, there is often only one starting candidate disease gene set with which to seed your network. And largely only one GO or Brainspan resource to compare it with. One can rerun the analysis to make sure the same result occurs (analogous to a 'technical replicate' at the bench), but this is not as reassuring as a true independent biological replicate would be. In general, replications of transcriptome analyses in independent samples have been difficult historically, and these discrepancies have been attributed to variations in study design, processing of samples, and/or computational methods (Gudjonsson et al., 2010; Suárez-Fariñas et al., 2010). Thus, assessing the reproducibility of a bioinformatic analysis is inherently challenging.

A fundamental question that must be faced is whether the inability to reproduce is due to systemic variations, such as those previously suggested, or due to failure to capture true biological signatures. A major obstacle for these studies is the difficulty in amassing large sample sizes and unfortunately, this issue is seldom addressed. Network construction is typically achieved by conducting some type of similarity or correlation tests across pairs of genes/proteins. Inadequate sample size can produce seemingly promising networks with strong community structure due to the clustering of false-positive correlations. Unfortunately, significant correlation thresholds are not one-size-fits-all and vary between datasets due to sample size, heterogeneity of samples and other factors. For this reason, we strongly recommend the use of a rigorous method for determining an appropriate threshold for edge placement during the construction of networks. For example, permutation trials provide a simple and robust method for determining appropriate

correlation thresholds. For each trial, the data values for each gene are permuted across individuals, thereby retaining all of the properties of each gene except for inherent correlations with other genes. After running an adequate number of trials, e.g., 1000 trials, the highest correlation values computed across the uncorrelated permuted data can be used to determine a threshold with a desired *p*-value.

Assuming *bona fide* network construction, there are at least three, albeit imperfect, options for replication. *First*, borrowing from machine learning or human genetics studies, the starting ASD-related gene sets could be broken into artificial 'discovery' and 'replication' subsets, or even K subsets, so some form of K-fold cross-validation of the results could be conducted (assuming adequate sample size). Then at least the robustness of the results with regards to sample selection could be assessed (Refaeilzadeh et al., 2009). *Second*, comparisons of results with those identified by independent groups using distinct analytical approaches may yield strong evidence of biological validity. To an extent, it is very reassuring that multiple groups have drawn fairly similar conclusions when applying these approaches to ASD (**Tables 1** and **2**), though of course these are not true replications because they are not independent – as they draw on similar comparison data resources (i.e., regardless of whether GO is accessed through DAVID, Panther, BinGO, GOrilla or other portal, the gene sets are largely identical). Further, the Brainspan, bacTRAP, and Cahoy datasets have seen similar widespread use (Cahoy et al., 2008; Doyle et al., 2008; Kang et al., 2011). Thus, it will be even more reassuring if similar results about these ASD-related gene sets hold when additional comparison data resources come online, such as single cell expression studies (e.g., Zeisel et al., 2015). The *third* option is replication through the increasing size of the ASD-related gene sets through time. In this regard it is reassuring that many of the patterns seen in the early Capstone analyses (e.g., neurite morphogenesis/cytoskeletal elements) have been reproduced in later discovered gene sets (**Table 1**).

Challenge 3: Converting Discovery-Driven Insights into Empirically Testable Hypotheses

How does one test a network result functionally? The recent application of a variety of these inductive discovery-driven approaches to ASD-related genes have highlighted potential molecular gene sets or cell types common to different genetic causes of ASD (**Tables 1** and **2**). As gene discovery and *post hoc* analyses are expected to continue apace, a key challenge is the conversion of these insights into clearly stated hypotheses from which we can *deductively* define a set of empirically testable predictions. This is not a straightforward endeavor – as a key facet of any good hypothesis is that it be falsifiable, and it is not clear that is the case with the insights emerging from capstone analyses. Assuming there are no artifacts in the analysis, how does one falsify the hypothesis that chromatin modifiers are important in ASD? If, following the discovery of many more ASD risk genes in the next rounds of sequencing there is no longer a significant enrichment of this class of genes, does that mean the chromatin modifiers are now unimportant? One could argue no. Because for that small subset of ASD cases who carry a mutation in a chromatin modifier like *Chd8*, chromatin modifiers still play an important role. Rather, it would argue that chromatin modifying genes play a role, but only in rare cases. Thus, the implications of the insights garnered from a properly conducted discovery-driven analysis can change in scale, but never really go away. Only if the assumptions of the analysis itself change (e.g., *Chd8* turns out not to be a chromatin modifier) can the insight be falsified.

Yet, sometimes discovery-driven analyses can lead to the generation of falsifiable hypothesis. In a simple example, one could mutate the chromatin modifying function of *Chd8* or other candidates and measure whether that phenocopies complete loss of function in a model system. Other predictions can also be made about increased ASD risk or shared biological functions for genes that share many edges in a network. Below we highlight some experiments and suggest others that might meet this challenge.

Recommendation 4: testing network predictions with human genetics

The networks described in several of the recent analyses cited above by design both included genes confidently associated with ASD, and included genes that were either less confidently associated from human genetics, or were implicated by 'guilt-by-association' (Quackenbush, 2003) in *post hoc* analyses: e.g., that were perhaps co-expressed, co-annotated (GO), co-published (Text mining), or co-immunoprecipitated (PPI) with the more confidently associated genes. One prediction of these networks might be that mutations or polymorphism in these guilt-by-association genes will also cause or contribute to ASD risk. This concept can be tested informatically (e.g., looking for an increased common variant risk near such genes, though controlling appropriately for gene length, etc.; Ben-David and Shifman, 2012; Parikshak et al., 2013). More directly, O'Roak et al. (2012a), tested their network's prediction by targeted resequencing of such genes in a large cohort of ASD patients, demonstrating novel statistical association for several of them, and showing the utility of the initial network analyses. Thus, these discovery-driven analyses can successfully serve to direct new studies in human genetics and additional studies may assist in more rapid identification of additional causative genes. This approach will continue to be useful for a few years, though eventually it is likely to be supplanted as sequencing costs decrease and targeted analyses are replaced by routine exome or whole genome sequencing. Likewise, inclusion in a particular gene set has been used to reweight the probability that a variant of unknown significance should be considered pathogenic (e.g., with the TADA algorithm De Rubeis et al., 2014), though the most compelling evidence continues to be the presence of recurrent mutations in cases of ASD.

Recommendation 5: phenotypic clustering in man and models

Another apparent feature of these analyses is that genes in the networks are somewhat clustered by function. Thus, these networks may be making testable predictions regarding the shared function of closely connected genes. If the genes do indeed

share some function at the molecular or cellular level, then the prediction is that genes that are closer in the network will be closer in their consequences in cell models, animal models, or potentially even patient symptoms.

A point of caution is that failing to identify a significant similar phenotype or any phenotype at all when investigating a set of genes highlighted by a network analysis does not necessarily reject that hypothesis that the genes share some function. There are a myriad of possible phenotypes to evaluate, and all cannot be exhaustively tested. However, considering the information used to create the network and the resulting gene set can provide limits to the scope of phenotypes to test and prioritize primary outcomes of interest. And, at least the hypothesis that these genes have shared impact on those particular phenotypes does become testable and falsifiable.

A second point of caution is that the novelty of these predictions depends on whether functional data (e.g., PPI), or functional annotations (GO) were not used to build the networks in the first place – a caveat that cannot be taken lightly. Otherwise the network is not really making new predictions, just redisplaying known relationships in a different form. Likewise, there are no generally enforced standards for how these networks are displayed, and in some cases the authors may have selected the presentation of the nodes that maximally illustrates the functional clustering they would like to discuss. Overall, care must be taken to assure that circular logic does not creep into the conclusions drawn from these analyses.

Nonetheless, a variety of methods exist which could test any novel predictions regarding shared impact on phenotype. Deeply phenotyped sets of patients (such as the Simons collection; Fischbach and Lord, 2010) could be studied to determine whether individuals with mutations in genes that are closely spaced in the network share more clinical features. Specific mutations could be isolated or introduced in IPSC derived neural cells and their consequences studied with data rich methods such as hi-content imaging or RNAseq, with the prediction that there will be more similar phenotypic consequences for genes that are closer in the network (**Figure 3**). However, cultured cells have limitations in terms of the cell types that can be generated. They also have a very limited behavioral repertoire. Thus, it is our opinion that there is also a strong need to study the commonalities in behavioral disruptions across a variety of mice modeling these mutations. Though mouse behaviors are not meant to be perfect proxies for human symptoms, behaviors are highly sensitive readouts of the functions of particular CNS circuits in an intact organism. Shared behavioral disruptions across these can indicate shared circuit level disruptions, and particularly cell-type predictions (Parikshak et al., 2013; Willsey et al., 2013; Xu et al., 2014; Chang et al., 2015) might be best tested in the context of a complex nervous system. Cre-Lox and optogenetic technologies

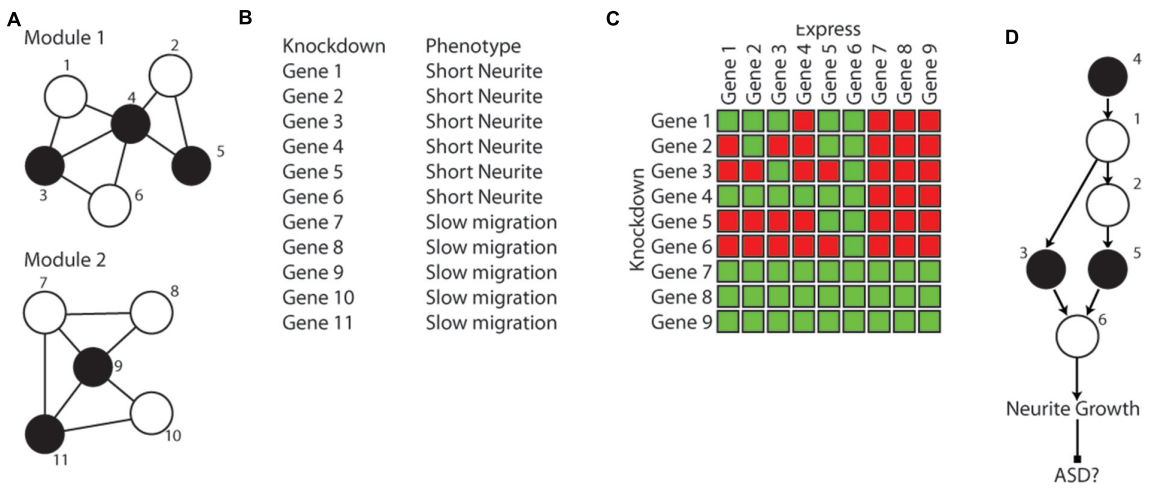


FIGURE 3 | Hypothetical example of phenotypical clustering and epistasis analysis in a culture model. (A) A hypothetical network constructed with five ASD genes (black) has resulted in two modules of 5–6 genes that are connected by expression and PPI data that include both ASD genes and tightly connected genes (white) not yet implicated in ASD. **(B)** The network result leads to a hypothesis that genes that are in the same module regulate the same phenotype. This is tested using single gene knockdown in iPSC derived neurons followed by high content imaging of neuronal morphology and behavior. Knockdown of the members of the two modules results in distinct cellular phenotypes, consistent with them potentially representing two distinct mechanisms of developing ASD (potentially two subtypes requiring different treatments). In this hypothetical example the tightly connected genes show the same phenotype. **(C)** Epistasis analysis for neurite length is used to test the hypothesis that all genes in module 1 are in the same pathway regulating neurite growth and are distinct from genes in module 2. Single gene knockdowns of all genes are transiently transfected with constructs expressing each individual gene. Green squares are normal neurite length, red squares are shortened neurites. These hypothetical results suggest several conclusions: (1) negative control genes from module 2 can't rescue short neurites, again indicating they are not in this genetic pathway. (2) As a control, expression of each gene in module 1 can rescue (complement) its own phenotype. (3) The pattern of complementation can be used to infer the functional relationship between the genes **(D)**. For example, gene 4 can be rescued by any other gene in the module, suggesting it must be before the others in the pathway, while gene 6 can rescue all others, but cannot be rescued by any of them, indicating it must be last. **(D)** The resulting pathway from the genetic analysis of neurite length. This result indicates that the original module did indeed represent a set of genes that regulate the same neurobiological phenomena. If indeed the shortened neurites lead to ASD, this also suggests that treatments targeting gene 6 (even though it was not itself an ASD gene), may be effective at treating individuals with ASD who have mutations in genes 3, 4, or 5.

in particular provide the opportunity to explicitly test shared contributions of particular circuits downstream of a genetic lesion. There is also a clear need for a more systematic approach to behavioral phenotyping, as the current one-lab-evaluates-one-model approach, often in different genetic backgrounds, makes careful and systematic *post hoc* comparisons across models nearly impossible.

Recommendation 6: epistasis analysis in models and mice
Genetic formalism has a lot to offer in the context of testing these networks. Genes that are closer in the networks (or perhaps co-expressed in the same cell type), may be in the same functional pathway. Studying compound mutations in humans might be informative (Pinto et al., 2014; Krumm et al., 2015). Indeed, examination of >100 million medical records has been used to test for epistatic effects of combinations of rare Mendelian diagnoses on risk of developing comorbid complex disease traits (Blair et al., 2013). But with currently available sample sizes for exome-sequenced cases, multiple ASD-related rare variants are unlikely to occur in the same individual frequently enough for formal testing of specific pairs of ASD-related genes. However, both in animals and in cell lines it is straightforward to make compound mutations and introduce rescue constructs with modern genome editing technologies. Thus, not only can we test whether network-associated genes have similar phenotypes (**Figures 3A,B**), we can also test whether any gene is in the same pathway and if it is dominant, complementary or suppressive of others (**Figures 3C,D**). Again, this could leverage both cell lines and mouse models for their relative strengths of either throughput or complexity.

Finally, all recommendations 4–6 might also provide the opportunity to subtype ASD cases into functionally distinct categories based on their molecular causes or cellular consequences – separate categories which may indeed be most amenable to different treatment strategies or that warrant stratification during clinical trials. Already results from exome studies are being used to define new subtypes of ASD starting from knowledge of the implicated gene (Bernier et al., 2014). Understanding commonalties in different subtypes of patients might be key to identifying routes to treatments for each.

Conclusion: Building New Cornerstones from Old Capstones

Over the last few years, discovery-driven bioinformatics analyses of ASD-related genes have moved from final figure capstone analyses to stand alone manuscripts. In architecture, the capstone is the coping, the final layer of finer, flat stone on the top of a wall of a that is somewhat functional (e.g., to end the structure, to protect from weather) but also somewhat decorative. Meanwhile, the cornerstones, classically, are the first stones placed in a new building – the seeds from which new buildings arise. Thus, the time has come to push these systems biology analyses away from capstones and toward cornerstones: studies from which we can derive empirically testable theories regarding commonalities of mechanism(s) for the diverse genetic risk factors contributing to ASD. The overall challenge now is to define criteria with which to systematically evaluate these discovery-driven insights, and to generate falsifiable hypotheses from these ideas. The hypotheses that survive rigorous empirical testing have the potential to become the foundations of new edifices rising toward ASD treatments.

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Characterizing autism spectrum disorders by key biochemical pathways

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The genetic and phenotypic heterogeneity of autism spectrum disorders (ASD) presents a substantial challenge for diagnosis, classification, research, and treatment. Investigations into the underlying molecular etiology of ASD have often yielded mixed and at times opposing findings. Defining the molecular and biochemical underpinnings of heterogeneity in ASD is crucial to our understanding of the pathophysiological development of the disorder, and has the potential to assist in diagnosis and the rational design of clinical trials. In this review, we propose that genetically diverse forms of ASD may be usefully parsed into entities resulting from converse patterns of growth regulation at the molecular level, which lead to the correlates of general synaptic and neural overgrowth or undergrowth. Abnormal brain growth during development is a characteristic feature that has been observed both in children with autism and in mouse models of autism. We review evidence from syndromic and non-syndromic ASD to suggest that entities currently classified as autism may fundamentally differ by underlying pro- or anti-growth abnormalities in key biochemical pathways, giving rise to either excessive or reduced synaptic connectivity in affected brain regions. We posit that this classification strategy has the potential not only to aid research efforts, but also to ultimately facilitate early diagnosis and direct appropriate therapeutic interventions.

Keywords: autism spectrum disorders (ASD), neurotrophic factors, mTOR pathway, ERK signaling, MAP kinase signaling system, protein synthesis

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Introduction

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders frequently characterized by impairments in social interactions, difficulties with language and communication, and the presence of repetitive, perseverative behaviors (Abrahams and Geschwind, 2008; Zoghbi and Bear, 2012). While ASD are generally highly heritable and can be defined by symptoms in core areas, there exists significant heterogeneity in genetics, phenotypes, clinical presentation, and associated comorbidities (Persico and Bourgeron, 2006). Recent advances have identified hundreds of genetic risk factors, including common and rare genetic variants, which can increase the likelihood of ASD (Ronemus et al., 2014). Many autism susceptibility genes are known to have important roles in brain development, with functions ranging from synaptic transmission to RNA processing and neurogenesis (Gilman et al., 2011; O'Roak et al., 2012; De Rubeis et al., 2014). However, the plethora of genetic targets has highlighted the need for the ASD research

community to understand whether genes implicated in ASD may converge on common cellular and developmental processes that can ultimately disrupt functions of brain circuits mediating language, cognition, and social behavior.

Attempts have been previously made to stratify ASD patients into smaller, more homogeneous subgroups by utilizing specific genetic signatures (Bernier et al., 2014) or behavioral and clinical endophenotypes (Spence et al., 2006; Eapen and Clarke, 2014). However, these strategies face difficulty encompassing the genetic and phenotypic heterogeneity of ASD, and may not assist in the identification of common neurobiological pathways underlying disease. In this review, we propose that genetically diverse forms of ASD may be usefully parsed into entities resulting generally from either synaptic and neural overgrowth or undergrowth, and the corresponding alterations in key biochemical pathways supporting these phenotypes. We review recent studies in patients and mouse models of ASD indicating convergence toward these two fundamental biological processes among genetically diverse causes of autism. We also discuss potential molecular signaling pathways that may contribute to these general alterations in growth and neural connectivity in ASD. The review primarily emphasizes data reported from earlier developmental stages to maintain a focus on alterations that are potentially causal, rather than secondary results of ASD pathology.

We propose that stratifying ASD based on readouts of over- or under-growth is an informative approach for establishing a more homogeneous sample. This will facilitate future research into the mechanisms underlying autism pathogenesis. This approach also has the potential to enable autism risk assessment, accelerate progress in pre-clinical research and clinical trials to facilitate an individualized approach to treatment, and avoid the discarding of potentially useful therapeutic strategies. **Figure 1** organizes over- and under-growth phenotypes seen in ASD models and patients, and pairs reviews and primary literature examining these phenotypes.

Brain Growth, Structure, and Connectivity in ASD

One of the earliest indications of aberrant brain growth during development in ASD came from measurements of head circumference among infants and young children with autism. Head circumference is posited as a reliable proxy for relative brain size during early postnatal ages (Bartholomeusz et al., 2002). These studies have provided important initial evidence for the presence of both over- and under-growth in ASD. Many studies have shown head circumference to be abnormally enlarged in children with ASD around the age of symptomatic diagnosis (Courchesne et al., 2003, 2007). A study examining 126 autistic children (2–16 years of age) reported a higher than expected incidence of both macrocephaly and microcephaly (Fombonne et al., 1999).

Reciprocal changes in growth as grossly measured by head size have also emerged as characteristic features of an increasing number of syndromic and non-syndromic forms of ASD. For example, de novo mutations in the dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 1 A (DYRK1A) gene are associated with a type of syndromic ASD and intellectual disability that presents with microcephaly (Bronicki et al., 2015; Van Bon et al., 2015). Conversely, macrocephaly has been shown to occur in a subset of ASD patients harboring disruptive mutations in the ASD-linked chromodomain helicase DNA binding protein 8 (Chd8) gene or deletions in 17q12 (Moreno-De-Luca et al., 2010; Bernier et al., 2014). This phenotype is further discussed in the context of specific, genetically defined causes of ASD later in this review. It is important to note that this early pathology of dysregulated brain growth tends to normalize later in childhood and adolescence, highlighting the necessity to focus on alterations that occur during the critical periods of prenatal and early postnatal development in ASD.

The application of neuroimaging methods to the study of ASD has provided unprecedented insights into the structure and intrinsic connectivity patterns of brain regions involved in complex social behavior and cognition. Due to technical constraints, previous assessments of functional brain connectivity in ASD were limited to task-based activation studies in adults with high-functioning autism, with relatively small sample sizes and varied methodologies (reviewed in Müller et al., 2011; Vissers et al., 2012). Brain changes observed in adulthood, however, may not reflect primary aberrations and may not generalize to children with severe low-functioning autism. Notably, it is now also understood that lowered task-based activation in fMRI could reflect cognitive performance deficits, rather than an intrinsic property of brain function. Recent studies have provided some clarification by taking advantage of technical advancements in imaging and analysis to conduct resting-state fMRI (R-fMRI) studies in younger ASD subject populations in order to provide a more accurate model of the developmental origins of the disease. These studies are useful for an initial assessment of the feasibility of categorizing ASD by over- or under-growth.

Supekar et al. (2013) and Keown et al. (2013) focused their R-fMRI analyses to young children and adolescents with ASD (7–14 years) to show increased long-range and short-range intrinsic connectivity across multiple brain regions. Moreover, the degree of functional hyperconnectivity was positively correlated with severity of social and repetitive behavioral symptoms (Keown et al., 2013; Supekar et al., 2013). Conversely, decreased functional connectivity has been observed in long-range interhemispheric projections and default mode networks that typically exhibit greater activity under resting conditions as opposed to tasks that require significant attention (Assaf et al., 2010; Anderson et al., 2011; Anderson, 2014). In certain cases, decreased functional connectivity has been inversely correlated to severity of core behavioral deficits in ASD (Assaf et al., 2010). Collectively, the fact that reciprocal changes in the intrinsic functional architecture of the brain may result in phenotypic outcomes associated with ASD supports the involvement of both under- and over-growth mechanisms in ASD pathophysiology.

Recent work by Ellegood et al. has shown that mouse models of ASD may be clustered in broad categories of brain under- and over-connectivity, providing further support for parsing the

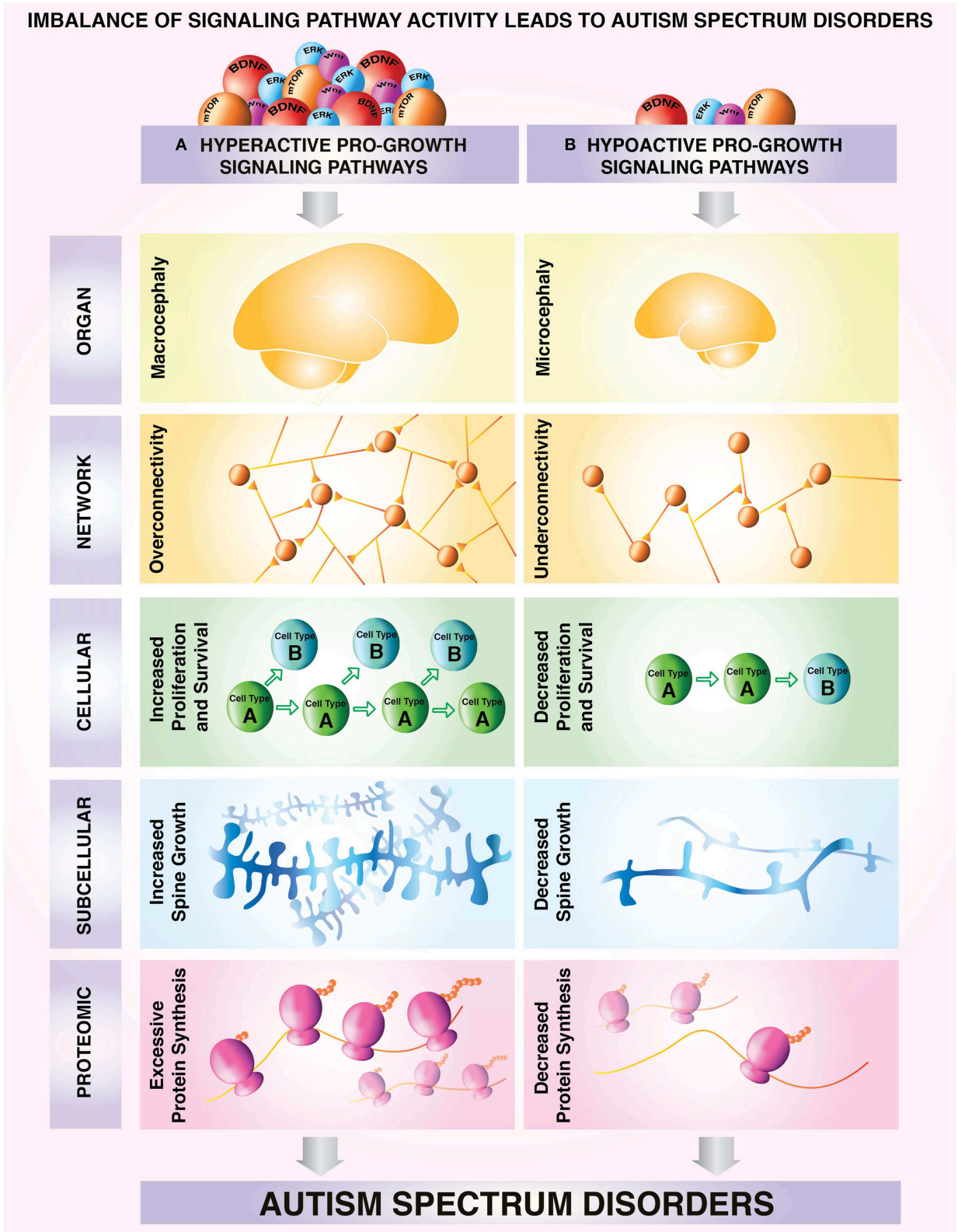

FIGURE 1 | Model depicting a proposed classification of different subtypes of ASD based on correlates of growth state. Neural overgrowth and undergrowth phenotypes have been associated with aberrant regulation of growth control pathways in autism spectrum disorders. Characteristic overgrowth and

(Continued)

FIGURE 1 | Continued
undergrowth phenotypes can be observed consistently from molecular to cellular and network levels. **(A)** Upregulation of pro-growth pathways can lead to: macrocephaly (Courchesne et al., 2003, 2007) aberrant overconnectivity of neuronal networks (Meikle et al., 2007; Keown et al., 2013; Supekar et al., 2013) increased survival and proliferation at the cellular level (Castrén et al., 2005; Callan et al., 2010) increased synaptic growth at the subcellular level (Irwin et al., 2001; Jaworski et al., 2005; Kwon et al., 2006; Tang et al., 2014), excessive protein synthesis (Osterweil et al., 2013; Santini et al., 2013) and/or a selective protein synthesis program enhancing production of growth-promoting proteins. **(B)** In contrast, hypoactive growth pathways lead to microcephaly, (Bronicki et al., 2015; Van Bon et al., 2015) underconnectivity of neuronal networks (Assaf et al., 2010; Anderson, 2014) decreased survival and proliferation at the cellular level (Yufune et al., 2015), decreased synaptic growth at the subcellular level (Cheng et al., 2014), decreased protein synthesis (Li et al., 2013; Tian et al., 2015) and/or a protein synthesis program that does not promote growth. These example phenotypes of undergrowth and overgrowth can be used as readouts for categorization of growth status in autism spectrum disorders.

heterogeneity of ASD using this approach (Ellegood et al., 2015). In patients, the majority of existing structural and neuroimaging studies have focused on delineating the effects of age on intrinsic connectivity patterns. As a result, while this approach holds promise, we must await further work in order to assess whether different genetic causes of autism may also classify into over- or under-growth based on structural and functional measures of brain connectivity.

Molecular Readouts of Growth

Mounting evidence has highlighted key growth signaling pathways that are frequently perturbed in patients with ASD as well as mouse models of autism. This section will cover these key molecular readouts of growth in autism, including dysregulated growth factor, mammalian target of rapamycin (mTOR), and extracellular signal-regulated kinase (ERK1/2) signaling. Characterizing how the regulators and downstream correlates of these growth pathways are dysregulated in ASD will allow us to better classify forms of autism into broad, but more homogeneous subtypes. This knowledge will be valuable for (i) diagnosis of under- or over-growth phenotypic subtypes of ASD, (ii) selection of homogeneous patient populations for more rigorously controlled studies, and (iii) a potential biomarker that can be used to potentially assess efficacy of clinical trials. We propose that broadly stratifying patient populations in terms of under- or over-growth phenotypes based on the following signaling pathways would facilitate mechanistic and therapeutic insights into ASD, without necessitating an impractical fine categorization by precise genetic etiology.

Growth Factor Signaling

Neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) were first identified as target-derived survival factors. However, growing body of evidence has indicated that neurotrophins regulate many aspects of neuronal structure and function, including neurodevelopment, differentiation, morphogenesis, and synaptic plasticity (Poo, 2001; Reichardt, 2006). Subsequent research over the past half century has revealed the existence of other canonical neurotrophins, including neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Each of the four mammalian neurotrophins binds to and activates one or more of the three members of the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (TrkA, TrkB, and TrkC), leading to subsequent activation of phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC), ERK1/2, and mTOR signaling (Chao et al., 2006; Reichardt, 2006). Additionally, appropriate control of neurotrophin signaling at multiple regulatory levels (epigenetic, transcriptional, post-transcriptional, and post-translational) is critical to physiological functions, such as cell fate decisions, axon growth, and dendritic growth and pruning, and overall neuronal network connectivity (Reichardt, 2006; Park and Poo, 2013). Genetic knockout strategies and pharmacological interventions reveal that many growth regulatory effects of neurotrophins depend upon Trk signaling pathways and subsequent activation of downstream cellular cascades (Reichardt, 2006; Park and Poo, 2013). Consistent with their potent roles as regulators of neuronal proliferation, survival, differentiation, and morphogenesis, dysregulated neurotrophin signaling has been implicated in neurodegenerative disorders, such as Alzheimer's and Huntington's disease, and also psychiatric disorders, including depression, substance abuse, as well as autism (Tsai, 2005; Chao et al., 2006; Martinowich et al., 2007; Nishimura et al., 2007; Gadow et al., 2009; Sadakata and Furuichi, 2009).

Other trophic factors, such as glial-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), and ciliary neurotrophic factor (CNTF), as well as insulin-like growth factor (IGF) also induce pro-growth signaling pathways. Like BDNF, NGF, NT-3, and NT-4, these additional trophic factors also signal through receptor tyrosine kinases to elicit downstream signaling cascades that promote growth in a variety of tissues, including neurons (Junger and Junger, 1998; Ferrara et al., 2003; Chao et al., 2006; Reichardt, 2006; Laviola et al., 2007). Dysregulation in these trophic factors has been documented in ASD, and extensive work in neurodegenerative disorders has characterized their trophic and pro-survival effects in the brain, including in motor neuron atrophy (IGF and CNTF), neuropathies of the peripheral nervous system (NGF and NT-3), Alzheimer's disease (NGF, BDNF, IGF), diabetic retinopathy (CNTF, BDNF), Huntington's disease (BDNF, NT-3, NT-4), Parkinson's disease (BDNF, GDNF), and amyotrophic lateral sclerosis (VEGF and IGF) (Hefti, 1994; Dawbarn and Allen, 2003; Gasparini and Xu, 2003; Zuccato and Cattaneo, 2009; Weissmiller and Wu, 2012; Ola et al., 2013; Keifer et al., 2014). The pronounced pro-growth and proliferative effects of these neurotrophins and their downstream effectors, such as mTOR and ERK1/2, make dysregulation in trophic factor cascades excellent readouts for stratification of ASD into generally overgrowth or undergrowth entities.

mTOR

mTOR is a highly conserved and ubiquitously expressed serine/threonine kinase that serves as an important regulator of cellular growth, metabolism, and survival in both developmental and disease states across a variety of tissue types. mTOR functions in two heteromeric and functionally distinct protein complexes, mTORC1 and mTORC2, which are embedded into complex signaling networks. In the brain, mTOR integrates inputs from a range of extracellular sources, including growth factors, guidance cues, and nutrients (Takei and Nawa, 2014).

mTOR regulates many processes that are essential for growth by serving as a nexus for controlling protein synthesis, energy homeostasis, metabolism and actin cytoskeletal dynamics. Studies of tumorigenesis strongly implicate mTOR activity as a correlate of growth and metabolic status. Many familial cancer syndromes result from mutations in genes encoding upstream proteins that influence mTOR activation, including Tsc1/2, PTEN, and neurofibromatosis type I (NF1) (Yuan and Cantley, 2008). Dysregulation of genes in this pathway has also been linked to multiple disease conditions, including ASD, type II diabetes, obesity, and neurodegeneration (Zoncu et al., 2011; Laplante and Sabatini, 2012; Takei and Nawa, 2014).

Importantly, bidirectional changes in mTOR signaling have also been shown to result in opposing downstream effects on neuronal growth and morphogenesis. In mice, ablation of mTOR or associated components that regulate mTORC1/2 assembly and signaling leads to embryonic lethality (Guertin et al., 2006; Shiota et al., 2006). Conditional deletion of both mTORC1 and mTORC2 in neural progenitors of the developing CNS can cause microcephaly due to an overall decrease in neuronal number and size as a result of reduced neuronal progenitor proliferation and suppressed differentiation of cortical neurons (Cloëtta et al., 2013; Thomanetz et al., 2013; Ka et al., 2014). Conversely, enhanced mTOR signaling following inactivation of upstream negative regulators (PTEN and TSC1/2), or constitutive activation of positive regulators (PI3K, Akt, and Ras), has been associated with macrocephaly, neuronal hypertrophy, and increased soma size and dendritic complexity of hippocampal neurons (Jaworski et al., 2005; Kwon et al., 2006). These effects of mTOR activation on neuronal and dendritic morphogenesis requires novel protein synthesis (Jaworski et al., 2005).

mTOR-mediated regulation of protein translation has garnered much interest in the field of ASD research. Substrates of mTOR that are critically involved in the translation initiation machinery, such as p70 ribosomal S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor 4E-binding proteins (4E-BPs), are emerging as key players in autism pathogenesis (Klann and Dever, 2004). Phosphorylation of S6K1 by mTORC1 promotes ribosomal biogenesis, translational initiation, and elongation through a variety of effectors (Ma and Blenis, 2009). Mice harboring a genetic deletion of S6K1 are significantly smaller than wild-type counterparts. Certain protein synthesis-dependent forms of synaptic plasticity which may represent correlates of growth in adulthood, such as mGluR-LTD, have been associated with increased phosphorylation and activation of S6K1 (Antion et al., 2008).

Unphosphorylated 4E-BP2 inhibits cap-dependent protein synthesis by sequestering the translation initiation factor eIF4E. Phosphorylation of 4E-BP2 by mTORC1 leads to its dissociation from eIF4E, thereby de-repressing its cap-binding activity and enabling formation of the eIF4F translation initiation complex. Deletion of 4E-BP2 or overexpression of eIF4E *in vivo* leads to increased eIF4F complex formation, facilitated protein synthesis-dependent long-term synaptic plasticity, elevated dendritic spine density, and behavioral abnormalities reminiscent of ASD (Banko et al., 2005, 2006; Gkogkas et al., 2013; Santini et al., 2013).

Taken together, bidirectional changes in mTOR signaling lead to opposite effects on protein synthesis, metabolism, and growth. Therefore, measuring activity levels of mTOR or downstream effectors of this cascade may serve as reliable indicators of altered pro- or anti-growth states in the brain.

ERK1/2

ERK1/2 are paralogous members of the MAPK signaling cascade with well-characterized roles in regulating growth at cellular and organismal levels. The canonical MAPK/ERK pathway transduces signals from cell surface receptors to the nucleus through sequential phosphorylation steps. This pathway is responsive to growth factors, chemokines, oxidative stress, and cytokines (Lu et al., 2005; Byts et al., 2006; Samuels et al., 2009; Hsieh et al., 2010). The ERK1/2 signaling cascade has important roles in cellular proliferation, differentiation, and apoptosis (Murphy and Blenis, 2006). In the nervous system, activation of this pathway generally promotes excitation and is involved in activity-dependent plasticity, long-term potentiation (LTP), long term depression (LTD) and memory formation (Satoh et al., 2011). Downstream targets of ERK1/2 signaling in neurons govern processes such as dendritic spine stabilization, modulation of ion changes and receptor insertion (Sweatt, 2004).

Recent genome-wide association studies (GWAS) and analysis of copy number variations (CNV) have identified an enrichment of MAPK/ERK signaling components in patients with autism (Pinto et al., 2010). Furthermore, ERK1/2 activation is required for the formation and stabilization of dendritic spines (Wu et al., 2001; Goldin and Segal, 2003) and activation of ERK1/2 by growth factors has well-characterized functions in regulating cell proliferation in the CNS (Sweatt, 2004; Samuels et al., 2009). ERK1/2 plays a critical role in corticogenesis through regulation of the cell cycle in proliferation of neural progenitor cells. While both ERK1 and ERK2 are expressed highly in the adult brain, ERK2 levels are higher than ERK1 (Samuels et al., 2009). Additionally, only loss of ERK2 results in early embryonic lethality in mouse models primarily through a failure of normal placental and trophoblast development (Samuels et al., 2009). Conditional knockout of ERK2 during the height of cortical neurogenesis results in a decrease in neuron number and subsequent increases in astrocyte number in the murine cortex. This decrease reflects alterations in growth and proliferation by a reduction in the number of cell divisions in intermediate progenitor cells leading to decreased cortical thickness (Samuels et al., 2008).

The strongest evidence implicating disruption of ERK1/2 signaling in ASD comes from a number of single gene mutations that are associated with autism including Tuberous sclerosis, Fragile X syndrome, 16p11.2 (discussed in future sections in this review) and NF1 all ultimately leading to activation of ERK1/2. NF1 is a GTPase-activating protein (GAP) for Ras, an upstream activator of ERK1/2. Mutations in NF1 cause neurofibromatosis type 1, an autosomal inherited disorder with a high frequency of hyper-proliferative schwannoma cancers. More than 50% of individuals with mutations in NF1 also have cognitive impairments and recent studies report a significant increase in the incidence of ASD in NF1 patients (Marui et al., 2004). In addition to the numerous monogenic forms of autism implicating ERK1/2 activity in disease pathophysiology, the inbred mouse strain BTBR, a model of non-syndromic autism, shows an increase in phospho-ERK1/2 levels in the pre-frontal cortex (Faridar et al., 2014).

ERK1/2 is a critical regulator of development and alterations in ERK1/2 activity, either increased active phospho-ERK1/2 or decreased phospho-ERK1/2, have been associated with autistic features (Wang et al., 2012; Yufune et al., 2015). Interestingly, a recent study conducted in mice suggests that there may be a critical window for alterations in ERK1/2 activity to lead to the development of ASD. The authors find that blockade of ERK1/2 signaling through administration of the MEK inhibitor SL327 at postnatal day 6 leads to adult mice exhibiting common autistic behavioral phenotypes, such as deficits in social interaction, as well as increased apoptosis in the forebrain (Yufune et al., 2015). However, inhibition of ERK1/2 at postnatal day 14 did not lead to the development of autistic behaviors in these mice, suggesting a critical window in development for regulation of ERK1/2 activity. Collectively, strong evidence from multiple biological settings links ERK1/2 pathways to the regulation of growth states, including proliferation and excitation.

Characterization of Genetically Defined Models of ASD Based on Growth Readouts

This section reviews ASD of known genetic cause and the mouse models of these ASD with reference to growth pathway correlates (**Table 1**). The presented mouse models recapitulate many phenotypes of human ASD, including dysregulation of neuronal growth and development, synaptic transmission, neuronal connectivity, and behavior. Although each mouse model does not perfectly genocopy the human genetic variants that predispose individuals to ASD, they present valuable resources for mechanistic investigations into the genetic pathways underlying aberrant neuronal growth. Moreover, given that several of these models involve copy number variants (CNVs) as well as deletions and duplications of a given chromosomal locus, these models provide insights into how gain or loss-of-function can impact the spectrum of neuronal growth phenotypes inherent in patients with ASD. Mouse models of ASD of known genetic etiology will be informative for diagnosis and future clinical studies that target pathways of cellular growth that are dysregulated in individuals with ASD.

TABLE 1 | Classification of well-established monogenic and copy number variant models of ASD based on correlates of neuronal growth and connectivity.

Genetic etiology	Associated syndrome	Synaptic or neuronal growth phenotype
Fmr1	Fragile X syndrome	Overgrowth
TSC	Tuberous sclerosis	Overgrowth
PTEN	N/A	Overgrowth
MeCP2 loss-of-function	Rett syndrome	Undergrowth
MeCP2 gain-of-function	MeCP2 duplication syndrome	Overgrowth
AUTS2	Syndromic intellectual disability	Undergrowth
16p11.2 microdeletion	Non-syndromic ASD	Overgrowth
16p11.2 duplication	Non-syndromic ASD	Undergrowth
22q11.2 microdeletion	Non-syndromic ASD	Undergrowth

Monogenic Models of ASD

Fragile X Syndrome

Fragile X syndrome (FXS) is the most common inherited cause of ASD and intellectual disability, affecting approximately 1 in 4000 males and 1 in 8000 females worldwide (Peprah, 2012). Accounting for 2–6% of all cases of autism, FXS is a neurodevelopmental disorder that is associated with an expansion in a CGG trinucleotide repeat element in the 5′ UTR of the Fragile X Mental Retardation 1 (FMR1) gene. The mutation leads to hypermethylation of the locus and transcriptional silencing of FMR1, which subsequently leads to a loss of production of its downstream gene product, Fragile X Mental Retardation Protein (FMRP) (Penagarikano et al., 2007). FMRP is localized in the soma and dendrites of neurons, where it functions predominantly to suppress the translation of a subset of mRNAs (Penagarikano et al., 2007). The anatomical, synaptic, and molecular features of FXS have made this monogenic disorder a classic model of developmental overgrowth in ASD.

FXS patients display intellectual disability and cognitive impairments, developmental delay and physical features consistent with overgrowth, such as macrocephaly, elongated facial morphology, and enlarged ears (Chudley and Hagerman, 1987). Additional growth deficits include an increase in body weight in a subset of FXS patients (Nowicki et al., 2007) and macroorchidism, or enlarged testicles, in males following the onset of puberty (Chudley and Hagerman, 1987). In postmortem brain tissue isolated from FXS patients, studies have found an overall increase in dendritic spine density as well as more immature spine morphology (Irwin et al., 2001). FXS patients and mouse models deficient in FMRP also display hallmarks of brain morphology and function found in other genetic causes of autism defined by overgrowth. These include an early overabundance of synapses, brain hyperconnectivity, and excessive basal and activity-responsive neuronal protein synthesis (Dölen et al., 2007; Gibson et al., 2008; Pan et al., 2010).

In the central nervous system and the testis, two tissues in which FMRP is known to be highly expressed (Penagarikano et al., 2007), loss of FMRP function causes gross morphological

abnormalities. For example, children with FXS and mice deficient in FMRP display significantly increased hippocampal volume (Kates et al., 1997; Shi et al., 2012). Specifically, structural MRI methods show an increase in hippocampal volume from P18–30 in Fmr1 KO mice (Shi et al., 2012). Another well-described and reproducible growth defect observed in patients and mouse models of FXS is macroorchidism. Fmr1 KO mice display an elevation in testes weight that has been linked to increased proliferation of supporting Sertoli cells during embryonic development (Slegtenhorst-Eegdeman et al., 1998; Dölen et al., 2007).

Accumulating reports further indicate a critical role for FMRP function in the proper control of developmental timing. FMRP deficiency leads to temporal delays in many developmental settings, including the perinatal critical period for barrel cortex plasticity (Harlow et al., 2010) and the switch in polarity of GABA signals from depolarizing to hyperpolarizing (He et al., 2014). Loss of FMRP also causes increased proliferation and abnormal differentiation of neural stem and progenitor cells (Castrén et al., 2005; Callan et al., 2010).

Abnormalities in dendritic spines are thought to be a central feature of FXS, but the precise nature of the defect is still controversial. Although there are several reports of altered spine density and morphology in the Fmr1 KO mouse model, the observed alterations appear to vary considerably depending on the culture/staining methods, developmental age, brain region, and mouse background strain (He and Portera-Cailliau, 2013). More recent studies using *in vivo* two-photon microscopy to image dendritic spine dynamics in intact neocortex from Fmr1 KO mice have revealed specific defects in spine turnover and maturation that reflect a pro-growth state during development. Cortical pyramidal neurons display abnormally high rates of spine turnover and delayed stabilization of spines during early postnatal development, coinciding with the critical period for spine formation and plasticity in the neocortex (Cruz-Martín et al., 2010; Pan et al., 2010).

At the functional level, Fmr1 KO mice exhibit network hyperexcitability and an imbalance between excitation and inhibition in neural circuits (Gibson et al., 2008; Gonçalves et al., 2013). While excessive neuronal growth and delayed synaptic maturation during critical periods for experience-dependent plasticity in the brain can influence the wiring and functional integrity of neural circuits, the exact outcome of such changes are likely mediated in a cell type-specific manner. Therefore, future studies need to examine molecular readouts of growth in different cell types in the brain, and in the context of specific functional circuits.

Several studies using neuronal and peripheral tissues from FXS patients and Fmr1 KO mice have investigated ERK1/2 phosphorylation status. Although results from these studies have shown some discrepant findings, possibly due to the labile nature of ERK1/2 phosphorylation, a number of reports have shown elevated basal phospho-ERK1/2 (pERK1/2) levels in brain tissues from Fmr1 KO mice and FXS patients (Michalon et al., 2012; Wang et al., 2012). Inhibition of the ERK1/2 pathway normalizes excessive hippocampal protein synthesis and also alleviates certain behavioral abnormalities observed in Fmr1 KO mice, including audiogenic seizure susceptibility (Wang et al., 2012; Osterweil et al., 2013). Further, a phase I clinical trial of the Ras-ERK1/2 inhibitor, lovastatin, conducted by Dr. Francois Corbin's group showed significant behavioral improvements in FXS patients (Çaku et al., 2014).

In addition to hyperactive ERK1/2, another critical molecular player that may contribute to pro-growth phenotypes in FXS is dysregulated mTOR signaling. Although most analyses so far have been conducted at older ages, elevated levels of phospho-AKT, phospho-S6K, and active phospho-eIF4E have been detected in brain tissue and peripheral blood lymphocytes from FXS patients (Hoeffer et al., 2012). Similar hyperactivation of several upstream and downstream components of the mTOR cascade have been observed in brains of Fmr1 KO mice (Sharma et al., 2010). Additionally, deletion of S6K in Fmr1 KO mice rescues exaggerated hippocampal protein translation, aberrant dendritic spine morphology and macroorchidism (Bhattacharya et al., 2012). Normalizing elevated levels of PI3K enhancer (PIKE), the upstream activator of PI3K-Akt-mTOR signaling, in Fmr1 KO mice also normalizes dendritic spine density and network hyperexcitability (Gross et al., 2015).

Hyperactivation of the ERK1/2 and mTOR pathways in FXS has been generally linked to excessive global protein synthesis in the brain. Auerbach et al. elegantly demonstrated that restoring the optimal balance of intracellular signaling and downstream protein synthesis may reverse synaptic and behavioral defects in mouse models of FXS and TSC (Auerbach et al., 2011; Bhakar et al., 2012). Changes in protein synthesis have become a focal point in the study of ASD, and are one of multiple key cellular readouts for growth state. It is worth noting, however, that global upregulation or downregulation of mRNA translation may not directly result in overgrowth or undergrowth phenotypes, which are likely to depend upon the identity of the affected mRNAs. For example, an overgrowth phenotype might also be achieved by an alteration in the specificity of protein synthesis to increase translation of pro-growth genes without any change in total protein synthesis. The regulation of gene target selectivity in translation may also play a role in the contribution of altered protein synthesis to pro- or anti-growth phenotypes observed in subtypes of ASD.

There is also evidence supporting dysregulation of upstream activators of the ERK1/2 and mTOR pathways in autism, including BDNF signaling through the TrkB receptor (Maija Castrén and Castrén, 2014). *TrkB* mRNA has been identified as a target for translational suppression by FMRP (Darnell et al., 2011). While it is clear that BDNF/TrkB signaling is altered in FXS, the precise effects of FMRP loss on spatiotemporal expression patterns or activity of BDNF and the TrkB receptors are currently unknown. Elevated catalytic TrkB expression has been observed in undifferentiated neural progenitor cells from Fmr1 KO mice (Louhivuori et al., 2011). BDNF expression in the hippocampus varies with age in Fmr1 KO mice, with levels significantly higher than wild-type controls at 2 months of age (Uutela et al., 2012). However, alterations in BDNF expression appear to differ by brain region (Louhivuori et al., 2011). Since BDNF is known to establish pro-growth programs of gene expression that are important for neuronal proliferation and

morphogenesis, elevations in BDNF signaling may significantly contribute to hypertrophic phenotypes observed in FXS (Poo, 2001; Reichardt, 2006).

Another line of evidence pointing to upregulated BDNF signaling in FXS comes from recent studies demonstrating aberrant increases in matrix metalloprotease-9 (MMP9) levels in brains of FXS patients and Fmr1 KO mice (Gkogkas et al., 2014). Elevated protein levels of MMP9 have also been detected in plasma and amniotic fluid derived from FXS and ASD patients (Dziembowska et al., 2013; Leigh et al., 2013). MMP9 is a protease that can cleave pro-BDNF to mature BDNF in the hippocampus (Mizoguchi et al., 2011) and developing neuromuscular junction (Je et al., 2012). Two studies demonstrated that genetic and pharmacological reduction of MMP9 levels ameliorates FXS-associated anatomical and behavioral abnormalities (Gkogkas et al., 2014; Sidhu et al., 2014). Treatment with a tetracycline derivative, minocycline, ameliorates enhanced MMP9 levels and significantly improves behavioral performance in Fmr1 KO mice (Bilousova et al., 2009). In clinical trials, treatment of FXS children and adults with minocycline has been found to be well-tolerated and results in significant behavioral improvements, as measured by the Clinical Global Impression Scale and ABC-C Irritability Subscale (Paribello et al., 2010; Leigh et al., 2013). Further, clinical responses to minocycline are correlated with changes in plasma MMP9 levels (Dziembowska et al., 2013). Together, these findings point to a potential involvement of the BDNF/MMP9 axis in FXS pathogenesis that would be worth exploring in future studies.

Collectively, work by several groups indicates that hyperactive ERK1/2, mTOR, and BDNF pathways may contribute to neuronal protein synthesis, growth, and connectivity defects in FXS. Therefore, the molecular and biochemical signatures of FXS are in accordance with its categorization as an overgrowth form of ASD.

Tuberous Sclerosis

Tuberous sclerosis (TSC) is a neurodevelopmental disorder that is caused by autosomal dominant mutations in the TSC1 or TSC2 tumor suppressor genes, which function as negative regulators of the mTOR signaling cascade. Loss of TSC1/2 function which enhances cell growth and promotes dysregulated metabolism leads to non-malignant tumor formation in the skin, brain, and other organs (Curatolo et al., 2008). Importantly, almost 50% of individuals with TSC also meet criteria for diagnosis of ASD or intellectual disability (Curatolo et al., 2008; Jeste et al., 2008). Most commonly used mouse models of TSC, such as mice harboring heterozygous genetic deletions of Tsc1 or Tsc2, exhibit phenotypes that recapitulate aspects of the human disease, including synaptic dysfunctions, deficits in learning and memory, and impaired social interactions (Goorden et al., 2007; Ehninger et al., 2008; Sato et al., 2012; Tang et al., 2014).

Homozygous deletion of Tsc1 in postnatal forebrain neurons using CaMKII-CRE $Tsc1^{flox/flox}$ mice leads either to lethality or severe brain enlargement and neuronal hypertrophy in animals surviving to 3 months of age (Ehninger et al., 2008). Initial studies characterizing the function of TSC1/TSC2 complexes in the brain identified important roles in regulating neuronal growth and neural network homeostasis. In particular, complete *in vivo* loss of Tsc1 in post-mitotic neurons induces ectopic axon formation (Choi et al., 2008), enlarged and dysplastic neuronal morphology (Meikle et al., 2007), and network hyperexcitability due to weakened functional synaptic inhibition (Bateup et al., 2013). Recent work has also demonstrated that Tsc1 and P20-P30 Tsc2 deficient mice display reduced pruning of dendritic spines during a critical period in development, giving rise to increased spine density at later postnatal ages. Tang et al. attributed the observed failure of developmental spine pruning in mouse models of TSC to a deficit in mTOR-dependent macroautophagy (Tang et al., 2014).

Importantly, many of the aforementioned hypertrophic phenotypes of TSC loss-of-function are ameliorated by treatment with the mTORC1 inhibitor, rapamycin, suggesting that hyperactive mTOR signaling plays a critical role in neural overgrowth and hyperconnectivity defects in TSC. For most studies conducted, rescue of synaptic or behavioral abnormalities has been most successful if rapamycin is administered chronically beginning at early postnatal ages. This further supports the possibility that early mTOR hyperactivation may be the primary insult that triggers downstream effects on neuronal growth, development, and excitability. Although mTOR signaling appears to be persistently increased following TSC1/2 deficiency, secondary pathways may be engaged to maintain synaptic and behavioral defects into adulthood. Therefore, it remains to be assessed whether synaptic overgrowth and associated dysfunctions may be reversed by mTOR inhibition later in adulthood.

In accordance with known functions of mTOR in the regulation of mRNA translation, hyperactivation of this pathway would be expected to enhance global protein synthesis. Unexpectedly, adult Tsc2 haploinsufficient mice exhibit basal suppression of hippocampal protein synthesis (Auerbach et al., 2011). While examination of mRNA translation at early postnatal ages is required, the reported reduction in basal protein synthesis in adult Tsc2 deficient mice could reflect the tight homeostatic regulation of mTOR signaling. Work in non-neuronal systems has found that long-term hyperactivation of mTOR can evoke compensatory downregulation of upstream signaling factors through an inhibitory feedback loop (Hay, 2005). For example, murine embryonic fibroblast cultures derived from Tsc2 null mice exhibit increased mTOR activation, but reduced upstream PI3K-Akt signaling (Zhang et al., 2003). The contribution of developmental and homeostatic changes in growth pathway readouts further underscore the importance of focusing on early developmental ages for the classification of ASD subtypes.

Recent studies have also demonstrated that TSC1/2 may interact with the MAPK/ERK signaling cascade. This relationship was first appreciated in clinical studies which revealed that components of the ERK1/2 pathway are constitutively activated in TSC-associated brain lesions and tumor cell lines (Govindarajan et al., 2003; Ma et al., 2005, 2007). Upregulated ERK1/2 signaling has been observed in the ΔRG model of TSC, which recapitulates human deletion mutations that disrupt TSC1/2 complex function by interfering with Tsc2 GAP activity (Chévere-Torres et al., 2012). The authors of the

same study reported no differences in ERK1/2 phosphorylation in hippocampi from Tsc1 (CamKII-CRE $Tsc1^{+/fl}$) and Tsc2 (CamKII-CRE $Tsc2^{fl/fl}$) mutant mice as compared to wild-type controls. However, this experiment was performed in adult animals where compensatory changes could have occurred, and therefore, more thorough analysis of phospho-ERK1/2 levels throughout development may be required.

TSC2 has, itself, also been implicated as a direct target of ERK1/2 kinase activity, wherein phosphorylation of TSC2 by ERK1/2 results in dissociation of the TSC1/2 tumor suppressor complex and subsequent disinhibition of mTOR-dependent effects on growth, proliferation, and protein synthesis (Ma et al., 2005). Although these studies were conducted in the context of tumorigenesis, this mechanism may be conserved in the nervous system as well. The fact that TSC1/2 serves as an important node of cross-talk and convergence between the ERK1/2 and mTOR signaling cascades might explain how changes in components of both pathways can produce a dysregulated growth state in many syndromic and non-syndromic forms of ASD. Based on the aforementioned phenotypes resulting from mutations in the TSC tumor suppressor, we propose that ASD patients with TSC loss-of-function represent an example characterized by neuronal overgrowth phenotypes.

Phosphate and Tensin Homolog (PTEN)

The tumor suppressor gene PTEN is a dual protein and lipid phosphatase critical for modulating cellular growth, proliferation and survival. PTEN is a major inhibitor of the highly conserved PI3K-Akt signaling pathways, and regulates diverse cellular processes, including metabolism, survival, proliferation, growth, and motility (Worby and Dixon, 2014). The tumor suppressor functions of PTEN have been most thoroughly described in the context of cancers, such as those of the skin, endometrium, prostate and central nervous system (Chalhoub and Baker, 2009). Mounting evidence has documented a role for PTEN in disorders of nervous system function, including ataxia (Backman et al., 2001), seizures (Backman et al., 2001), mental retardation (Varga et al., 2009; McBride et al., 2010), and autism (Varga et al., 2009; Zhou and Parada, 2012). Individuals with germline PTEN mutations are not only prone to cancer, but many of these individuals are also at risk of developing autism with comorbid macrocephaly (Butler et al., 2005; Clipperton-Allen and Page, 2014).

To better understand the cellular and molecular mechanisms by which dysregulated PTEN signaling may predispose individuals to macrocephaly and ASD, multiple groups have genetically engineered PTEN-deficient mice. These studies have shown that mice lacking PTEN in differentiated neurons exhibit macrocephaly, regional hypertrophy, increased soma, dendritic and axonal growth, ectopic axons and dendrites, and increased spine density (Backman et al., 2001; Kwon et al., 2001; Greer and Wynshaw-Boris, 2006; Fraser et al., 2008; Amiri et al., 2012). Additionally, these mice display classic autism-related behavioral abnormalities including impaired social interaction, learning deficits, hypersensitivity to acoustic stimuli, increased seizure susceptibility, and exaggerated anxiety-like behaviors (Kwon et al., 2006). Additionally, loss of PTEN in adult hippocampal stem cell populations was shown to accelerate stem cell proliferation rates and activate PI3K/AKT/mTOR/GSK3B signaling pathways to promote a pro-growth phenotype. Neurons differentiated from these stem cells exhibit dendritic and axonal hypertrophy (Amiri et al., 2012), consistent with PTEN's role as a suppressor of growth in multiple systems. Many of the neuronal hypertrophic phenotypes and behavioral abnormalities associated with loss of PTEN can be rescued by pharmacological inhibition of downstream mTOR signaling with rapamycin, but surprisingly not by loss of S6K1, a downstream effector of ERK1/2 (Kwon et al., 2003; Chalhoub et al., 2006; Zhou et al., 2009). Thus, future research is required to determine the precise mechanisms by which rapamycin-mediated inhibition of mTOR is able to rescue the pro-growth phenotypes observed in PTEN deficient neurons.

Methyl-CpG-binding protein

Methyl-CpG-binding protein (MeCP2) is a regulator of gene expression which is present in cells throughout the body, but is particularly abundant in the brain. MeCP2 provides an interesting case in which the available evidence indicates that loss of MeCP2 (Rett Syndrome) can produce an "undergrowth" entity, while excess MeCP2 (MeCP2 duplication syndrome) conversely results in a presentation consistent with underlying "overgrowth." Rett Syndrome (RTT), in 95% of cases, is caused by loss-of-function mutations in the X-linked MeCP2 and is predominately seen in females (around 1/10,000 live births) as these mutations in males lead to perinatal lethality (Amir et al., 1999). Behaviorally, children with Rett syndrome display some core features of autism, including repetitive behaviors and poor communication skills. MeCP2 duplication syndrome, which is caused by duplication of the *MeCP2* gene, presents with intellectual disability, seizures, motor dysfunction, developmental delay and autistic behavior (Ramocki et al., 2010).

Patients with Rett Syndrome appear to develop normally until 6–18 months of life and then exhibit dramatic motor/autonomic deterioration, and deceleration of head growth with microcephaly evident by the second year of life. MeCP2 plays a role in synaptic maturation and pruning in development as well as the maintenance of dendritic arbors in adulthood (Schüle et al., 2008; Matijevic et al., 2009; McGraw et al., 2011; Baj et al., 2014). Characterization of postmortem brain tissue obtained from patients with RTT found a 12–34% reduction in brain weight and volume. There is a decrease in hippocampal spine density, decreased dendritic branching, reduction in neuronal size, and reduced white matter volume. However, there is no obvious degeneration present in RTT brains suggestive of MeCP2's role in regulation of postnatal neuronal growth and not in neurodegeneration (Reiss et al., 1993; Jellinger, 2003). A number of these phenotypes are recapitulated in the male Mecp2-null mice which display normal development until 3–8 weeks of age but begin a sharp decline, exhibiting tremors, difficulty in locomotion and hypotonia. While there are no obvious structural abnormalities present in the MeCP2 null mice, their brains are reduced in size and have a smaller, denser composition of neurons as compared to wild-type controls. While male MeCP2 null mice often die within 6–10 weeks,

female heterozygotes remain viable until 4–6 months and at this point begin to exhibit RTT-like symptoms (Guy et al., 2001; Ricceri et al., 2008; Chen et al., 2013). In an additional mouse model of RTT, a truncation of MeCP2 at amino acid 308 (MeCP2308/Y) leads to a less severe progression of neurological phenotypes but also mirrors deficits found in RTT patients. Due to the rare occurrence of MeCP2 duplication syndrome, very little analysis on the morphology of post-mortem brain tissue has been reported; however, some evidence suggests macrocephaly in patients with MeCP2 duplication syndrome (Lugtenberg et al., 2009). The MeCP2 duplication mouse model (2-fold overexpression of human MeCP2) displays an initial enhancement of synaptic plasticity and motor and contextual learning. At around 20 weeks of age, however, these transgenic mice develop seizures and motor deficits and eventually die around 1 year of age (Collins et al., 2004; Luikenhuis et al., 2004). While there are conflicting reports about the effects of MeCP2 overexpression on synaptic density, there is some evidence to suggest that MeCP2 duplication leads to an increased density of excitatory synapses (Zhou et al., 2006; Chao et al., 2007; Chapleau and Pozzo-Miller, 2012). Misexpression of MeCP2 is also associated with other neuropsychiatric disorders ranging from intellectual disability, schizophrenia, Angelman-like syndrome and autism (Chahrour and Zoghbi, 2007).

MeCP2 plays a complex role in regulating the gene expression of multiple growth control pathways, and the cellular outcomes downstream of these genes. Current research, which is detailed in other reviews (Cheng and Qiu, 2014), has shown that MeCP2 can regulate transcription both as a transcriptional repressor, as well as a transcriptional activator through CREB1 recruitment (Chahrour and Zoghbi, 2007). Induced neurons (IN) have been used recently to show that MeCP2-deficiency particularly reduces the levels of activity-dependent neuronal genes which are important for the promotion of growth and plasticity (Li et al., 2013), including BDNF and IGF1 (Castro et al., 2014). *In vitro* MeCP2 has been shown to bind the BDNF promoter region and repress its transcription when the neuron is not active. Following neuronal activity, MeCP2 is phosphorylated at serine 421 and removed from the promoter region, allowing for transcription of BDNF to occur (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006). The story *in vivo*, however, is more complex. In the MeCP2 -/y mice, Chang et al., discovered that overall BDNF levels are reduced, most likely due to reduced cortical activity present in the MeCP2 -/y brain (Dani et al., 2005; Chang et al., 2006). Importantly, the authors demonstrated that increasing expression of BDNF within the forebrain of MeCP2 -/y mice rescues motor function and increases lifespan, suggesting an important functional interaction between this neurotrophic factor and MeCP2. In accordance with this result, loss of MeCP2 has also been reported to depress levels of activated pERK1/2, and activation of the Ras-ERK1/2 pathway by MeCP2 to be required for the promotion of neuronal differentiation by MeCP2 (Sweatt, 2004). MeCP2-deficiency is also reported to lower ribosomal RNA levels and to reduce protein synthesis (Li et al., 2013) consistent with an undergrowth phenotype. Interestingly, recent work provides evidence that MeCP2 interacts with DGCR8, a component of nuclear microRNA (miRNA) processing, and leads to suppression of pri-miRNA processing (Cheng et al., 2014). Effects on miRNA biogenesis could provide an additional regulatory mechanism by which loss of MeCP2 alters not only the quantity, but also the specificity of protein synthesis. Collectively, the cellular and molecular signature of Rett syndrome is consistent with its classification as an undergrowth type ASD, while MeCP2 duplication syndrome is more appropriately categorized as an overgrowth phenotype based on available data.

Autism Susceptibility Candidate 2

The autism susceptibility candidate 2 (AUTS2) was initially suspected to be associated with ASD found in a pair of monozygotic twins which had a *de novo* balanced translocation which disrupted the AUTS2 locus (Sultana et al., 2002). It has been reported that a subset of patients harboring deletions within exonic regions of AUTS2 present with a short stature, facial dysmorphism, and microcephaly (Beunders et al., 2013). Given these phenotypes, AUTS2 mutations are thought to lead to predominately "undergrowth" phenotypes. Since the initial identification of the AUTS2 locus, there have been over 30 additional individuals with cognitive disorders ranging from ASD to epilepsy, attention deficit disorder, intellectual disability and developmental delay harboring disruptions in both the coding and noncoding regions of the AUTS2 locus (Kalscheuer et al., 2007; Bakkaloglu et al., 2008; Glessner et al., 2009a; Elia et al., 2010; Pinto et al., 2010; Ben-David et al., 2011). Given that alteration of AUTS2 levels results in a myriad of neuronal deficits, it is not surprising that AUTS2 is highly expressed in developing brain regions important for higher order cognitive functions.

The function of AUTS2 had preliminarily been explored in the model system *Danio rerio* while the recent development of a mouse loss-of-function model has begun to elucidate the function of the *AUTS2* gene product. The function of AUTS2 has been recently addressed in animal models of perturbation in AUTS2. In zebrafish, reductions of AUTS2 by morpholinos knockdown results in microcephaly, reduced mobility and decreases in the number of neurons (Beunders et al., 2013; Oksenberg and Ahituv, 2013). The Auts2 KO mouse model also results in reduction in size and these mice also display developmental delays, motor deficits, and altered communications as assayed by pup ultrasonic vocalizations (USVs) (Gao et al., 2014). This microcephaly phenotype is also observed in mice and human patients with AUTS2 disruptions suggesting a critical role for AUTS2 in neurodevelopment and growth (Beunders et al., 2013).

Previous research has shown AUTS2 to be highly expressed in neurons. The cytoplasmic pool of AUTS2 has been shown to regulate neurite outgrowth and migration through the regulation of the Rho family of GTPases (Hori et al., 2014). Bidirectional alterations of AUTS2 levels in cortical neurons lead to opposing phenotypes in neurite growth. Cortical neurons which have shRNA knockdown of AUTS2 (introduced through in utero electroporation at E15.5 and cultured to DIV 2-6) display decreases in dendrite length and branch number whereas overexpression in primary hippocampal neurons (at DIV 4)

leads to increase in neurite elongation (Hori et al., 2014). Nuclear populations of AUTS2 have been shown to play a role in regulation of chromatin dynamics through its interaction with PRC1, highlighting its potential role in regulation of transcription (Gao et al., 2014). One interesting finding from Gao et al. hints at a potential interaction between AUTS2 and signaling pathways involved in growth. The authors find that AUTS2 binds to the promoter regions of factors implicated in growth including TSC2, IGFR, and k-Ras as seen through a ChIP-seq performed in mouse brains. This is suggestive of a mechanism by which disruption of AUTS2 could lead to disruption of neuronal growth and the "undergrowth" phenotype described in human patients and animal models of AUTS2 mutations. While current research has started to address the function of AUTS2 in neuronal growth and development, much work needs to be done to determine the molecular mechanism by which AUTS2 leads to the pathogenesis of autism.

Copy Number Variant Models of ASD

16p11.2 Copy Number Variation

CNV at the human chromosome 16p11.2 locus is among the most common risk variants associated with ASD, accounting for approximately 0.5–1% of all cases (Malhotra and Sebat, 2012). In fact, duplications and deletions in this ∼600 kB region of 16p11.2 have been linked to a number of neurodevelopmental and psychiatric conditions, including intellectual disability, schizophrenia, epilepsy, bipolar disorder, and obesity (Weiss et al., 2008; McCarthy et al., 2009; Shinawi et al., 2010; Walters et al., 2010; Zufferey et al., 2012). Several reports indicate that reciprocal phenotypes occur as a result of deletion or duplication of the approximately 27 protein-coding genes found in the affected region. Therefore, 16p11.2 CNVs present a unique model to explore the effects of altered dosage of genes within this region on underlying growth pathway correlates.

In humans, there is accumulating evidence supporting the presence of pro-growth phenotypes in deletion carriers and anti-growth features in duplication carriers. For instance, 16p11.2 microdeletion is linked to macrocephaly, whereas duplication of this locus has been associated with microcephaly (Shinawi et al., 2010). A recent study comparing global brain differences between child carriers of 16p11.2 deletion (8.1 ± 3 years) and duplication (7.8 ± 5 years) identified a strong dose-dependent influence of CNV on brain volume. In deletion carriers, global measures of gray and white matter volume, along with volumes of certain subcortical structures such as the thalamus, were found to be significantly elevated as compared to typically developing controls. In contrast, these measures were altered in the opposite direction in the case of duplication carriers (Qureshi et al., 2014).

CNVs at 16p11.2 also confer highly penetrant and opposing effects on body mass index (BMI). Parallel to the effects observed with head size, deletion of this region often results in early-onset obesity, while duplication is associated with significantly reduced postnatal body weight and BMI (Jacquemont et al., 2011). Importantly, a study that analyzed the co-occurrence of head size and BMI phenotypes observed a higher incidence of the 16p11.2 deletion among cohorts ascertained for both developmental delay and obesity (2.9%), as opposed to cohorts assessed for either outcome alone (0.6 and 0.4%, respectively) (Walters et al., 2010). A potential avenue for future investigation in mouse model systems is to test the involvement of common molecular pathways in both neuronal and somatic growth defects in 16p11.2 CNVs.

In order to systematically assess the effects of this CNV on development and behavior, two independent groups, Horev et al. and Portmann et al., utilized chromosome engineering approaches to develop mutant mouse models harboring deletion (*df*/+) and/or duplication (*dp*/+) of the conserved syntenic region on chromosome 7 (Horev et al., 2011; Portmann et al., 2014). Characterization of adult *df*/+ and *dp*/+ mutants has revealed dose-dependent effects on expression of most genes within the engineered interval, as well as opposing phenotypes in certain neuroanatomical measures, including mild reciprocal volumetric changes across multiple brain regions in adulthood (Horev et al., 2011).

However, currently available mouse models of 16p11.2 CNVs may not accurately recapitulate head and body size phenotypes observed in human carriers. Despite expected changes in expression of genes in the deleted interval, *df*/+mutants display decreases in postnatal body weight (Portmann et al., 2014; Pucilowska et al., 2015) and mild reductions in overall brain volume that persist into adulthood (Horev et al., 2011; Portmann et al., 2014; Pucilowska et al., 2015). Therefore, more detailed phenotypic characterization of 16p11.2 CNV mouse models is required in order to pinpoint the cause for this discrepancy.

The gene, *MAPK3*, which encodes ERK1 is located within the 16p11.2 region and has garnered interest with respect to molecular phenotypes associated with this CNV. However, there have been conflicting reports on the effect of 16p11.2 deletion, and consequent ERK1 deficiency, on overall ERK1/2 activity. When ERK1/2 activity is measured as a ratio of phosphorylated to total ERK1/2 levels, studies have reported contradicting findings. One group observed decreases in ERK1/2 activity, which were correlated with decreased overall protein synthesis (Tian et al., 2015). On the other hand, Pucilowska et al., found a paradoxical upregulation of ERK1/2 activation (Pucilowska et al., 2015). Inconsistencies in reports may necessitate the use of ERK1/2 reporters to accurately assay ERK1/2 catalytic function in mouse models of 16p11.2 CNVs. Elucidating ERK1/2 pathway readouts in 16p11.2 CNVs is further complicated by the fact that a gene known to function as a negative regulator of ERK1/2, known as major vault protein (MVP), is also found within the 16p11.2 locus (Liang et al., 2010). Therefore, further studies need to be conducted in order to ascertain the impact of 16p11.2 CNVs on readouts of ERK1/2 activity.

22q11.2 Copy Number Variation

Several reports have described the co-occurrence of autism in subjects with chromosome 22 abnormalities including trisomy 22, translocation 20/22, 22q11.2 deletion, 22q11.2 duplication, ring chromosome 22, and 22q13.3 deletion (Mukaddes and Herguner, 2007). In 22q11.2 microdeletion (22q11.2DS;

velocardiofacial syndrome, Digeorge syndrome), a syndrome characterized by either a 1.5 or 3 megabasepair deletion of 22q11, roughly one third of individuals develop a form of schizophrenia and a smaller fraction (∼10–20%) display developmental delays and learning disabilities characteristic of autism. In addition to microdeletion at the 22q11.2 locus, genome-wide assays for copy number variants have also identified significant enrichment of 22q11.2 duplication in unrelated ASD patients (Marshall et al., 2008; Glessner et al., 2009b).

Although neuroimaging studies of patients with 22q11.2DS report a wide range of abnormalities in both brain structure and function, which are consistent with neuronal undergrowth phenotypes, the literature remains relatively inconsistent regarding differences in specific neuronal regions or structures. Thus, direct correlations between these structural changes and behavior are unable to be made at this time. These different conclusions may be due to the use of different protocols for measuring or normalizing volumes in studies that should be replicated with larger cohorts (Karayiorgou et al., 2010). However, in a meta-review on 22q11.2DS, when Karayiorgou et al. examined those findings that have been replicated by at least two independent research groups in at least two separate and independent studies, they observed a pattern of neuroanatomical abnormalities associated with undergrowth phenotypes in patients with 22q11.2DS, both with and without psychosis (Karayiorgou et al., 2010). These changes include larger ventricles, reduced brain size, and volumetric reductions of parietal lobes, hippocampus, cerebellum, reduced cortical thickness, and lateral cortical thinning (Xu et al., 2010; Drew et al., 2011a). Recent data modeling the 22q11.2 deletion in rodents has revealed that a significant number of these anatomical findings from 22q11.2 patients, including impaired circuitry and reduced regional volumes of brain structures that are critical to autism, are reliably recapitulated in the brains of mice modeling this human disorder (Sigurdsson et al., 2010; Drew et al., 2011a; Ellegood et al., 2014).

The 22q11.2DS mouse displays neuronal undergrowth, dysregulated synaptic plasticity, and impaired neuronal circuit formation, which are associated with abnormal behavioral outcomes (Karayiorgou et al., 2010; Drew et al., 2011b; Ellegood et al., 2014); however, the precise genetic mechanisms underlying this phenotype remain unknown. This region of human chromosome 22q11 contains 16 protein coding genes, many of which have been shown to regulate cellular growth and proliferation, such as *TBX1*, *RTN4R*, and *ZDHHC8* (Yagi et al., 2003; Mukai et al., 2008; Borrie et al., 2012); however, for the purposes of this review, we will focus on another gene found within this locus, *DGCR8*, since deletion of *DGCR8* in mouse models has revealed neuronal undergrowth that is able to be rescued by enhanced trophic signaling.

DGCR8 positively regulates the maturation of miRNAs, a recently discovered class of small, highly evolutionarily conserved regulatory RNAs, which inhibit the translation of near-complementary target mRNAs (Han et al., 2006). Individuals with hemizygous expression of DGCR8, as found in 22q11.2 deletion patients, would thus have impaired miRNA biogenesis and reduced inhibition of target protein synthesis, while those with 22q11.2 microduplication will have increased DGCR8 gene dosage (Xu et al., 2010). In mice with DGCR8 haploinsufficiency, there is reduced basal dendritic complexity (fewer branch points, less intersections, and decreased overall dendritic length), decreased hippocampal neurogenesis, and significant cognitive and behavioral deficits, consistent with the neuronal undergrowth phenotype present in individuals with 22q11.2 microdeletion (Schofield et al., 2011; Ouchi et al., 2013). Although it remains unknown as to whether or not these neuronal growth deficits and behavioral abnormalities are a direct consequence of reduced miRNA levels and enhanced translation of these miRNA target genes, subsequent experiments revealed that many of these deficits can indeed be rescued by exogenous application of insulin-like growth factor 2 (Igf2), a gene that is reduced in DGCR8-deficient animals by an unknown mechanism (Ouchi et al., 2013). It should also be noted that while expression of a phosphomimetic DGCR8 (which mimics phosphorylation by kinases such as MAPK/ERK) induces a pro-growth miRNA signature in a heterologous cell line (Herbert et al., 2013), it remains unknown whether or not the phosphorylation status of DGCR8 could serve as a diagnostic biomarker for cases of autism characterized by aberrant growth.

Conclusions

Relevance for Future Clinical Research and Trials

A compelling avenue of investigation is to design therapeutics that effectively target pathways controlling neuronal growth in ASD. Recent studies have allowed us to make significant progress in understanding the nature of neuronal growth defects in ASD. It remains a significant question in the field as to whether growth defects are causal to the development and pathogenesis of ASD. There are two lines of evidence that suggest that this may be the case (i) growth abnormalities emerge early in the pathogenesis of the disease, and (ii) manipulations and interventions that target growth-related pathways not only ameliorate growth defects but also lead to improvements in behavior.

For therapies to yield maximal success, the initial identification and design should consider whether the form of ASD being treated could be generally classified as overgrowth or undergrowth. This sub-classification of growth phenotypes in ASD is critical as the same drug is unlikely to be effective under both circumstances. Aside from obvious growth defects such as macrocephaly and microcephaly, more subtle growth phenotypes might prove to be difficult to diagnose early in development. Ultimately, development of a biomarker that serves as a readout for growth status in ASD would be necessary in order to facilitate early detection and proper intervention. Possible development of biomarkers could include components of growth pathways highlighted in this review, such as ERK1/2, mTOR, and neurotrophic factors.

Relevance for Future Research Efforts

Proper growth of functional neural circuits requires highly orchestrated control of intracellular signaling cascades and gene expression. These changes in neuronal growth depend upon

the specific composition of gene regulatory networks present not only within each cell type, but within subcellular regions of a given neuron that give rise to the various neuronal processes, such as axons, dendrites, and spines, structures that are often dysregulated in forms of autism characterized by over- or undergrowth. Although CRE-driver lines have been highly informative for our understanding of cell type specific changes in behavior, gene expression, patterns of neuronal growth, and connectivity across various brain regions between wild-type mice and disease models, development of CRE-drivers requires a priori knowledge of the specific gene expression patterns unique to each cell type. Even within cells identified by such a CRE-driver system, there exists considerable heterogeneity in terms of gene expression. Each of the human brain's billions of neurons is unique not only in terms of its activity signature but also within the complex three-dimensional spatial environment of the brain that permits unique connectivity for each neuron. For these reasons, we predict that future single cell analyses of gene expression across ensembles of cells from wild-type controls and disease models will transform our understanding of these highly complex cells and gain better mechanistic insights into the signaling pathways that underlie over- or under-growth phenotypes in ASD.

Many large-scale ASD studies have now been conducted, which have included mixed patient populations, with variations in multiple critical factors including age, diagnosis, and symptom severity. However, it has remained difficult to extract meaningful conclusions from these studies, in part, since molecular changes are likely to be different among forms of ASD with differing underlying etiologies. Identifying common molecular pathways that are dysregulated among various genetic causes of ASD is an important step in effectively stratifying ASD into endophenotypes from which therapeutic responses can be more readily anticipated. Shrinking the heterogeneity of the disorder would also make the study of ASD more tractable, allowing investigations in monogenic mouse models to be more appropriately applied to other genetic forms of ASD. Utilizing overgrowth and undergrowth phenotypes (**Figure 1**) as a basis for this stratification is useful since these phenotypes are present across multiple genetic causes of ASD and occur early in the development of disease.

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MINI REVIEW
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From UBE3A to Angelman syndrome: a substrate perspective

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Angelman syndrome (AS) is a debilitating neurodevelopmental disorder that is characterized by motor dysfunction, intellectual disability, speech impairment, seizures and common features of autism spectrum disorders (ASDs). Some of these AS related phenotypes can be seen in other neurodevelopmental disorders (Williams, 2011; Tan et al., 2014). AS patients commonly carry mutations that render the maternally inherited *UBE3A* gene non-functional. Duplication of the chromosomal region containing the *UBE3A* gene is associated with ASDs. Although the causative role for *UBE3A* gene mutations in AS is well established, a long-standing challenge in AS research has been to identify neural substrates of UBE3A, an E3 ubiquitin ligase. A prevailing hypothesis is that changes in UBE3A protein levels would alter the levels of a collection of protein substrates, giving rise to the unique phenotypic aspects of AS and possibly *UBE3A* associated ASDs. Interestingly, proteins altered in AS are linked to additional ASDs that are not previously associated with changes in *UBE3A*, indicating a possible molecular overlap underlying the broad-spectrum phenotypes of these neurogenetic disorders. This idea raises the possibility that there may exist a "one-size-fits-all" approach to the treatment of neurogenetic disorders with phenotypes overlapping AS. Furthermore, while a comprehensive list of UBE3A substrates and downstream affected pathways should be developed, this is only part of the story. The timing of when UBE3A protein functions, through either changes in UBE3A or possibly substrate expression patterns, appears to be critical for AS phenotype development. These data call for further investigation of UBE3A substrates and their timing of action relevant to AS phenotypes.

Keywords: Angelman syndrome, neurodevelopmental disorders, autism, ubiquitin ligase, UBE3A

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Angelman syndrome (AS) is a neurodevelopmental disorder that affects one in 15,000 individuals (Williams et al., 2006). Angelman syndrome is characterized by lack of speech, cognitive impairments, unusually happy demeanor, motor deficits and seizures, among other symptoms (Bird, 2014). Notably, Angelman syndrome shows symptomatic overlap with multiple other neurodevelopmental diseases, including Rett syndrome and Pitt-Hopkins syndrome (Forrest et al., 2013). While the phenotypic overlap of these diseases is discussed elsewhere (Tan et al., 2014; Margolis et al., 2015), it is an intriguing hypothesis to consider that converging molecular mechanisms contribute to a subset of their underlying symptoms.

Maternal loss of chromosomal region 15q11-13 is one cause of Angelman syndrome. While there are multiple genes in this region, it became apparent that mutations in *UBE3A*, a paternally-imprinted gene, are sufficient for causing Angelman syndrome (Kishino et al., 1997; Matsuura et al., 1997). Phenotype severity is correlated with

the type of mutation, with the full deletion of 15q11-13 the most severe and point mutations in *UBE3A* less severe (Gentile et al., 2010; Valente et al., 2013). Moreover, while UBE3A is expressed off the maternal allele in mature neurons, it is biallelically expressed in most peripheral tissues, in glia, and in newly born neurons (Albrecht et al., 1997; Gustin et al., 2010; Judson et al., 2014). Despite this systemic reduction in UBE3A expression of AS individuals, much research has been focused on the central nervous system, ignoring peripheral contribution of reduced UBE3A expression to AS-associated phenotypes. Interestingly, chromosomal region 15q11-13 is found to be duplicated in 1–2% of all autism spectrum disorder (ASD) cases, providing additional evidence for the importance of this region in developing a functional nervous system (Cook et al., 1997; Sutcliffe et al., 1997). Indeed, duplications in the chromosomal region containing only UBE3A have been associated with developmental delay (Noor et al., 2015).

Mouse models with a maternally-inherited *Ube3a* deletion display many Angelman-like phenotypes, including learning and memory deficits, motor phenotypes, and seizures (Jiang et al., 1998; Miura et al., 2002). The phenotypes listed here are far from exhaustive, but have been reviewed elsewhere (Margolis et al., 2015). These phenotypes are only present when the deletion is maternally-inherited, with little to no phenotype in the paternally-deleted animals. In mouse models, not only are reductions in UBE3A protein expression capable of inducing neurological deficits, but duplications in UBE3A also show autism-like phenotypes, such as social and learning and memory deficits (Smith et al., 2011). The combination of mouse and human data suggests that UBE3A plays a fundamental and critical role in regulating pathways important for autism-like disorders.

UBE3A is an E3 ubiquitin ligase that functions to conjugate ubiquitin groups to a unique set of proteins (Scheffner et al., 1993; Huang et al., 1999). Ubiquitinated proteins are then, generally, targeted for degradation through the ubiquitin-proteasome system (Ciechanover and Schwartz, 1998). Since mutations in the catalytic domain of UBE3A are sufficient for development of Angelman syndrome (Kishino et al., 1997; Matsuura et al., 1997; Cooper et al., 2004), the lack of ubiquitination and degradation of UBE3A substrates is predicted to increase these substrate protein levels. Conversely, increases in UBE3A are expected to decrease levels of its substrates. It is hypothesized that this alteration in substrate levels contributes to the variety of phenotypes associated with AS and, potentially, ASDs.

Given the many neurological phenotypes associated with changes in UBE3A expression, one major task in the field has been to identify brain-derived targets as disease-relevant substrates. A previously published substrate of UBE3A is Pbl/ECT2, a RhoA guanine nucleotide exchange factor (RhoA GEF), although the contribution of AS phenotype has not been interrogated (Reiter et al., 2006). Another published substrate of UBE3A is the negative synaptic regulator Ephexin5, another RhoA GEF (Margolis et al., 2010). By reducing Ephexin5 in AS mice, a recent study found that Ephexin5 does not contribute to AS related cortical and cerebellar phenotypes such as vocalization deficits, seizure activity, or motor deficits (Mandel-Brehm et al., 2015). These results are not surprising considering that, in the brain, high Ephexin5 expression is restricted to hippocampus compared to surrounding brain regions when measured by *in situ* hybridization (Margolis et al., 2010). Another substrate reported recently is GAT1, a GABA transporter that is upregulated in the absence of UBE3A in the cerebellum. Treatment with THIP, a selective extrasynaptic $GABA_A$ receptor agonist showed the capacity to rescue electrophysiological and motor deficits (Egawa et al., 2012). Arc, a cytoskeleton-associated protein known to regulate trafficking of AMPA receptors to the membrane, is reported to be a substrate of UBE3A (Greer et al., 2010). Consistent with Arc's role in contributing to AS related phenotypes recent data demonstrate that reduction of Arc levels is capable of ameliorating recovery time after audiogenic seizures without rescue of ultrasonic vocalizations or motor behavior deficits (Mandel-Brehm et al., 2015). Despite several groups having observed varying results regarding Arc's status as a direct substrate of UBE3A (Greer et al., 2010; Kühnle et al., 2013; Mabb et al., 2014; Mandel-Brehm et al., 2015), these data establish reduction in Arc levels are capable of mitigating symptoms. Given that these substrates contribute to only a subset of phenotypes associated with AS, they cannot be the sole enactors of phenotypic change in AS.

Despite this paucity of confirmed substrates, many non-substrates, i.e., proteins which are reported to not directly be regulated by UBE3A *in vivo* in mammalian tissue, have been shown to be altered in AS mouse model brains, either at the level of protein expression or activity, including α1-Na^+/K^+-ATPase and CaMKII phosphorylation (Weeber et al., 2003; van Woerden et al., 2007; Kaphzan et al., 2013; Mandel-Brehm et al., 2015). It should be noted that the drosophila homolog to α1-Na^+/K^+-ATPase was identified as an interactor with dUBE3A (Jensen et al., 2013), however this result was not replicated in mouse brain (Kaphzan et al., 2011). Rescue experiments modulating these misregulated proteins were done using genetic deletion of the target proteins or modification of CaMKII phosphorylation sites. In these cases, a subset of behavioral phenotypes, mostly related to learning and memory, were ameliorated (van Woerden et al., 2007; Kaphzan et al., 2013; Mandel-Brehm et al., 2015). Despite the amelioration of certain phenotypes, none of these studies reported rescuing all phenotypes observed in AS mouse models. While the molecular understanding for these disrupted pathways and their rescuing capacity remains to be fully elucidated, these data indicate that the development of AS can arise from a myriad of secondary and tertiary changes downstream of altering the expression of UBE3A (**Figure 1**). Interestingly, some of the proteins shown to be misregulated in AS and/or interact with UBE3A have also been shown to be disrupted in other neurodevelopmental disorders, including ASDs in which UBE3A is not reported to be misregulated (**Table 1**). These data raise the possibility that phenotypic overlap between AS and other neurodevelopmental disorders can be explained by the molecular overlap in downstream pathways important for nervous system development. Such overlapping molecular discoveries will be the first targets toward developing "one size fits all" type therapies

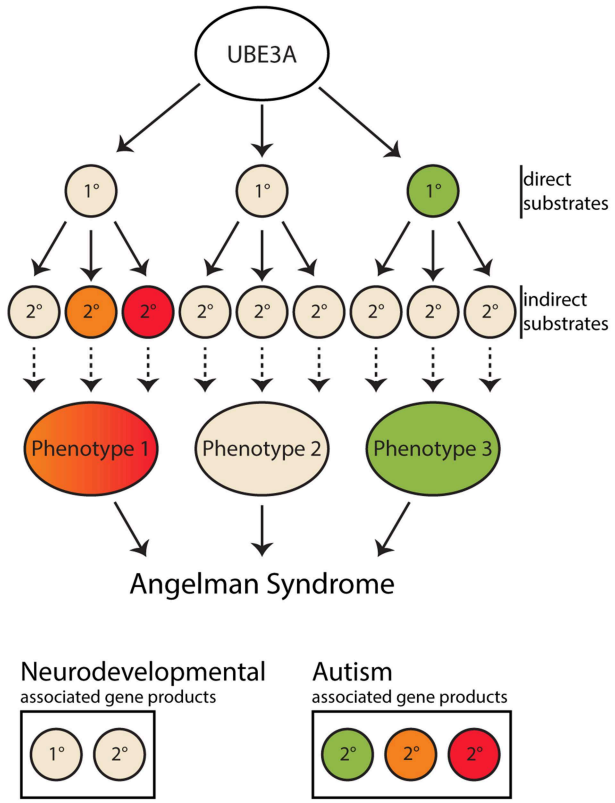

FIGURE 1 | Diagram of UBE3A substrate cascade to Angelman syndrome. Angelman syndrome is caused by the pathological loss of maternal UBE3A protein expression, resulting in the loss of regulation of its pool of downstream substrates. UBE3A direct interactors/substrates are labeled as a 1° inside a circle. Indirect substrates/changes are labeled as 2° inside a circle. Gene products associated with neurodevelopment are in tan colored circles and associated with Autism spectrum disorders are colored. Following the loss of UBE3A changes to these 1°substrates are the initiators of cascade of events which lead to 2° changes. These 2° changes can be in the form of protein-protein interactions, cell biological, electrophysiological, etc. The convergence of these 2° and downstream changes produce phenotypes characteristic of Angelman syndrome. Given some proteins known to be misregulated in ASDs are primary or secondary interactors in this UBE3A-dependent pathway, as indicated by the colored substrates, the phenotypic overlap between Angelman Syndrome and autism spectrum disorders it is not surprising. Through the study of UBE3A substrates, more overlapping molecular changes underlying shared phenotypes may become apparent.

TABLE 1 | Possible mammalian UBE3A substrates.

Substrates/Interactors	Disease	Citation
ANXA1	ASD	Shimoji et al., 2009; Correia et al., 2014
AR	ASD	Khan et al., 2006; Henningsson et al., 2009
Arc	AS, Fragile X	Park et al., 2008; Greer et al., 2010; Mandel-Brehm et al., 2015
CDKN1B	ASD	Mishra et al., 2009; Grey et al., 2013
DLG1	ASD	Matsumoto et al., 2006; Li et al., 2014
Ephexin5	Epilepsy	Margolis et al., 2010; Veeramah et al., 2013
ESR2	Asperger syndrome, ASD	Picard et al., 2008; Chakrabarti et al., 2009
Herc2	AS, ASD	Kühnle et al., 2011; Puffenberger et al., 2012; Harlalka et al., 2013
mGluR5	AS, Fragile X	Dölen et al., 2007; Pignatelli et al., 2014
SOD1	ASD	Mishra et al., 2013; Kovac et al., 2014
TSC1	AS, TS, ASD	Smalley, 1998; Sun et al., 2015
TSC2	AS, TS, ASD	Smalley, 1998; Zheng et al., 2008; Sun et al., 2015
UBE3A	AS, ASD	Schwarz et al., 1998; Nurmi et al., 2001

AS, Angelman Syndrome; TS, Tuberous sclerosis; ASD, Autism spectrum disorder.

with the potential of treating similar phenotypes of many neurodevelopmental disorders associated with AS.

While a comprehensive list of UBE3A substrates and downstream effected pathways should be developed, this is only part of the story. The functional interaction between UBE3A and these pathways will likely vary based on both the expression profile of UBE3A and the pathways in question.

Both the total protein level and subcellular localization of UBE3A is altered over development in neurons (Dindot et al., 2008; Williams et al., 2010; Judson et al., 2014). As previously mentioned, pyramidal cells in both cortex and hippocampus express UBE3A from the maternal allele. In intact brains, maternal UBE3A expression is much higher in mature neurons as opposed to newly born neurons, both early in development as well as in adult-born neurons (Dindot et al., 2008; Judson et al., 2014). Over time, this adult level of expression is reduced in the mammalian brain during advanced aging (Williams et al., 2010). Despite this robust age-related decrease in UBE3A expression, older individuals do not develop Angelman syndrome. This seemingly contrary idea highlights the possibility of separating UBE3A functions between the development of the CNS and in the adult CNS. Not only are levels of UBE3A consistently reported as changing across development, the localization of UBE3A shifts to the nucleus as development progresses. Maternal UBE3A-YFP shows a shift toward nuclear and synaptic localization later in development (Judson et al., 2014). To add another layer of complexity, neuronal activity in culture leads to increased localization of UBE3A to the nucleus and plasma membrane (Filonova et al., 2014). Since this change in localization is also activity-regulated, this indicates that UBE3A interaction with specific substrates will be activity-regulated as well. Due to neuronal activity and compartment-specific localization we suggest that sensitivity of each individual substrate to UBE3A will be altered based on these parameters, making it possible to miss functionally-relevant interactions in development- or activity-dependent scenarios. In fact, measurement of UBE3A's activity toward

its substrates in these developmentally-restricted windows, in various compartments, and under alternative neuronal activity scenarios has not been done and could elucidate a more complex layer of regulation by UBE3A not yet appreciated.

The changes in UBE3A localization, expression, and possibly activity toward distinct targets over development hints that the timing of treatment in AS will be crucial to the success of the intervention. The importance of timing is further emphasized by experiments which re-express UBE3A at different times in development in AS mouse models: despite correcting the loss of UBE3A, the number and particular subset of phenotypes that are ameliorated is variable. It is difficult to ignore the potent role timing of UBE3A expression may be playing in neuronal development.

Recent work from the Elgersma lab has returned the wildtype UBE3A allele to a mouse model of AS at different times in development using an inducible Cre model (Silva-Santos et al., 2015). The work indicates that later and later UBE3A expression resulted in fewer rescued phenotypes. For instance, marble burying and rotarod deficits were returned to wild type levels when UBE3A was re-expressed at birth. However, UBE3A no longer rescued these phenotypes when expressed in adolescence or adulthood. Interestingly, despite the lack of behavioral rescue, LTP in the Schaffer collateral was reinstated in both juveniles and adults. A similar experiment to reactivate UBE3a expression utilized the mechanism of *UBE3A* imprinting to induce UBE3A expression from the paternal allele. The paternal allele is silenced by the expression of an antisense RNA (*UBE3A-ATS)* that is highly expressed to prevent overexpression of UBE3A. When this transcript is silenced, paternal expression is reinstated up to 90% of control level. Utilizing antisense oligonucleotides to disrupt the *UBE3A-ATS* and induce paternal expression of UBE3A protein, some phenotypes, such as fear conditioning and body weight, were ameliorated even upon treatment as juveniles. However, many phenotypes, including the robust and reproducible motor phenotypes and marble burying, were not rescued (Meng et al., 2014). Pharmacological methods of expressing paternal UBE3A, while successful at initiating protein expression, have not been characterized to ameliorate Angelman syndrome phenotypes *in vivo* (Huang et al., 2012; King et al., 2013; Powell et al., 2013).

These studies, among others, have illustrated the importance of the timing of UBE3A expression in the intact animal. While re-expression of UBE3A later in development is capable of positively impacting synaptic plasticity, it less effectively rescues behavioral deficits. Such results question whether the clinically relevant phenotypes in AS mice are those related to cellular or behavioral changes. Given the complex nature by which UBE3A affects nervous system development it is likely that studying UBE3A interactions and downstream effects relevant to AS pathogenesis can be best understood through *in vivo* studies. Systems such as neuronal culture are more apt to answer questions about general UBE3A function in the neuron, as developmental dependent UBE3A effects are stripped of meaning in context of *in vivo* AS pathogenesis.

UBE3A is a protein capable of mediating a variety of effects due to its consistent expression across many cell types and ages. However, while all functions are potentially important for fulfilling UBE3A's role in the body, only a subset of these functions is especially relevant to AS. Determining the latest possible point at which UBE3A re-expression is sufficient to rescue all phenotypes, including behavior, cell biology, and electrophysiology, will define the crucial window for UBE3A's function in AS. This window is the point in which UBE3A is formative for AS. Therefore, the large list of all UBE3A substrates can be narrowed to a short list of substrates altered in this time window and thus more likely to be AS-relevant. Perhaps more importantly, it is in this time window, and not in others, that research should be focused in order to determine the most disease-relevant pathways and mechanisms. This approach for studying UBE3A contribution to AS provides an opportunity to explore the overlap with other ASDs, as it is likely that the developmental restrictions on UBE3A's contribution to AS relevant substrates are similar to constraints present in other ASDs pathogenic proteins.

Mouse models of AS are an important tool for determining the UBE3A interactors and mechanisms in this critical window of AS development. As is well known, studying AS in mouse models is insufficient for complete understanding the human disease. In order for the UBE3A-dependent mechanisms elucidated in mice to have meaning, they must be translated from mouse to humans, both in respect to subtle changes in mechanism and to developmental age at which these mechanisms are occurring.

In humans, many changes likely occur from the loss of UBE3A at conception to the point at which an affected individual is diagnosed, and we are very much unaware of the true extent of these alterations. Therefore, a key question is: when is AS actually developing in humans? Work done in mouse models will give a framework for discussing these questions in the context of human AS, but the timing of human AS still needs to be established. Reports up to this point indicate that AS begins in early in life (Bird, 2014), and one hypothesis is that this early development of AS in mouse will translate to early development of AS in humans. Given that AS may be taking hold early in human development, early treatment will be crucial to efficacy of potential interventions. Therefore, improving methods of AS detection and removing the delay to diagnosis will be a key component to taking advantage of these mechanisms for the successful treatment of AS.

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REVIEW
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Oxytocin and vasopressin: linking pituitary neuropeptides and their receptors to social neurocircuits

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Oxytocin and vasopressin are pituitary neuropeptides that have been shown to affect social processes in mammals. There is growing interest in these molecules and their receptors as potential precipitants of, and/or treatments for, social deficits in neurodevelopmental disorders, including autism spectrum disorder. Numerous behavioral-genetic studies suggest that there is an association between these peptides and individual social abilities; however, an explanatory model that links hormonal activity at the receptor level to complex human behavior remains elusive. The following review summarizes the known associations between the oxytocin and vasopressin neuropeptide systems and social neurocircuits in the brain. Following a micro- to macro- level trajectory, current literature on the synthesis and secretion of these peptides, and the structure, function and distribution of their respective receptors is first surveyed. Next, current models regarding the mechanism of action of these peptides on microcircuitry and other neurotransmitter systems are discussed. Functional neuroimaging evidence on the acute effects of exogenous administration of these peptides on brain activity is then reviewed. Overall, a model in which the local neuromodulatory effects of pituitary neuropeptides on brainstem and basal forebrain regions strengthen signaling within social neurocircuits proves appealing. However, these findings are derived from animal models; more research is needed to clarify the relevance of these mechanisms to human behavior and treatment of social deficits in neuropsychiatric disorders.

Keywords: oxytocin, vasopressin, vasopressin receptor subtype 1a, OXTR, autism

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Introduction

Oxytocin and arginine vasopressin (AVP) are neuropeptides synthesized in the hypothalamus and secreted from the posterior pituitary gland. Oxytocin was first described for its important role in stimulating uterine contractions and milk let down after birth, while AVP is central to water homeostasis by regulating urine concentration at the level of the kidney. In addition to these physiologic functions, both peptides are now understood to mediate numerous social behaviors in mammals.

The role of the oxytocin and vasopressin systems in social functioning has developed out of a large body of animal research, focusing primarily on rodents. This literature has been extensively reviewed elsewhere (Wang et al., 1998; Insel, 2010). For example, oxytocin has been

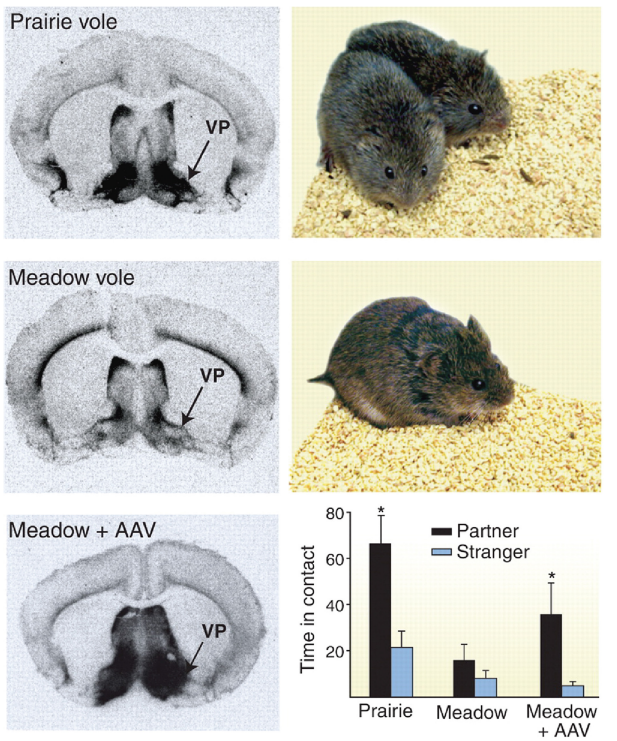

FIGURE 1 | Example of receptor autoradiography study in voles showing higher density of vasopressin receptor 1a staining in monogamous prairie voles (top) as compared to polygamous meadow voles (middle). When receptor expression was increased using an adeno associated viral (AAV) vector, polygamous meadow voles demonstrated more preferential contact with their partners **(bottom).** Figure reproduced with permission from *Science*, (Donaldson and Young, 2008) adapted from research presented by Lim et al. (2004). *$p < 0.05$.

shown to be an important regulator of maternal behavior in female rats, with central injection of this molecule triggering protective and nursing behavior toward pups. Similarly, both peptides mediate affiliative behavior in prairie voles, although effects differ by sex. In male prairie voles, for example, manipulation of this system with AVP receptor antagonists attenuates preferential association with a partner after mating, while central administration of this peptide triggers pair bonding even in the absence of mating behavior (see **Figure 1**) (Wang et al., 1998; Cho et al., 1999). Accordingly, there is growing interest in oxytocin and vasopressin as modulators of social behavior and functioning in humans; either as a potential explanatory factor for social differences in typically developing individuals, or as a possible precipitant of and/or treatment for social deficits in neurodevelopmental disorders such as autism spectrum disorder (ASD).

Indeed, there is ample research to suggest to that common genetic variation in the receptor structure for these molecules may impact on some aspects of social functioning in humans; additionally, central administration of oxytocin seems to encourage certain social behaviors and cognitive capacities (Meyer-Lindenberg et al., 2011). Functional neuroimaging studies further support a link between these neuropeptides and activity in specific brain regions implicated in social communication and behavior. It remains unclear whether disruption of the oxytocin/vasopressin system contributes to the etiopathogenesis of ASD, however. A single case report describes a family in which a rare mutation in the oxytocin receptor was detected in an individual with ASD (Gregory et al., 2009), and a recent meta-analysis suggests certain common genetic variants may be over represented in autism (Loparo and Waldman, 2014). However, new research has shown that both peripheral oxytocin levels, and common genetic variation in the oxytocin receptor affect social communication abilities in family members of individuals with ASD as well, irrespective of diagnosis (Skuse et al., 2014; Parker et al., 2014). Accordingly, dozens of trials in which oxytocin or vasopressin are manipulated with pharmacotherapy are underway, showing early evidence as a potential treatment for social deficits (reviewed by Baribeau and Anagnostou, 2014). Despite this growing interest, a model that links the molecular and cellular activity of these peptides to social neurocircuits detectable on neuroimaging remains elusive.

Accordingly, the following review intends to summarize the current literature with respect to underlying mechanisms via which neuropeptides affect social processes in humans, focusing on the oxytocin and vasopressin systems. We aim to provide a sequenced narrative review of research evidence, following a micro- to macro- level trajectory. Specifically, we begin by summarizing what is known about the synthesis and secretion of these peptides, followed by a discussion on the distribution, structure, and activity of their respective receptors. Next, current models associating these peptides to specific effects on neurons, neurotransmitters, and microcircuits will be reviewed. We will then correlate this research with current functional neuroimaging literature examining responses to experimental manipulation of these systems. Potential implications for, and associations with ASD are included throughout. The aim is to provide a non-technical overview of this field, to synthesize results from overlapping yet distinct areas of science, and to identify knowledge gaps in need of further exploration.

Oxytocin and Vasopressin Molecules: Synthesis and Release

Oxytocin and vasopressin are related pituitary non-apeptides; they consist of nine amino acids in a cyclic structure. These molecules differ by only two amino acids, at position 3 and 8 (isoleucine and leucine in oxytocin are replaced by phenylanine and arginine in vasopressin, respectively). Related peptides are detectable in all vertebrate species and are thought to have evolved from similar parent compounds. Both oxytocin and vasopressin are coded in a precursor form on chromosome 20 (Gimpl and Fahrenholz, 2001).

Both molecules are synthesized in overlapping regions of the hypothalamus, primarily in large magnocellular neurons situated in the supraoptic and paraventricular nuclei. These neurons project their axons to the posterior pituitary, where the peptides are stored in vesicles until action potentials trigger their release into the peripheral circulation (for example during labor, or

imbalance of water homeostasis) (Ludwig and Leng, 2006) (see **Figure 2**). Oxytocin and vasopressin molecules that have been released in this way, through the axon projections, are for the most part prevented from re-entering the central nervous system (CNS) via the blood brain barrier; however, very small amounts of peripherally administered peptides (e.g., < 1%) do appear to cross over into the cerebral spinal fluid (CSF) (Mens et al., 1983; Opacka-Juffry and Mohiyeddini, 2012). It has been shown that oxytocin and vasopressin concentrations can be up to 1000X higher in the brain than the peripheral blood, indicative of a potentially important role for both molecules in the central nervous system (CNS) (Ludwig and Leng, 2006). While earlier studies suggested potentially lower oxytocin and vasopressin levels in the plasma of children with ASD as compared to typical children (Modahl et al., 1998; Al-Ayadhi, 2005), subsequent research has shown that plasma oxytocin levels tend to be similar within members of the same family, irrespective of a diagnosis of autism, although do correlate with social communication abilities overall (Parker et al., 2014). Of note, the methodology used to quantify plasma oxytocin levels in humans has varied across studies, which may have affected the reliability of results (Szeto et al., 2011).

Oxytocin has a single receptor (OXTR) encoded on chromosome 3, whereas vasopressin has three types of receptors, AVPR1a and AVPR1b (also called V3) and V2, on chromosome 20 (De Keyzer et al., 1994; Thibonnier et al., 2002). AVPR1a is present primarily on vascular smooth muscle, in the liver, and on neurons; AVPR1b/V3 is detectable in the anterior

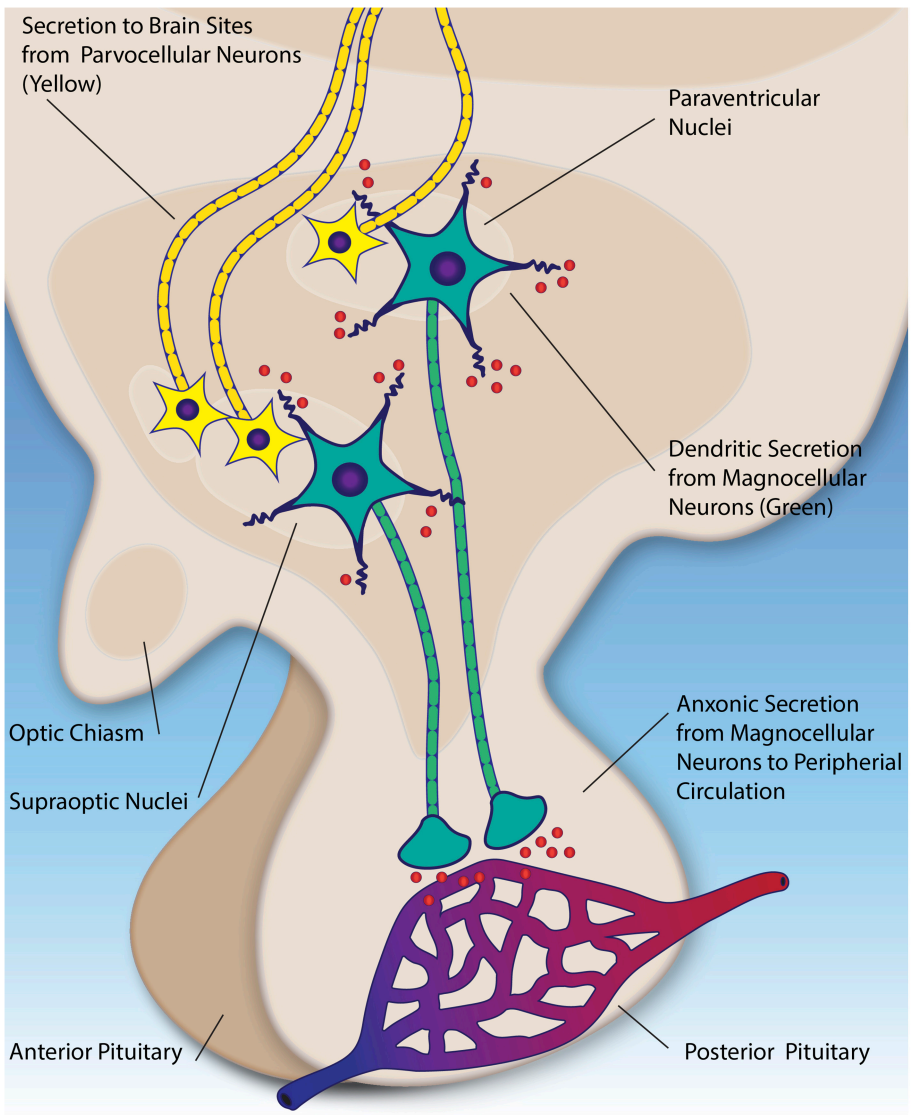

FIGURE 2 | Parvocellular neurons (yellow) secrete oxytocin and vasopressin (red) to numerous brain regions, including the amygdala, brainstem, and anterior pituitary. Magnocellular neurons (green) in the hypothalamic nuclei secrete oxytocin and vasopressin into the peripheral circulation via the posterior pituitary (axonic secretion). Additionally, they secrete these peptides into the extracellular fluid the hypothalamus (dendritic secretion).

pituitary; and the V2 receptor is found primarily in the kidneys. Information on the central distribution, structure, and function of these receptors will be discussed further in subsequent sections. Outside of the brain, oxytocin receptors are detectable in humans in high concentrations in the uterus, gradually increasing in number over the course of pregnancy. Tissue taken from hysterectomy or cesarean section at different gestational time points in pregnant women has shown a significant and rapid up regulation (e.g., 200-fold) of oxytocin receptor expression around the onset of labor, facilitating uterine contractions (Fuchs et al., 1984). Many other tissues and organs, including ovaries, testis, mammary glands, kidneys, thymus, pancreas, adrenal, and even adipose tissue, have been shown to express oxytocin and/or vasopressin receptors in different species; some studies even suggest exogenous synthesis of oxytocin can take place at certain peripheral sites (see Gimpl and Fahrenholz, 2001 for a detailed review). As well, both oxytocin and vasopressin can exert effects on the cardiovascular system by affecting blood pressure, vasodilation, diuresis, and water intake (Pittman et al., 1982; Petersson et al., 1996). This finding may be of relevance in ASD given emerging evidence of aberrant autonomic functioning and heart rate reactivity in this condition (Ming et al., 2005; Kushki et al., 2013).

The synthesis and release of oxytocin and vasopressin in the CNS is of primary importance for models associating these peptides with social behavior. Our knowledge of these processes stems almost exclusively from research in animal models, primarily in rodents (see Ludwig and Leng, 2006, for an extensive review). Central release appears to be mediated through two pathways, distinct from the peripheral secretion described above (see **Figure 2**). First, both peptides are produced in in hypothalamic neurosecrotor neurons (parvocellular neurons), whose axons project to the anterior pituitary and other brain regions in rodents (Castel and Morris, 1988; Ludwig and Leng, 2006). Parallel to this, the magnocellular neurons (mentioned in the previous section) also have been shown to secrete oxytocin and vasopressin from their dendrites (as opposed to the axons) in the hypothalamus (Pow and Morris, 1989). This mechanism appears to be separate and distinct from the axonal secretion in the posterior pituitary, and potentially contributes feedback to the overall system (Ludwig et al., 2002). Following secretion, these peptides are thought diffuse throughout the extracellular space, serving a neuromodulatory effect on surrounding brain tissue (Landgraf and Neumann, 2004).

Across histological studies, many investigators have highlighted extensive axonic and dendritic projections extending from the oxytocin and vasopressin neurons in the hypothalamus. In mice, for example, dendrites arising from hypothalamic magnocellular neurons were shown to display a corkscrew morphology, projecting posteriorly toward the third ventricle, and also extending beneath the pia layer of the base of the brain (Castel and Morris, 1988). Similarly, oxytocin neurons in the paraventricular nucleus of the hypothalamus have been shown to project axons long distances across the basal forebrain in rats, with extensive branching and three dimensional orientations extending potentially as far as the nucleus accumbens, amygdala, hippocampus, and into the somatosensory cortex (Knobloch et al., 2012; Grinevich et al., 2015). Interestingly, these axonal projections are only detected in adult animals, and are absent in prenatal and early postnatal studies. Dendritic secretion has been shown to be of central importance in animal models of social stress (Ludwig and Leng, 2006), while axonic secretion has been shown to effect fear responses in mice (Knobloch et al., 2012). As summarized in **Table 1**, oxytocin receptors have been detected on cell fibers in the hypothalamus, brainstem, and throughout the limbic system in human brains (Boccia et al., 2013).

In animal models, the central vs. peripheral secretion of these peptides have been shown to follow distinct timelines in their responses, with brain levels peaking later, and lasting for longer than elevations in blood levels. For example, injection of a hypertonic solution into the peripheral circulation of rats triggered elevations in peptide levels that peaked at 150 min in the CNS as opposed to 30 min in the periphery (Ludwig et al., 1994). When either oxytocin or vasopressin was injected into the rat peripheral circulation, central elevations in the CSF were also shown to persist for much longer, and were cleared more slowly than peripheral levels (Mens et al., 1983). In order to deliver oxytocin directly to the central nervous system for therapeutic uses in ASD, intranasal sprays have been developed which show rapid rises in CSF oxytocin levels within 10 min of application in macaques (Dal Monte et al., 2014). In humans, single intranasal administration of oxytocin led to elevated plasma levels for approximately 90 min afterwards (Gossen et al., 2012). In primates, levels of both oxytocin and vasopressin have been shown to fluctuate over the course of the day, with different patterns in the plasma vs. the CSF; specifically, CSF levels tended to correspond with periods of daylight, while plasma levels did not (Perlow et al., 1982).

Interestingly, oxytocin and vasopressin neurons have receptors for their own secreted neuropeptides on their cell surfaces, and are able modulate their own release, respectively, without necessarily triggering action potentials (Gouzenes et al., 1998; Ludwig et al., 2002). Evidence derived from mouse models indicates that a transmembrane glycoprotein called CD38 must be present to facilitate depolarization-induced oxytocin secretion in the pituitary, and that blockade of this molecule interrupts mouse maternal and social behavior (Jin et al., 2007; Lopatina et al., 2012). Specific common genetic variants in CD38, and reduced CD38 expression on lymphoblastoid cells, have been associated with ASD (Higashida et al., 2010; Lerer et al., 2010). Once released via either central pathway, the peptides then diffuse throughout the extracellular space, and can be detected across the brain, where they act on their respective receptors (Ludwig and Leng, 2006). The mechanism of action of peptide binding and central distribution of these receptors will be discussed in the next sections.

Neuropeptide Receptors: Structure and Function

Both oxytocin and vasopressin receptors are G-protein coupled, each with seven transmembrane alpha-helices connected via extra and intracellular loops (Kimura et al., 1992) (for a detailed

TABLE 1 | Distribution of oxytocin receptors in the central nervous system.

	Non-human Primates				Humans	
References	Boccia et al., 2001	Boccia et al., 2007	Freeman et al., 2014a	Freeman et al., 2014b	Loup et al., 1989, 1991	Boccia et al., 2013
Species	Macaque	Macaque	Macaque	Titimonkey	Human	Human
Method (Right)	Monoclonal antibody	Oxytocin antagonist	Autoradiography	Autoradiography, mRNA	Autoradiography	IHC
Brain regions (Below)						
CORTICAL AREAS						
Frontal cortex		+			–	±
Temporal cortex					–	–/–
Parietal cortex		–				–/–
Cerebellar cortex		–			–	
Occipital cortex		–		++		
Retrosplenial cortex/Subcallosal area						+/+
Cingulate cortex						+++/+
BASAL FOREBRAIN						
Diagonal band of Broca					+++	+++
Basal nucleus of Meynert			+++	++	+++	
Septal nuclei	++	+++			+++	+++/+++
BASAL GANGLIA						
Caudate nuclei		–			–	
Globus pallidus					+	
Nucleus accumbens				–	–	–/–
Putamen					–	
LIMBIC SYSTEM						
Amygdala		+		–	–	+++/+++
Hippocampus		++			–	–/–
Parahippocampus/ Hippocampal formation				+++	–	+++/+++
Olfactory system					–	+/+
Thalamus				++		
HYPOTHALAMUS						
Anterior Hypothalamus	++	+++			++	++/++
Posterior Hypothalamus					++	+++/+++
Tuberal Hypothalamus			+++		+	++/–
BRAIN STEM						
Midbrain		–	+++		++	–/–
Pons		–	+++	++	+	–/–
Medulla		–		++	++	+++/+++

Cell bodies/Cell fibers. IHC, immunohistochemistry; +++, high density binding; ++, moderate density binding; +, low density binding; –, no binding. Boxes left blank not explicitly described in manuscript. Note that tracts and nuclei have been grouped by brain region. See respective reference for more details.

review of the receptor structure see (Zingg and Laporte, 2003) or Gimpl and Fahrenholz, 2001). There is cross reactivity in binding of each peptide with its respective receptor; oxytocin binds to the oxytocin receptor with only 10x greater affinity than vasopressin, for example (Kimura et al., 1994). The strength of binding of the neuropeptide into its specific binding pocket can be manipulated by mutational analyses; for example, single amino acid substitutions at key structural areas can significantly reduce or eliminate peptide binding (Hausmann et al., 1996; Postina et al., 1996). A natural example of this occurs in nephrogenic diabetes insipidis, where a point mutation affecting arginine disrupts the V2 receptor structure, resulting in an inability to concentrate urine in affected individuals (Bichet et al., 1993; Birnbaumer et al., 1994; Rosenthal et al., 1994). The genetic sequences coding for mouse, rat, and human oxytocin/vasopressin receptor genes are conserved across species, with over 80% identical amino acid residues. There are subtle differences in receptor function across species, however. For example, in mice and rats, there are two N-glycosylation sites in the extracellular NH2 region, while in humans and primates, there appear to be three (Gimpl and Fahrenholz, 2001). *In vitro* studies suggest that the binding affinity of endogenous

oxytocin for its receptor is comparable in humans, rats, and mice. However, there is significant variability the affinity constants of the synthetic oxytocin analog TGOT for OXTR across different species (Busnelli et al., 2013). As such, animal models using synthetic OXTR agonists may not necessarily reflect human physiology. Although numerous studies have associated common genetic variation in the human oxytocin receptor with ASD or social deficits (Bakermans-Kranenburg and van Ijzendoorn, 2014; Loparo and Waldman, 2014), the impact of these genetic changes on the structure and function of the oxytocin receptor *in vivo* is unclear at the present time.

The neuropeptide binding to its respective receptor triggers a conformational change in the receptor structure, leading to downstream activation of G proteins, and subsequent Ca^{2+} release from intracellular stores. Potential downstream effects include phosphorylation of intracellular proteins, activation of nitric oxide synthase leading to vasodilation, smooth muscle contraction, gene transcription, and increased excitability of neurons. The specific effects of receptor activation seem to vary by organ and tissue (Zingg and Laporte, 2003). For both OXTR and V2, there is evidence for rapid receptor desensitization, via receptor internalization. *In vitro* studies suggest this desensitization effect may be present minutes to hours after exposure to the peptide, and can result in >50% internalization of receptors (Gimpl and Fahrenholz, 2001). Internalized receptors are not degraded, however; approximately 85% of receptors return to the cell surface within 4 h (Conti et al., 2009). This internalization process may impact on social functioning. In mice, for example, chronic twice-daily administration of intranasal oxytocin reduced oxytocin receptor expression in the brain, and decreased some social behavior, while acute administration increased social behaviors, although findings varied by dose (Huang et al., 2014). The mechanism driving receptor internalization appears to involve receptor congregation with beta-arrestin into clathrin-coated pits (Oakley et al., 2001). Both oxytocin and vasopressin receptors show capacity to form hetero- homo- or oligo-dimers *in vitro*; it is unclear to what extent formation of receptor complexes is a biologically important process *in vivo* (Cottet et al., 2010).

Mediators of Peptide and Receptor Transcription, Synthesis, and Secretion

Several mediators of transcription, synthesis, and secretion/expression of the oxytocin and vasopressin peptides and their respective receptors have been described in various species (Burbach et al., 1995; Jorgensen et al., 2002; Weiser et al., 2008). Specifically, activation of transcriptional promoters upstream of the oxytocin or vasopressin genes via estrogen receptor binding, thyroid hormone receptor binding or retinoic acid receptor binding has been shown *in vitro* (Richard and Zingg, 1990, 1991; Shapiro et al., 2000; Pak et al., 2007). Sex steroids, including estrogen, progesterone, and testosterone, and pro-inflammatory cytokines, such as interleukin-6 and interleukin 1-beta, have been shown to impact on OXTR expression levels in various tissues in animal models (Kimura et al., 2003). Of note, in ASD, abnormal levels of inflammatory cytokines have been described (Croonenberghs et al., 2002). Various neurotransmitters, including noradrenaline and serotonin have also been shown to play a role in modulating neuropeptide secretion in both the central and peripheral circulation (Vacher et al., 2002). Restraint of a rat induced elevation in oxytocin and vasopressin levels, which could be inhibited by blocking specific serotonin receptors, for example (Jorgensen et al., 2002). The discussion on the relationship between sex steroids, neurotransmitters, and the behavioral effects of oxytocin and vasopressin is elaborated in Section Association with Neurotransmitters and Social Circuits.

Receptor Distribution in the Central Nervous System

The specific distribution of oxytocin and vasopressin receptors in the human brain has been difficult to study precisely. Unlike other neurotransmitter systems, a positron emission tomography (PET) radioligand has yet to be identified with adequate receptor specificity and CNS penetration for use in humans. Early trials testing tentative oxytocin PET ligands are currently underway in animal models, however (Smith et al., 2013a,b).

Accordingly, investigators have relied on post-mortem tissue analysis via receptor autoradiography and immunohistochemistry (IHC) in small samples of human subjects; alternatively, inferences can be drawn from data derived using similar techniques in animal studies. Both approaches have associated limitations. For example, certain oxytocin autoradiographic receptor ligands have been shown to have significant cross reactivity with AVP receptors (Toloczko et al., 1997). Acquisition of post-mortem brain tissue for analysis can prove challenging. Only typical adult brains have been studied so far. Additionally, receptor distribution in animal models has been shown to vary significantly depending on the age of the animal (Tribollet et al., 1989), and the species studied (Raggenbass et al., 1989; Gimpl and Fahrenholz, 2001). Translating information on receptor distribution in rodents to humans is particularly problematic, as patterns vary profoundly even between related rodents species.

Autoradiography uses a radioactive ligand tracer applied to mounted tissue sections and analyzed under a microscope, circumventing any difficulties with receptor penetration of the blood brain barrier. Tribollet and colleagues were some of the first investigators to apply this approach to the rat brain (Tribollet et al., 1988), suggesting that AVP and OT receptor distributions were sufficiently distinct. AVP receptors were detected primarily in the limbic system and hypothalamus; oxytocin receptors were also detected in the hypothalamus, as well in the olfactory tubercle and hippocampus. The relevance and translation of this information to the human brain was unclear at the time.

Loup and colleagues subsequently applied autoradiography to study oxytocin receptor distributions in 12 post-mortem human brains in typical adults, free of psychiatric illness (Loup et al., 1989). They used $[^3H]OT^{9-11}$ and a newly synthesized OXTR ligand $[^{125}I]OTA$, applied to tissue sections. In their first

publication on receptor distribution in the brain stem and spinal cord, they identified oxytocin binding in numerous overlapping tracts involved in sensory, motor, and autonomic function (e.g., the substantia nigra, the substantiae gelatinosa of the spinal trigeminal nucleus, the dorsal horn of the upper spinal cord, as well as the nucleus of the solitary tract) (Loup et al., 1989).

The same investigators subsequently applied this technique to the entire brain, while adding an AVP receptor ligand ([^{3}H]AVP) to distinguish AVP binding from binding to the oxytocin receptor, in both cortical and subcortical regions. Oxytocin and AVPR1a binding was detected in numerous limbic and autonomic pathways, with some distinct areas and some overlapping. In the cortical sections, oxytocin binding was strongest in the basal forebrain and nearby structures, including (1) specific cholinergic tracts (i.e., the basal nucleus of Meynert and the nucleus of the vertical limb of the diagonal band of Broca); (2) in the ventral part of the lateral septal nucleus (which relays between the hippocampus, thalamus, and midbrain); and (3) in parts of the hypothalamus and basal ganglia. Importantly, no OXTR binding was identified in the nucleus accumbens, caudate, putamen, hippocampus, amygdala, or in the frontal, temporal, or cerebellar cortices (see **Table 1**). The areas with strongest AVP binding were non-overlapping as compared to oxytocin, in the dorsal part of the lateral septal nucleus, and in certain thalamic nuclei as well (Loup et al., 1991). Many other areas showed weaker binding for AVP, including the hippocampal formation, parts of the basal ganglia, and specific brainstem nuclei (e.g., nucleus of the solitary tract and spinal trigeminal nucleus). However, the ligand used to test for oxytocin receptor binding in these studies (^{125}I-OTA), was subsequently shown to also bind AVPR1a receptors with equal strength as to the oxytocin receptor, calling into question the reliability of previous findings (Toloczko et al., 1997).

Recent literature is limited with respect to more concise localization of oxytocin and vasopressin receptors in the central nervous system in humans (see **Table 1**). One study in human brains used immunohistochemistry with a monoclonal antibody targeted to the oxytocin receptor (Boccia et al., 2013). As with earlier work, oxytocin receptors were identified in the hypothalamic and limbic areas, including the vertical limb of the diagonal band. The authors specifically commented on a lack of oxytocin receptor detection in the raphe nucleus of the brainstem. Unlike in previous human autoradiographic studies, however, oxytocin receptors were also detected in the anterior cingulate, amygdala, and in the olfactory nucleus. Of note, IHC staining of OXTR receptors was detected on both the cell membrane and in the cytoplasm of the cell body. The authors of this paper and of others (e.g., Yoshida et al., 2009) have described difficulty with reliability using immunostaining for the OXTR receptor, however, with variable results with each lot of antiserum.

Recent research in non-human primate brains may help clarify potential inconsistencies in the limited literature on human subjects. For example, Freeman et al., applied novel autoradiographic ligands for both OXTR and AVPR1a to coppery titi monkey brains, a socially monogamous species (Freeman et al., 2014b). They found AVPR1a receptors diffusely throughout the brain, with oxytocin receptors more localized to specific areas (e.g., the hippocampus and surrounding areas, nucleus basalis, thalamus, visual cortex, and brainstem structures). They confirmed their findings regarding OXTR by measuring mRNA expression levels, which overlapped with autoradiographic binding for OXTR (Freeman et al., 2014b). Similarly, Freeman et al., applied the same technique to macaque brains, and again detected more diffuse AVPR1a binding, with more localized OXTR binding. In the macaques, regions where OXTR bound most strongly involved sensory processing of visual and auditory stimuli, (e.g., nucleus basalis, pedunculopontine tegmental nucleus, superior colliculus, trapezoid body in the brainstem, hypothalamus) and seemed to overlap with many cholinergic pathways of the basal forebrain (Freeman et al., 2014a).

Overall, limited human data in control subjects only, inconsistencies and criticisms regarding methodology, and lack of a specific PET ligand for either receptor, highlight a need for further investigation into the distribution of these receptors in the CNS. However, by looking across existing human studies, and extrapolating from primate data, several conclusions can be drawn: (1) Oxytocin and vasopressin receptors are consistently detected in the hypothalamus. (2) AVPR1a expression appears to occur more diffusely throughout the central nervous system, while oxytocin receptor expression appears more localized. (3) Oxytocin receptors have been inconsistently identified in the limbic system, with conflicting evidence regarding the amygdala. (4) Oxytocin receptor staining occurs most prominently in the basal forebrain, in certain cholinergic tracts (e.g., nucleus basalis, diagonal band of Broca) and specific brainstem nuclei (e.g., the pedunculopontine tegmental nucleus). The basal forebrain consists of a group of structures situated anterior and inferior to the striatum, including the nucleus basalis of Meynert, the diagonal band of Broca and the medial septal nuclei. It provides extensive cholinergic input to all layers of the cortex, and receives input from prefrontal regions, the nucleus accumbens and the ventral tegmental area. GABAergic basal forebrain projections to the amygdala have also been shown to modulate inhibitory signals in this region (McDonald et al., 2011). The basal forebrain is thought to play an important role in visual attention, memory, and learning, and undergoes degeneration in conditions such as Alzheimer's dementia. Future studies examining differences in the expression and distribution of these receptors in neurodevelopmental and neuropsychiatric disorders will be of particular interest moving forward.

Association with Neurotransmitters and Social Circuits

The complex relationships between oxytocin, vasopressin, and monoamine neurotransmitter systems have been studied in various animal models. The translation of this information to human social networks remains speculative. Below, we discuss the relationship between oxytocin, vasopressin, and various neurotransmitter systems and brain circuits.

Serotonin

As mentioned in previous sections, serotonin activity may also contribute to the effects of oxytocin and vasopressin on social functioning, either by modulating peptide secretion, or for downstream effects on fear responses and anxiety. For example, functional activation of specific serotonin receptor subtypes was necessary to facilitate elevations in oxytocin and vasopressin levels in response to stress in rodents (Jorgensen et al., 2002). Likewise, application of serotonin to tissue sections from the hypothalamus/pituitary of the rat brain increased oxytocin and vasopressin secretion (Galfi et al., 2005). Data in rodent models suggest that the aggressive behavior stimulated as a result of AVP administration can be blocked via serotonergic activity (Delville et al., 1996a; Ferris, 1996). Oxytocin receptors are expressed on the neurons of the serotonin raphe nuclei in rats; infusion of oxytocin facilitated serotonin release from these cells and had an anxiolytic effect on rat behavior (Yoshida et al., 2009). However, a recent study in which oxytocin receptors were knocked out of the raphe nuclei in mice found deficits only in males with respect to aggression; all other social and parenting behaviors remained intact (Pagani et al., 2015). In macaques, serotonin transporters co-localized to the regions of the hypothalamus expressing oxytocin receptors (Emiliano et al., 2007). In human subjects with personality disorders, CSF levels of AVP correlated with aggression, while one of two serotonin proxy-measures was inversely associated with aggressive behavior (Coccaro et al., 1998). In children with ASD, oxytocin and serotonin plasma levels were inversely correlated with each other in one study (Hammock et al., 2012). In adults with ASD, serotonin transporter binding was lower throughout the brain on PET (Nakamura et al., 2010). Overall, animal studies suggest that serotonin receptor activation can trigger, and may be necessary to facilitate oxytocin and vasopressin secretion, while oxytocin may also stimulate serotonin release. Simultaneously, serotonin and vasopressin may have opposing effects with respect to aggressive behavior.

Hypothalamic Pituitary Axis

Other investigators have shown that oxytocin and vasopressin may affect behavior by regulating stress responses through the HPA axis (Neumann, 2002). For example, rats put under a forced swim test showed central elevation of both oxytocin and vasopressin. Peripheral blood levels of oxytocin but not vasopressin, increased as well (Wotjak et al., 1998). Rats exposed to a noise stress had a dose dependent reduction in corticosteroid levels when treated with centrally administered oxytocin. Anxious behavior when exploring an unfamiliar maze was also less (Windle et al., 1997). In response to restraint, oxytocin but not vasopressin administration reduced adrenocorticotropic hormone (ACTH) and cortisol levels in rats (Windle et al., 2004). This effect resulted from reduced neuronal activity in the hypothalamus, hippocampus, and ventrolateral septum, as indicated by absence of elevations c-fos mRNA expression in these regions in the rats who were restrained and treated with oxytocin; no such effect was seen with vasopressin. In squirrel monkeys, chronic oxytocin administration reduced ACTH, but not cortisol secretion in response to social isolation (Parker et al., 2005). In humans, oxytocin administration enhanced the stress buffering effect of social support in response to a social stress paradigm, as indicated by increased calmness and reduced salivary cortisol levels in participants (Heinrichs et al., 2004). Similarly, oxytocin enhanced positive communication and reduced salivary cortisol levels during couple conflicts (Ditzen et al., 2009).

Vasopressin has been shown in animals to enhance corticotrophin releasing factor (CRF) mediated elevations in ACTH (Rivier and Vale, 1983). This process appears to be mediated via the AVPR1b receptor (Stevenson and Caldwell, 2012). It may be that oxytocin attenuates the stress response, while vasopressin might facilitate it (Bisagno and Cadet, 2014). In a rodent model, each peptide was shown to activate a different set of neurons in the amygdala, having opposite regulatory effects on excitatory input (Huber et al., 2005; Viviani et al., 2011). Interest in using AVPR1b antagonism to treat anxiety disorders has been investigated showing potential benefits in rodent models (Iijima et al., 2014), while commercial human studies are underway for AVPR1a receptor antagonists as a potential treatment for ASD (e.g., RG7314, clinical trials.gov ID: NCT01793441).

Sex Hormones

Sex hormones have been shown to be of particular importance to the central neuropeptide effects in animal models. Estrogen receptor beta (ER-β), for example, was found to co-localize in the hypothalamus with cells expressing oxytocin and vasopressin receptors in rodents (Alves et al., 1998). Female rats treated with exogenous sex hormones in the neonatal period showed higher levels of oxytocin receptor binding in the brain (Uhl-Bronner et al., 2005). Castrated hamsters had lower levels of AVPR1a receptor in their brains, unless they were treated with testosterone replacement (Delville et al., 1996b). In an animal model using ovariectomised rats, the HPA axis showed elevations in stress hormones when rats were restrained; this effect was buffered by administration of oxytocin only in the presence of exogenous estradiol replacement (Ochedalski et al., 2007).

Sex steroids have also been shown to be important to the behavioral effects of these neuropeptides. For example, knockout of either the oxytocin gene, or estrogen receptors α or β led to deficient social abilities in mice (Choleris et al., 2003). Central administration of vasopressin triggered aggression in rats, but the effect was lessened if they had been castrated, thereby lowering testosterone levels (Korte et al., 1990). The relevance of these findings to research looking at behavioral effects in humans remains unclear; a single intranasal dose of oxytocin in humans led to slight augmentation in peripheral testosterone levels, but no change in progesterone or estradiol levels (Gossen et al., 2012). The relationship between sex hormones and neuropeptides is supported, yet further complicated by literature showing that single doses of estradiol and testosterone administered in humans can lead to behavioral effects on social functioning and threat/reward perception that overlap with effects of oxytocin or vasopressin administration (Bos et al., 2012). While autism is significantly more common in males than females, there is no clear understanding of whether sex hormones, oxytocin,

or vasopressin contribute to this difference, although various hypotheses have been proposed (Baron-Cohen et al., 2011).

Dopamine

Dopamine is thought to contribute to the effects of oxytocin and vasopressin on social processes, potentially via its impact on the reward pathway. Specifically, research in prairie voles has highlighted the importance of dopamine in facilitating the partner preference formation via oxytocin and vasopressin manipulation. For example, dopamine receptor 2 (D2) blockade using various agents including haloperidol blocked partner preference behavior in prairie voles, while D2 agonists facilitated partner preference formation (Wang et al., 1999). This mechanism appeared to be mediated via dopaminergic activity in the nucleus accumbens, as evidenced by increased dopamine turnover in this area, and specificity of the effects of D2 blockade injected into this region in particular (Gingrich et al., 2000; Aragona et al., 2003). Activation of both the D2 receptor and oxytocin receptor was necessary to facilitate partner preference formation in voles; blockade of either receptor eliminated partner selection (Liu and Wang, 2003). Similarly, artificial up regulation of AVPR1a in voles using a viral vector led to increased partner preference formation, an effect that was blocked by D2 antagonism (Lim et al., 2004). Oxytocin neurons in the hypothalamic nuclei in rats have also been shown to express dopamine receptors (Baskerville et al., 2009).

Dopamine activity in the nucleus accumbens is central to behavioral reinforcement, reward, and motivation. Dopamine neurons which originate the ventral tegmental area (VTA) project to the medial prefrontal cortex, amygdala, and nucleus accumbens, while glutamatergic neurons in the medial prefrontral cortex project back to the nucleus accumbens and serve a regulatory function. Glutamate antagonists or oxytocin injected into the VTA in rodents decreased dopamine release in the frontal cortex, while increasing dopamine release in the nucleus accumbens, suggesting differential inhibitory regulation within this system (Takahata and Moghaddam, 2000; Melis et al., 2007). This regulation of dopamine in the nucleus accumbens by the ventral tegmental area via the prefrontal cortex in voles provides an example of a circuit driving social behavior. Inhibition of either $GABA_A$ (gamma hydroxyl butyric acid) receptors or AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in the ventral tegmental area also led to a decrease in dopamine activity in the prefrontal cortex, and an increase in dopamine in the nucleus accumbens, which was associated with increased partner preference formation (Curtis and Wang, 2005). Oxytocin, vasopressin and dopamine receptors are co-expressed in the medial prefrontal cortex in voles; higher D2 concentration and OXTR binding in this region is associated with greater monogamous behavior (Smeltzer et al., 2006). Simultaneously, variation in the density of OXTR in the nucleus accumbens of prairie voles (through viral mediated over-expression) accelerated partner preference formation (Ross et al., 2009). Along these lines, humans with ASD have been shown to have aberrant dopamine transporter distribution and function (Nakamura et al., 2010; Hamilton et al., 2013), while oxytocin administration in humans may be able to enhance the saliency of certain social cues (discussed further in subsequent sections).

In summary, oxytocin's effects on reward pathways, (including the nucleus accumbens, VTA, and prefrontal cortex) likely modulate the saliency of social stimuli. Given that oxytocin receptors have not been detected in the nucleus accumbens in human and primate brains, other areas may indirectly mediate this effect. Notably, the basal forebrain which stains densely with oxytocin receptors in humans receives many inputs from the prefrontal cortex, VTA, and nucleus accumbens, and indeed projects to various cortical regions (Sarter et al., 2009). It would follow that pituitary neuropeptides may selectively modulate signaling within the basal forebrain and surrounding areas, contributing to signaling within classic reward pathways involving dopamine in humans.

Interneurons

A separate body of research proposes that the neuropeptide effects occur specifically on fast spiking interneurons. Interneurons serve a local regulatory function within microcircuits by impacting on the firing of principal neurons. Principal neurons drive the dominant signals and outputs propagated to other brain regions (Freund and Buzsaki, 1996). There have been numerous and varied attempts to classify interneurons based on their structure and function. Generally speaking, interneurons exert inhibitory signals on principal neurons through GABA. Fast spiking neurons are classified as such due to their low threshold to quickly deliver inhibitory signals.

Recent research in hippocampal cells *in vitro* has shown that oxytocin receptor agonism effectively strengthens the signal to noise ratio via its impact on interneurons. When exposed to oxytocin, these fast-spiking interneurons in the hippocampus increased their inhibitory output, thereby lowering background firing (noise) in the principal cell circuits. Simultaneously, the balance of excitatory to inhibitory transmission within the circuit was altered, such that the strength and fidelity of firing within the principal cells was increased and made more efficient, effectively enhancing the coordinated signal in the overall network (Owen et al., 2013). In a mouse model *in vivo*, interneurons expressing the oxytocin receptors in the frontal cortex were shown to be involved in social and sexual behavior (Nakajima et al., 2014). A similar mechanism may also exist with respect to the actions of vasopressin. It has previously been shown that application of vasopressin to hippocampal cells can enhance neurotransmission, leading to long-term potentiation (Rong et al., 1993; Chepkova et al., 1995). In rats, application of AVP to hippocampal tissue sections increased the frequency of inhibitory signals; this process was shown to occur due to AVP binding to AVPR1a, which through a G-protein mediated cascade, increased the excitability of interneurons, leading to increased GABA release. At the same time, AVP had an excitatory effect on principal neurons (of pyramidal type) in this circuit (Ramanathan et al., 2012). OXTR and AVPR1a were also shown to mediate reciprocal inhibitory effects in different regions within the rat amygdala via GABA activity (Huber et al., 2005). Of note,

networks of GABAergic interneurons have also be characterized in the basal forebrain in primates (Walker et al., 1989).

Recent literature using optogenetic methods further support an association between the inhibitory GABA system, oxytocin, vasopressin, and behavior. Specifically, Knobloch et al. used an adenovirus vector to insert light sensitive channelrhodopsin molecules into the oxytocin axons of rat brains. They demonstrated that hypothalamic neurons have axonic projections extending directly to the amygdala, among other locations. When endogenous oxytocin was released as triggered by blue light, there was a local increase in GABAergic interneuron signaling within the amygdala, which was associated with a reduction in freezing behavior in fear conditioned rats (Knobloch et al., 2012). Cortical and brainstem networks may also be impacted in this way; a recent optogenetic study showed that oxytocin secretion via hypothalamic projections to the piriform cortex was necessary for social learning around both salient and aversive stimuli (Choe et al., 2015), while oxytocin projections to brainstem autonomic nuclei mediated heart rate variability (Pinol et al., 2014).

An imbalance of excitatory/inhibitory signaling during critical periods of development is an appealing explanatory theory for autism (Yizhar et al., 2011). A potential mechanism of action of oxytocin on GABA transmission occurs via modulation of chloride channel activity. In fetal rats, oxytocin increased intracellular chloride concentration in GABA neurons, thereby reducing neuronal excitation; this process was thoughts to protect the neonate from anoxic injury (Tyzio et al., 2006, 2014). Accordingly, chloride importer antagonists are currently being investigated as a potential treatment for ASD (Lemonnier et al., 2012).

Section Summary

In summary, data derived from animal studies have begun to tease apart a complex social network involving multiple brain structures potentially impacted by pituitary neuropeptides. Specifically, dopamine and serotonin appear to be important to encoding social information, potentially via their impact on reward pathways and anxiety, while serotonin may be involved in peptide secretion. Sex hormones appear to impact on the density of peptide receptor expression from early life, while both oxytocin and vasopressin can modulate stress responses in the hypothalamic-pituitary (HPA) axis. Recent research suggests that oxytocin and vasopressin may mediate their effects by activating inhibitory interneurons across subcortical and potentially cortical networks, including reward pathways. This model provides an appealing example of how these molecules may impact diffuse brain regions to strengthen signal outputs or fine tune inhibitory control. Further research using more advanced methods like optogenetics is anticipated to clarify these networks.

Functional Neuroimaging

Functional neuroimaging studies investigating the neural correlates of social processing began to emerge in the late 1990s and early 2000s. Investigators used blood oxygen level dependent imaging (BOLD) imaging to quantify local brain regions with increased activity in response to various social stimuli. Together, this research provides evidence for a large-scale social network in the human brain spanning multiple regions, including the amygdala, prefrontal, and orbitofrontal cortex, the insula, temporoparietal junction, and fusiform gyrus (Stanley and Adolphs, 2013). In the following section, we review functional neuroimaging literature in response to acute peptide administration in humans. Note that most studies include fMRI scans performed approximately 1 h after intranasal application, with data analyzed primarily via a region of interest approach. Neuroimaging correlates of common genetic variation in oxytocin and vasopressin receptors are not included in this review, but have been summarized elsewhere (Zink and Meyer-Lindenberg, 2012).

Oxytocin

Kirsch et al. (2005) were one of the first groups to employ functional neuroimaging technology in order to try to better understand the mechanism by which oxytocin exerts behavioral effects in humans following acute administration. They administered intranasal oxytocin to 15 male subjects, and then had them watch fear inducing stimuli, including fearful faces, in an fMRI scanner. Using a region of interest approach focused on the amygdala, they found that oxytocin significantly reduced amygdala activation, and also reduced coupling between the amygdala and brainstem regions involved in autonomic arousal (see **Figure 3**) (Kirsch et al., 2005). The authors proposed that oxytocin attenuated the fear response at the level of the amygdala.

Domes et al. (2007) subsequently showed that this attenuated activity in the amygdala from oxytocin persisted in response to viewing other facial expressions also (e.g., happy, angry) (Domes et al., 2007). On exploratory whole brain analysis, they also identified reduced activation in many other brain regions including areas of the temporal lobe, thalamus, and frontal lobe. Subsequent investigators have showed a similar pattern of attenuated fMRI activity in the amygdala when treated with oxytocin during games of trust (Baumgartner et al., 2008), and in response to the sound of an infant crying (Riem et al., 2011) (see **Table 2**). Further studies suggested that the pattern of amygdala activation might vary within specific subsection of the amygdala, or depending on the valence of the emotional cue. For example, more than one study has shown that oxytocin increased amygdala activation in response to positive social information (Gamer et al., 2010; Rilling et al., 2012). However, there is significant variability within the functional neuroimaging literature, with bidirectional, and at times conflicting findings regarding amygdala activation in response to social cues (see **Table 2**). A recent meta-analysis of data suggested left insular hyperactivation emerges most consistently (Wigton et al., 2015).

One potential explanation for observed differences across studies is the seemingly differential effects of oxytocin administration depending on the sex of the participant. Prior to 2010, most fMRI studies of oxytocin recruited only male participants. Domes et al. (2010) were the first to investigate oxytocin's effects on face processing in a group of females only;

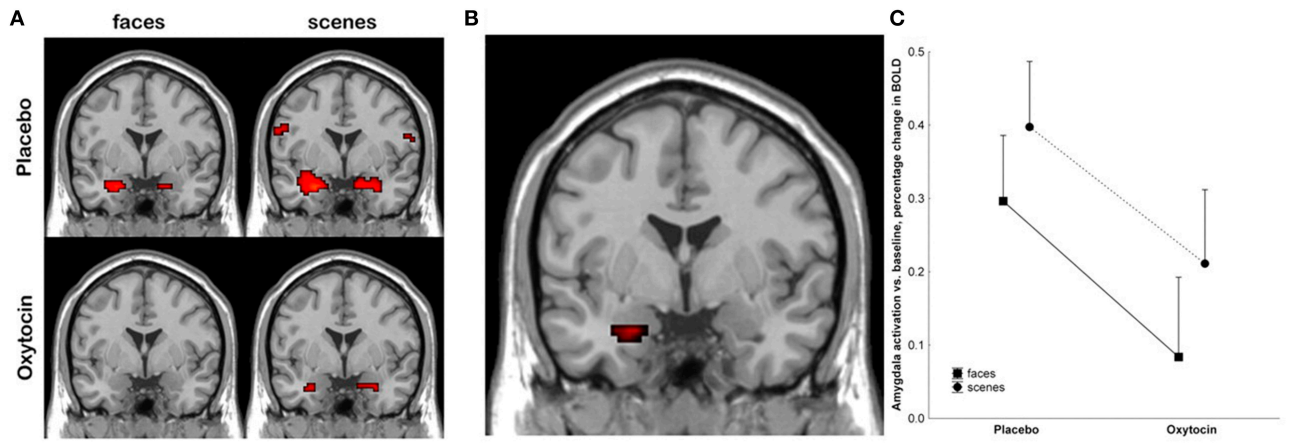

FIGURE 3 | Kirsch et al. (2005) showed that oxytocin attenuated amygdala activation in response to fearful stimuli. (A) shows activation at the amygdala, with neural responses to fearful faces shown on the left, and to fearful scenes on the right, under placebo conditions (top), and after treatment with oxytocin (bottom). **(B)** shows the main effect of the drug in the left amygdala, where the signal was strongest. **(C)** plots BOLD levels at the amygdala using a region of interest analysis. Reproduced with permission from *J. Neurosci.* (Kirsch et al., 2005).

they detected a pattern somewhat opposite to that observed in males, with increased amygdala activity while observing negative facial expression in response to oxytocin treatment (Domes et al., 2010). This effect persisted despite controlling for estradiol and progesterone levels. Increased amygdala activation in females in response to negative or threatening social information was replicated in a small sample (Lischke et al., 2012), although subsequent studies did not find the same effect (see **Table 2**).

Rilling and colleagues have published several manuscripts attempting to tease apart the sex effects of this response, with increasingly large sample sizes, using the prisoner's dilemma game. In this task, participants must choose whether to risk cooperating to achieve the best outcome for both participants, or defect against their partner and achieve a positive outcome for themselves only (Declerck et al., 2014). In males, treatment with either AVP or oxytocin increased brain activation in the basal forebrain, amygdala, hippocampus and striatum, and treatment with AVP increased cooperative behavior in the game (Rilling et al., 2012). In women, however, neither peptide led to activation in these brain regions; oxytocin instead decreased amygdala activity (Rilling et al., 2014). Plasma estrogen levels did not modulate this effect. A subsequent paper confirmed differential sex effects in the same, but larger group of participants; findings were more specific, however, with increased activity in the frontal pole, medial prefrontal cortex, and caudate/putamen in men, but decreased or no activity in these regions in women, in response to reciprocal cooperation while being treated with oxytocin (Feng et al., 2014). The amygdala was no longer implicated with the larger sample size. Other investigators recently showed that women who scored lower on a social perception task (reading the mind in the eyes), performed better in response to oxytocin administration, an effect that was associated with enhanced activation in the superior temporal gyrus and insula (Riem et al., 2014). The ventral tegmental area showed increased activation in response to oxytocin administration in another group of women (Groppe et al., 2013).

Subsequent studies have attempted to tease apart the differential fMRI findings in response to oxytocin administration by looking at brain connectivity specifically (for a more detailed review Bethlehem et al., 2013). Some have found, for example, that oxytocin can both reduce amygdala activation in response to negative stimuli (Striepens et al., 2012), but also increase connectivity of the amygdala to other regions including the insula, and prefrontal cortex, and anterior cingulate, potentially facilitating memory of social information (Striepens et al., 2012; Sripada et al., 2013). Resting state MRI data showed increased connectivity between the posterior cingulate and brainstem in response to oxytocin treatment (Riem et al., 2013). Others have shown that oxytocin reduced connectivity between the amygdala and precuneus (Kumar et al., 2014).

Oxytocin in ASD

Recent data have used fMRI technology to attempt to understand the potential effects of oxytocin administration in individuals with ASD. In a small pilot study, Domes et al., showed that participants with ASD had lower activity as compared to controls in the right amygdala, fusiform gyrus, and occipital region during face processing, and that intranasal oxytocin administration increased the right amygdala activity in the affected group (Domes et al., 2013). The same investigators subsequently showed that oxytocin improved emotion recognition abilities in adults with ASD, and that this effect correlated with increased left amygdala activation on fMRI. Note that under placebo conditions, amygdala reactivity was comparable in both the ASD and control group in this sample (Domes et al., 2014). In another study in adults with ASD, oxytocin increased the otherwise decreased brain activation in the medial prefrontal cortex (Watanabe et al., 2014). Increased activity in the right anterior insula in response to oxytocin treatment coincided with

TABLE 2 | fMRI changes in response to acute administration of oxytocin or vasopressin.

Study	Subjects	Task Description	Task Valence	Amygdala	Temporal lobe	Insula	Anterior cingulate	Prefrontal	Other
OXYTOCIN									
Kirsch et al., 2005	15 M	Fearful stimuli, including face showing fear	Negative	↓L					↓Coupling between amygdala and brainstem
Domes et al., 2007	13 M	Looking at fearful, angry, happy faces	Negative	↓R	↓*L*			↓*L*	↓*L cerebellum, medulla, thalamus, R pre- and L post-central gyrus (negative valence),* ↓*L paracentral gyrus (negative and positive valence)*
			Positive	↓R	↓				
Baumgartner et al., 2008	49 M	Financial game involving trust	Neutral	↓		↓L			↓Brainstem L (midbrain), ↓caudate, ↓L post-central gyrus
Petrovic et al., 2008	27 M	Neutral faces previously paired with negative experiences	Negative	↓R	↓		↓R	↓vmPFC ↓vlPFC ↓L OFC	↓R fusiform face area
Singer et al., 2008 (AH)	20 M	Observing pain inflicted on another	Negative						No difference on social/ empathy tasks
Gamer et al., 2010	46 M	Classifying fearful and happy faces	Negative	↓L (ant)					↑Superior colliculus, and connectivity of superior colliculus and posterior amygdala
			Positive	↑L (ant)					
Domes et al., 2010	16 F	Rating fearful, happy, angry faces	Negative	↑L	↑L	↑L		↑	↑R brainstem (fear), ↑ fusiform gyrus,
			Positive		↑L	↑L			
Riem et al., 2011	42 F	Sound of infant crying	Negative	↓R		↑		↑	
Rilling et al., 2012	60 M	Prisoners dilemma game (Reciprocated or un-reciprocated cooperation)	Negative					↑L	↑L caudate (positive) ↑Amygdala -insula and -temporal connectivity, ↓Amygdala-brainstem connectivity
			Positive	↑L					
Lischke et al., 2012	14 F	Viewing threatening and non-threatening scenes	Negative	↑	↑*L*				↓*R SMA for positive stimuli*
Wittfoth-Schardt et al., 2012	21 M	Viewing pictures of child faces (own vs. other)	Positive						↓Activity and functional connectivity in globus pallidus, ↓ *L hippocampus*
Striepens et al., 2012	70 M	Aversive social stimuli	Negative	↓R		↑L (recall)			↑Coupling between amygdala, insula and PFC
Groppe et al., 2013	28 F	Social incentive delay task (reward vs. punishment anticipation)	Positive		↑*L*				↑ VTA activity, ↑*L occipital,*
			Negative						↑ VTA, ↓ *precentral gyrus,* ↓*L cuneus,* ↓*R post-central gyrus,*

(Continued)

TABLE 2 | Continued

Study	Subjects	Task Description	Task Valence	Amygdala	Temporal lobe	Insula	Anterior cingulate	Prefrontal	Other
			Both			↑			↑VTA, ↑*L thalamus, striatum, brainstem,* ↑*R thalamus,*
Sripada et al., 2013	15 M	Resting state	N/A					↑mPFC	↑Amygdala connectivity to mPFC and ACC
Riem et al., 2013	42 F	Resting state while recalling love withdrawal	Negative						↑Connectivity between posterior cingulate cortex and brainstem
Rilling et al., 2014	87 F	Prisoners dilemma game (Reciprocated and un-reciprocated cooperation)	Negative						Note lack of findings as previously seen in male cohort on same task/ protocol (Rilling et al., 2012)
			Positive	↓L					
Voorthuis et al., 2014	50 F	Emotion recognition in infant faces	Both		↑L			↑L IFG	
Riem et al., 2014	50 F	Reading the mind in the eyes task	Both		↑L	↑L			
Feng et al., 2014	153 M 151 F	Prisoners dilemma game (reciprocated cooperation)	Positive					↑M ↓F	↑M ↓F caudate/putamen
Kanat et al., 2015b	49 M	Detection of angry, happy, or neutral expression	Negative	↓					↓*Fusiform, brainstem and striate cortex* ↓*connectivity amygdala and fusiform (for angry and fearful stimuli)*
			Positive	↓				↓	
Kumar et al., 2014	15 M	Resting state	N/A						↓Connectivity between amygdala and precuneus with OT
Eckstein et al., 2015	62 M	Conditioned fear response followed by extinction	Negative	↓				↑	↑*Connectivity from PFC to precuneus, and precuneus to amygdala*
Kanat et al., 2015a	50 M	Fearful faces, and eyes only (asked to assess gender)	Negative	↓R	↓*L*		↓*L*		↓R pulvinar (trend)
Chen et al., 2015	153 M 151 F	Prisoners dilemma game (unreciprocated cooperation)	Negative	↓M		↓M			
OXYTOCIN IN PARTICIPANTS WITH ASD									
Gordon et al., 2013	17 ASD	Reading the mind in the eyes task vs. vehicle classification task	Both		↑L				↑R precentral gyrus, ↑ striatum and nucleus accumbens, ↑ cerebellum and pons, ↑Posterior cingulate, precuneus, ↑L parahippocampal region, ↑ L inferior parietal lobule,
Domes et al., 2013	28 M 14 ASD	Face matching and house matching task	Neutral	↑R (in ASD)					

(Continued)

TABLE 2 | Continued

Study	Subjects	Task Description	Task Valence	Amygdala	Temporal lobe	Insula	Anterior cingulate	Prefrontal	Other
Domes et al., 2014	28 M 14 ASD	Face emotion recognition task	Both	↑L (ASD)	↑*R*				↑*IFG, SMA, cerebellum, and superior parietal lobe*
Watanabe et al., 2014	40 M ASD	Making decisions about social information	Both	↓			↑	↑mPFC	↑Connectivity from mPFC to ACC
Aoki et al., 2014	20 M ASD	Sally-Anne task (inferring emotions)	Both			↑ R (ant)			
VASOPRESSIN									
Zink et al., 2010	20 M	Face matching task	Negative					↓mPFC	↓Subgenual cingulate region ↓Connectivity in this area
Zink et al., 2011	20 M	Face matching task (familiar vs. unfamiliar)	Negative		↓TPJ				
Rilling et al., 2012	60 M	Prisoners dilemma game (Reciprocated and un-reciprocated cooperation)	Both						↑BNST, lateral septum and stria terminalis. ↑Amygdala insula connectivity
Brunnlieb et al., 2013	42 M	Black and white drawings of social situations without facial information	Negative	↑R					Increased functional connectivity between amygdala and mPFC
			Positive						
Feng et al., 2014	153 M 151 F	Prisoners dilemma game (reciprocated cooperation)	Positive			↑M ↓F			↑R SMG M ↓R SMG F
Chen et al., 2015	153 M 151 F	Prisoners dilemma game (unreciprocated cooperation)	Negative	↓M		↓M			

Table Includes studies with tasks related to social processing, or resting state, in control subjects or those with ASD. fMRI, functional magnetic resonance imaging. Boxes for regions left blank were either not studied or indicated in the respective manuscript, or differences were non-significant. Findings in italics are from secondary/exploratory analyses. Task valence refers to the emotional quality (e.g., positive = happy; negative = fearful) of the task in which the fMRI findings are described. L, left side only; R, right side only; M, male subjects; F, female subjects; N/A, not applicable; ↑, increased activity; ↓, decreased activity; vlPFC, ventrolateral prefrontal cortex; vmPFC, ventromedial prefrontal cortex; mPFC, medial prefrontal cortex; IFG, inferior frontal gyrus; Ant, anterior; VTA, ventral tegmental area; ASD, autism spectrum disorder; SMG, supramarginal gyrus; SMA, supplementary motor area; TPJ, temporoparietal junction; BNST, basal nucleus of stratum terminale; ACC, anterior cingulate cortex. Most studies used intranasal oxytocin administration, of 16–24 IU, or intranasal vasopressin administration of 20–40 IU.

increased accuracy in inferring others' emotions (Aoki et al., 2014). Gordon et al., also found that oxytocin enhanced fMRI activity in regions including the amygdala, nucleus accumbens, and orbitofrontal cortex during social tasks in a group of children with ASD (Gordon et al., 2013).

AVP

Far fewer studies have been conducted looking at AVP administration in humans on fMRI. One of the first trials found no effects of AVP administration on amygdala activity, although there was a corresponding decrease in medial prefrontal cortex hyperactivity in the treatment group as compared to placebo (Zink et al., 2010). A subsequent paper by the same group also showed that a region which showed increased activity in the temporoparietal junction in response to unfamiliar faces, was no longer hyperactive in participants treated with AVP (Zink et al., 2011). Notably, behavioral and symptom surveys did not identify any neuropsychiatric effects of AVP administration in these studies. Rilling et al. compared fMRI signals and functional connectivity measures in male participants during the prisoner's dilemma game, following administration of either oxytocin or vasopressin. Interestingly, both peptides led to behavioral changes, with more cooperative behavior. Oxytocin increased caudate activation in response to positive cooperation and also increased left amygdala activity on whole brain analysis. AVP increased activity in the bed nucleus of the stria terminalis and lateral septum. Both peptides reduced amygdala connectivity to the brainstem (Rilling et al., 2012). Subsequent studies also found sex effects in response to AVP administration; bilateral insula, and right supramarginal gyrus activity was increased in men, while decreased in women, during reciprocated cooperation (Feng et al., 2014).

Summary

Overall, functional neuroimaging literature following acute administration of oxytocin and vasopressin support their potential role in social information processing as evidence by neural activation in regions implicated in social brain networks. Findings in this regard are complicated by (1) significant heterogeneity in the tasks studied, (2) the potential of differential effects of these peptides depending on the sex of the participants and the valence of the emotional stimuli, and (3) the large number of studies of relatively small sample size. A few themes stand out overall: (1) Brain activation patterns in response to peptide administration span several different regions; the amygdala, prefrontal cortex, insula and temporal lobe emerge most frequently across studies. Some studies have also detected differential activation patterns in the basal forebrain and brainstem. (2) Many investigators have shown changes in functional connectivity between various structures in the above listed regions in response to peptide administration. (3) A small number of studies including participants with ASD suggest that aberrant functional activation patterns in response to social stimuli may be partially corrected following acute treatment with oxytocin. The implications of this information as it relates to previous sections are discussed in the next section.

Discussion, Synthesis, and Implications in Autism

In summary, oxytocin and vasopressin are neuropeptides synthesized in the hypothalamus and secreted into the peripheral vasculature through the posterior pituitary. In rodents, a functionally separate process also mediates central release of these peptides from hypothalamic neurons into central nervous systems; it is presumed that a similar mechanism is at play in humans as well. Centrally secreted neuropeptides are thought to diffuse through the extracellular fluid into surrounding tissue, where they exert their neuromodulatory effects; specific axons also deliver peptides directly to distant brain regions. The oxytocin and vasopressin receptors are G-protein linked, and activate various downstream pathways, which vary by cell type and organ system.

Limited literature on the distribution of these neuropeptide receptors in the central nervous system implicates the basal forebrain (including the nucleus basalis, and diagonal band), brainstem, and potentially the limbic system as areas of oxytocin binding in humans and primates, while AVP receptors appear more diffusely throughout the brain. The basal forebrain has previously been described as serving a regulatory function, consolidating various external inputs and amplifying signals in relevant downstream cortical targets (Givens and Sarter, 1997). It is functionally connected within a reward network involving the nucleus accumbens and ventral tegmental area (Sarter et al., 2009). Indeed, various neurotransmitter systems and brain regions that have afferent or efferent connections with the basal forebrain and brainstem regions have been implicated in studies investigating the mechanisms behind the neurobehavioral effects of these peptides. Blocking dopamine within the reward pathways described above can eliminate many of the behavioral effects of oxytocin in rodents. Recent literature demonstrating that oxytocin and vasopressin can increase the signal to noise ratio and enhance coordinated signaling via their activity on interneurons could tentatively link these concepts together. In essence, could these pituitary neuropeptides act on interneurons within the basal forebrain to consolidate and strengthen signaling to relevant downstream cortical and subcortical regions via cholinergic, glutamatergic, or monoamine neurotransmitter pathways in response to external social stimuli?

Functional neuroimaging can provide information on brain activation patterns. Earlier studies proposed that the neural effects of oxytocin and vasopressin occurred as a result of attenuated amygdala activity in response to fearful stimuli. Subsequent studied found differences in BOLD signaling more diffusely, although clustering within specific social brain areas (including the prefrontal cortex, insula, amygdala, and temporal lobe). Importantly, significant heterogeneity across studies highlight how the neural effects of oxytocin and vasopressin likely depend on the type of social stimulus, the sex of the participant, and other contextual factors. While oxytocin and vasopressin receptors have been detected in the insula, amygdala, and cerebral cortex in rodents, data are inconsistent in these regions in primates and humans. The neuroimaging literature

would instead support a model in which peptide effects on subcortical networks subsequently impact social appraisals and downstream cortical activation patterns. Indeed, numerous studies highlight how the functional connectivity between the amygdala, brainstem, anterior cingulate, insula, temporal lobe, and prefrontal cortex is altered under the influence of these pituitary neuropeptides. Along these lines, the cholinergic basal forebrain has prominent connections to the cortex, as well as other subcortical and brainstem structures; it can also regulate amygdala activity (Power, 2004).

Tentatively, many associations can be drawn between the putative mechanisms of action of oxytocin and vasopressin and hypotheses regarding the pathophysiology of ASD. For example, numerous immune system differences have been detected in autism, while specific inflammatory cytokines have been shown to alter the expression of oxytocin receptors *in vitro*. Sex differences in the manifestation and incidence of autism have been well-described; at the same time, the oxytocin and vasopressin systems have been shown to interact with sex hormones in numerous ways. Imbalances in excitatory/inhibitory neurotransmission have increasingly been characterized in neurodevelopmental disorders such as ASD, while cellular physiology research suggests that both oxytocin and vasopressin can alter this balance by acting on interneurons. Aberrant functional and structural connectivity in ASD has been detected using various neuroimaging modalities and both oxytocin and vasopressin may be able to alter connectivity within brain social networks. Although at present, data do not appear to suggest that disruption of the oxytocin or vasopressin systems necessarily contributes to the etiology of ASD (e.g., only a single case of a rare variant disrupting OXTR has been described), these molecules do seem to impact on social functioning, presenting a potential therapeutic target.

In the context of advancing technology, important next steps for this field include determining more precisely the anatomic location of CNS receptor expression via radiolabeled ligands, in typical individuals, and importantly, in those with ASD and other neurodevelopmental disorders. While rodent models permit elegant proof of concept experiments, results must be confirmed and replicated in primates, if not humans. For example, a recent neuroimaging paper in macaques provided evidence of functional overlap with humans in brain activation patterns in response to oxytocin (Liu et al., 2015). Quantification of the level of inter individual variation in receptor expression will be important as well. Characterization of the three dimensional and long-range patterns of receptor expression within nervous tissue in humans may prove informative, and may be possible with novel techniques (Chung et al., 2013). Functional connectivity analyses focusing on brainstem and forebrain regions, at different and more proximal time point following peptide administration may help to better characterize the sequence of changes taking place in the CNS. Investigations to support or refute an interneuron mediated increase in the signal to noise ratio in specific networks in humans in response to peptide administration will prove interesting and informative to the field. Excitingly, emerging research efforts are underway hoping to harness the therapeutic potential of these molecules with respect to treating social deficits in neurodevelopmental disorders. Given that peptide penetrance of the blood brain barrier has proven to be a challenge, exploration of other compounds that act on peptide receptors may also prove beneficial. Additionally, it is hoped that clinical biomarkers within this system (e.g., common genetic receptor subtypes) may be able to predict variable subgroup responses, in order to optimize the therapeutic potential of these peptides.

Author Contributions

DB authored the manuscript. EA developed the research topic, provided guidance, editing and supervision.

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REVIEW
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Brain-specific transcriptional regulator T-brain-1 controls brain wiring and neuronal activity in autism spectrum disorders

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T-brain-1 (*TBR1*) is a brain-specific T-box transcription factor. In 1995, *Tbr1* was first identified from a subtractive hybridization that compared mouse embryonic and adult telencephalons. Previous studies of *Tbr1*$^{-/-}$ mice have indicated critical roles for TBR1 in the development of the cerebral cortex, amygdala, and olfactory bulb. Neuronal migration and axonal projection are two important developmental features controlled by TBR1. Recently, recurrent *de novo* disruptive mutations in the *TBR1* gene have been found in patients with autism spectrum disorders (ASDs). Human genetic studies have identified *TBR1* as a high-confidence risk factor for ASDs. Because only one allele of the TBR1 gene is mutated in these patients, *Tbr1*$^{+/-}$ mice serve as a good genetic mouse model to explore the mechanism by which *de novo TBR1* mutation leads to ASDs. Although neuronal migration and axonal projection defects of cerebral cortex are the most prominent phenotypes in *Tbr1*$^{-/-}$ mice, these features are not found in *Tbr1*$^{+/-}$ mice. Instead, inter- and intra-amygdalar axonal projections and NMDAR expression and activity in amygdala are particularly susceptible to *Tbr1* haploinsufficiency. The studies indicated that both abnormal brain wiring (abnormal amygdalar connections) and excitation/inhibition imbalance (NMDAR hypoactivity), two prominent models for ASD etiology, are present in *Tbr1*$^{+/-}$ mice. Moreover, calcium/calmodulin-dependent serine protein kinase (CASK) was found to interact with TBR1. The CASK–TBR1 complex had been shown to directly bind the promoter of the *Grin2b* gene, which is also known as *Nmdar2b*, and upregulate *Grin2b* expression. This molecular function of TBR1 provides an explanation for NMDAR hypoactivity in *Tbr1*$^{+/-}$ mice. In addition to *Grin2b*, cell adhesion molecules—including *Ntng1*, *Cdh8,* and *Cntn2*—are also regulated by TBR1 to control axonal projections of amygdala. Taken together, the studies of *Tbr1* provide an integrated picture of ASD etiology at the cellular and circuit levels.

Keywords: amygdala, axonal projection, autism, cerebral cortex, intellectual disability, neural circuit, neurodevelopmental disorders, TBR1

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INTRODUCTION

Autism spectrum disorders (ASDs) are heterogeneous and highly heritable neuropsychiatric disorders. Hundreds of genes with *de novo* copy-number variations or *de novo* point mutations have been identified in thousands of patients with ASDs (Gilman et al., 2011; Neale et al., 2012; O'roak et al., 2012a,b; De Rubeis et al., 2014; Iossifov et al., 2014). Although this variety of ASD-associated genes reflects the high heterogeneity of ASDs, ∼26 high-confidence risk genes for ASDs have been summarized from large scale whole-exome sequencing (O'roak et al., 2012a; De Rubeis et al., 2014; **Table 1**). Among these high-confidence risk genes, 11 encode either transcription factors or chromatin remodeling factors, indicating that the dysregulation of gene expression is a common pathogenic mechanism for ASDs (**Table 1**). To date, *T-BRAIN-1* (*TBR-1*) is the best studied transcription regulator among the high-confidence risk genes for ASDs. In this review, we summarize the physiological functions of TBR1 and the currently understood mechanisms by which *TBR1* mutations cause ASDs. Based on the data accumulated from the mouse model, we suggest that abnormal brain wiring and reduced neuronal activity in the amygdala are the primary causes for *TBR1*-dependent ASDs.

IDENTIFICATION OF TBR1 IN THE REGULATION OF BRAIN DEVELOPMENT

TBR1 contains a T-box DNA binding domain (**Figure 1**) and belongs to the T-box transcription factor family (Papaioannou, 2014). Twenty years ago, Dr. John Rubenstein's laboratory first identified *Tbr1* from a subtractive hybridization screen using cDNA libraries made from mouse embryonic day 14.5 (E14.5) and adult telencephalons (Bulfone et al., 1995). *Tbr1* mRNA levels were approximately 10-fold higher in E14.5 telencephalons than in adult telencephalons (Bulfone et al., 1995), suggesting a role for TBR1 in brain development. *In situ* hybridization and immunofluorescence staining indicate that *Tbr1* is expressed in the postmitotic neurons of the cerebral cortex, hippocampus, olfactory bulb and amygdala at the embryonic stages (Bulfone et al., 1995, 1998; Remedios et al., 2007; Huang et al., 2014). Using markers of projection neurons, including glutamate and CaMKII, TBR1 has been found to be further restricted to the projection neurons of the cerebral cortex, amygdala and olfactory bulb (Bulfone et al., 1998; Hevner et al., 2001; Huang et al., 2014). In the cerebral cortex, layer 6 neurons express the highest levels of TBR1. Projection neurons in the remaining layers also express TBR1, though the expression levels are lower

TABLE 1 | High-confidence risk factors for ASDs.

Gene symbol	Gene Name	Molecular function	References
ADNP	Activity-dependent neuroprotector homeobox	Transcription regulator	De Rubeis et al., 2014; SFARI
ANK2	Ankyrin 2, neuronal	Cytoskeleton interactor	De Rubeis et al., 2014; SFARI
ARID1B	AT rich interactive domain 1B (SWI1-like)	Transcription regulator	De Rubeis et al., 2014; SFARI
ASH1L	Ash1 (absent, small, or homeotic)-like (Drosophila)	Transcription regulator	De Rubeis et al., 2014; SFARI
ASXL3	Additional sex combs like transcriptional regulator 3	Transcription regulator	De Rubeis et al., 2014; SFARI
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	Transcription regulator	De Rubeis et al., 2014
CACNA2D3	Calcium channel, voltage-dependent, alpha 2/delta subunit 3	Ion channel	De Rubeis et al., 2014
CHD8	Chromodomain helicase DNA binding protein 8	Transcription regulator	De Rubeis et al., 2014; O'roak et al., 2012a; SFARI
CTTNBP2	Cortactin binding protein 2	Cytoskeleton interactor	De Rubeis et al., 2014
CUL3	Cullin 3	Protein degradation	De Rubeis et al., 2014
DYRK1A	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	Signaling	De Rubeis et al., 2014; O'roak et al., 2012a; SFARI
GABRB3	Gamma-aminobutyric acid (GABA) A receptor, beta 3	Ion channel	De Rubeis et al., 2014
GRIN2B	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	Ion channel	De Rubeis et al., 2014; O'roak et al., 2012a; SFARI
KATNAL2	Katanin p60 subunit A-like 2	Cytoskeleton interactor	De Rubeis et al., 2014
MIB1	Mindbomb E3 ubiquitin protein ligase 1	Protein degradation	De Rubeis et al., 2014
MLL3	Lysine (K)-specific methyltransferase 2C	Transcription regulator	De Rubeis et al., 2014
POGZ	Pogo transposable element with ZNF domain	Enzyme	De Rubeis et al., 2014; SFARI
PTEN	Phosphatase and tensin homolog	Phosphatase	De Rubeis et al., 2014; O'roak et al., 2012a; SFARI
RELN	Reelin	Signaling	De Rubeis et al., 2014
SCN2A	Sodium channel, voltage-gated, type II, alpha subunit	Ion channel	De Rubeis et al., 2014; SFARI
SETD5	SET domain containing 5	Transcription regulator	De Rubeis et al., 2014; SFARI
SHANK3	SH3 and multiple ankyrin repeat domains 3	Postsynaptic adaptor	SFARI
SUV420H1	Suppressor of variegation 4–20 homolog 1 (Drosophila)	Transcription regulator	De Rubeis et al., 2014; SFARI
SYNGAP1	Synaptic Ras GTPase activating protein 1	Signaling	De Rubeis et al., 2014; SFARI
TBL1XR1	Transducin (beta)-like 1 X-linked receptor 1	Transcription regulator	O'roak et al., 2012a
TBR1	T-box, brain, 1	Transcription regulator	De Rubeis et al., 2014; O'roak et al., 2012a; SFARI

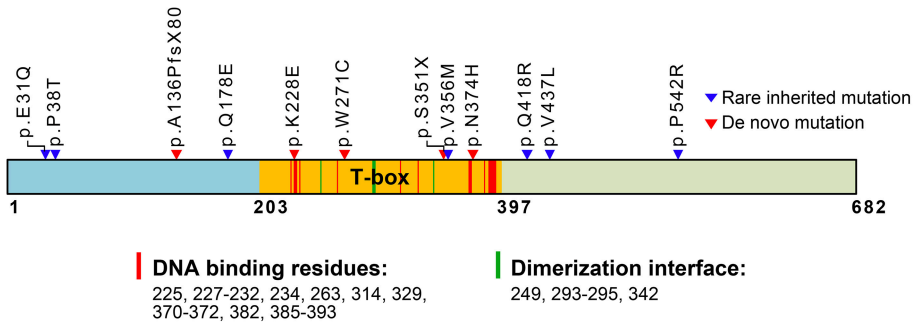

FIGURE 1 | Schematic domain structure of TBR1 and identified mutations in patients with ASDs. The T-box DNA binding domain extends from amino acid (aa) residue 203–397. The predicted aa residues for DNA binding and dimerization based on the T-box structure of Brachyury (T protein) are also indicated and labeled with red and green strips in the T-box. The positions of *de novo* mutations are labeled with red triangles; the positions of rare inherited mutations are labeled with blue triangles. The functions of the residues in the T-box are predicted based on comparison with the Brachury T-box (pfam00907: T-box, http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=250216).

(Hevner et al., 2001). In the amygdala, TBR1 is only expressed in the projection neurons of the lateral and basal amygdala (Huang et al., 2014). These studies clearly show that TBR1 is a projection neuron-specific T-box factor highly enriched in embryonic telencephalons.

Dr. John Rubenstein and colleagues generated *Tbr1*$^{-/-}$ mice to investigate the physiological function of *Tbr1* (Bulfone et al., 1998; Hevner et al., 2001). A homozygous deficiency of *Tbr1* results in neonatal lethality within 1–2 days after birth, indicating that *Tbr1* is essential for survival. Most projection neurons in the olfactory bulb, including mitral and tufted cells, and axonal output to the lateral olfactory tract are lost in *Tbr1*$^{-/-}$ mice (Bulfone et al., 1998). In the cerebral cortex, the inside-out pattern of neuronal migration is completely disrupted, as the six-layer laminar structure of the cortex is disorganized. Moreover, the contralateral axonal projections of the cerebral cortex and both corticothalamic and thalamocortical axonal projections are also defective, since they end mid-way to their final destinations in *Tbr1*$^{-/-}$ mice (Hevner et al., 2001). TBR1 is also required for neuronal migration in the amygdala. A portion of the dorsal pallium that migrates from the caudal telencephalon pole toward the rostral telencephalon forms the basal and lateral amygdala. In *Tbr1*$^{-/-}$ mice, this caudal-to-rostral migration is disrupted and thus impairs amygdala development (Remedios et al., 2007). Although TBR1 is also expressed in the hippocampus, its importance in hippocampus development and function remains unclear.

Based on the studies using *Tbr1*$^{-/-}$ mice, it is clear that TBR1 is critical for development of projection neurons in the cerebral cortex, olfactory bulb and amygdala.

TBR1 DOWNSTREAM TARGET GENES

Target genes of the TBR1 transcription factor were first identified by searching a database using the target sequence of the T-box DNA binding domain (Hsueh et al., 2000; Wang et al., 2004a,c). Because members of the T-box protein family share a DNA binding sequence, this method cannot ensure that the target genes are specific for TBR1. Because TBR1 is neuron-specific, neuronal expression is the first criterion to further screen the TBR1 target genes identified from sequence analysis. Results of an electrophoretic mobility shift assay, chromatin immunoprecipitation and a luciferase reporter assay have shown that TBR1 directly binds to the promoters and regulates the promoter activity of *Grin2b* (*Glutamate receptor, ionotropic, N-methyl-aspartate 2b*, also known as *Nmdar2b*) and *Reln* (*Reelin*) (Hsueh et al., 2000; Wang et al., 2004a,c). Changes in RELN and NMDAR2B protein levels have also been confirmed in *Tbr1*$^{-/-}$ mice (Hevner et al., 2001; Wang et al., 2004c). Because *Reln* encodes an extracellular protein that is critical for neuronal migration (Martinez-Cerdeno and Noctor, 2014; Ohshima, 2014; Sekine et al., 2014), regulation of *Reln* expression by TBR1 could explain the migration phenotype in *Tbr1*$^{-/-}$ mice. Regulation of *Grin2b* expression by TBR1 is critical for neuronal activation, which we discuss further in a later section.

Both our and Dr. Robert Hevner's laboratories independently applied microarray analyses to identify TBR1 downstream genes. Using E14.5 and P0.5 mouse brains, Dr. Hevner's laboratory focused on the arealization and lamination of the cerebral cortex (Bedogni et al., 2010). *Tbr1* exhibits a high rostral and low caudal expression pattern in the cortex (Bulfone et al., 1995). At both E14.5 and P0.5, a *Tbr1* deletion noticeably alters the expression of regional markers. In general, rostral genes are downregulated in *Tbr1*$^{-/-}$ brains, while caudal genes are upregulated. For cortical layer markers, most markers of layer 6, subplate and Cajal–Retzius cells exhibit noticeably reduced expression levels in *Tbr1*$^{-/-}$ brains. The majority of layer 2–5 markers are upregulated (Bedogni et al., 2010). The markers of lamination and arealization whose expression levels are altered in *Tbr1*$^{-/-}$ brains are listed in **Table 2**. These studies indicate that TBR1 is critical for controlling the neuronal specification of the cerebral cortex.

In our laboratory, we have identified more than 124 genes that are regulated by TBR1 at E16.5 (Huang et al., 2014; Chuang et al., 2015). The 16 region- or layer-specific genes presented in work by Bedogni et al. (2010) from Dr. Hevner's

TABLE 2 | *Tbr1* deletion alters expression of genes with layer- or region-specific distribution in the cerebral cortex.

Gene symbol*	Gene name	Layer	Region
Bhlhb5 (BHLHE22)	Basic helix-loop-helix family, member e22	L5	Caudomedial
Calb2	Calbindin 2	Cajal–Retzius	All
Cdh8*	Cadherin 8, type 2	L5	Rostral + caudal
Cdh9	Cadherin 9, type 2 (T1-cadherin)	L6	Rostral
Cntn3	Contactin 3 (plasmacytoma associated)	L5	Caudal
Cntn6*	Contactin 6	L5 SCPN	Caudal
Cplx3	Complexin 3	Cajal–Retzius	All
Crim1*	Cysteine rich transmembrane BMP regulator 1 (chordin-like)	L5 corticospinal motor neuron	All
Crym	Crystallin, mu	L5 corticospinal motor neuron	Caudal
Ctgf*	Connective tissue growth factor	Subplate	All
Ctip2 (Bcl11b)	B-cell CLL/lymphoma 11B (zinc finger protein)	L5 SCPN	All
Cux1	Cut-like homeobox 1	L2–4	All
Cux2	Cut-like homeobox 2	L2–4	All
Dkk3	Dickkopf WNT signaling pathway inhibitor 3	CPN	Caudomedial
Drd1a*	Dopamine receptor D1	L6	All
Dtx4	Deltex 4, E3 ubiquitin ligase	L2–4	All
Etv1 (Er81)	Ets variant 1	L5	All
Fezf2	FEZ family zinc finger 2	L5 SCPN	All
Flrt3*	Fibronectin leucine rich transmembrane protein 3	L2–3	Caudal
Foxp1	Forkhead box P1	L5	All
Foxp2	Forkhead box P2	L6	All
Inhba	Inhibin, beta A	L2–4, CPN	All
Lhx5	LIM homeobox 5	Cajal–Retzius	All
Limch1	LIM and calponin homology domains 1	L2–3, CPN	All
Mdga1	MAM domain containing glycosylphosphatidylinositol anchor 1	L2–3	Caudal
Mef2c	Myocyte enhancer factor 2C	L2–3	All
Nefm*	Neurofilament, medium polypeptide	L2–3	Caudal
Nfe2l3	Nuclear factor, erythroid 2-like 3	L6	All
Ngfr	Nerve growth factor receptor	L6	Caudal
Nhlh2*	Nescient helix loop helix 2	Cajal–Retzius	All
Npy	Neuropeptide Y	L6	All
Nr4a2 (Nurr1)*	Nuclear receptor subfamily 4, group A, member 2	L6	Caudal
Nr4a3	Nuclear receptor subfamily 4, group A, member 3	L6	Caudal
Nrgn*	Neurogranin	L2–4	All
Nxph4	Neurexophilin 4	Subplate	All
Oma1	OMA1 zinc metallopeptidase	L5 SCPN	All
Pcdh11x	Protocadherin 11 X-linked	L2–4	All
Pcdh19	Protocadherin 19	L5	All
Pcdh20	Protocadherin 20	L2–4	Rostral
Pcdh8	Protocadherin 8	L2–3	Caudal
Pcp4	Purkinje cell protein 4	L5	All
Pou3f2 (Brn-2)	POU class 3 homeobox 2	L2–4	All
Pou3f3 (Brn-1)	POU class 3 homeobox 3	L2–4	All
Ppp1r1b*	Protein phosphatase 1, regulatory (inhibitor) subunit 1B	L6	Rostral
Ptn	Pleiotrophin	CPN	All
Ptprz1	Protein tyrosine phosphatase, receptor-type, Z polypeptide 1	L2–3	Caudal
Pvrl3	Poliovirus receptor-related 3	L2–3, CPN	Caudomedial
Reln*	Reelin	Cajal–Retzius	All
Rgs8	Regulator of G-protein signaling 8	L2–3	All
Rorb*	RAR-related orphan receptor B	L4	Rostral
S100a10	S100 calcium binding protein A10	L5 corticospinal motor neuron	All

(Continued)

TABLE 2 | Continued

Gene symbol*	Gene name	Layer	Region
Satb2	SATB homeobox 2	CPN	All
Sorl1	Sortilin-related receptor, L(DLR class) A repeats containing	L2–3	All
Sox5*	SRY (sex determining region Y)-box 5	L6	All
Tle1	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	L2–3	All
Tle4*	Transducin-like enhancer of split 4	L6	All
Tox	Thymocyte selection-associated high mobility group box	L5	Rostromedial
Trp73	Tumor protein p73	Cajal–Retzius	All
Unc5c	Unc-5 homolog C (C. elegans)	L4	All
Unc5d (Svet1)	Unc-5 homolog D (C. elegans)	L2–4	All
Wnt7b*	Wingless-type MMTV integration site family, member 7B	L6	All
Wscd1	WSC domain containing 1	L6	Rostral
Zfpm2 (FOG2)	Zinc finger protein, FOG family member 2	L6	All

**Genes also identified in our microarray analysis (Huang et al., 2014). CPN, callosal projection neurons; SCPN, subcerebral projection neurons.*

laboratory are also included in our gene list (**Table 2**). Moreover, based on a literature search and the database of the Simons Foundation Autism Research Initiative (https://gene.sfari.org/autdb/Welcome.do), 23 ASD-associated genes and a dyslexia causative gene, Kiaa0319, have also been found to be regulated by TBR1. Changes in the expression of these ASD- and dyslexia-associated genes (**Table 3**) provide support for the influence of TBR1 in ASDs. Tbr1 might act as master gene controlling the expression of a panel of ASD-associated genes and thus influence neural development and function (Chuang et al., 2015).

Moreover, the expression levels of 15 transcriptional regulators are reduced, while those of three transcription factors are upregulated in *Tbr1*$^{-/-}$ brains compared with wild-type littermates (**Table 4**). These changes suggest that, in addition to directly regulating gene expression, TBR1 also controls transcriptional networks to influence neuronal development. Indeed, evidence has indicated that TBR1 directly binds to the locus of *Fezf2*, a layer 5-specific transcription factor, and represses *Fezf2* expression in layer 6 to specify the corticothalamal projections of layer 6 neurons (Han et al., 2011; McKenna et al., 2011). The second transcriptional regulator directly controlled by TBR1 is autistic susceptibility gene2 (*Auts2*). TBR1 binds to the region around the *Auts2* transcriptional start site and activates expression of the *Auts2* gene (Bedogni et al., 2010). AUTS2 is part of polycomb repressive complex I (PRCI) that catalyzes the monoubiquitination of histone H2A and epigenetically represses gene expression, particularly during the developmental stage (de Napoles et al., 2004; Wang et al., 2004b). In contrast to the canonical role of PRCI in gene repression, the PRCI–AUST2 complex activates neuronal gene expression by recruiting casein kinase 2 and p300 to chromatin (Gao et al., 2014). The activation of *Auts2* expression by TBR1 supports the influence of TBR1 on global gene expression in neurons.

In addition to using a transcriptional cascade to indirectly control gene expression, TBR1 may also alter the relative number of projection neurons and interneurons in the brain and influence the total expression levels of certain genes, such as *Gad1*, which encodes glutamate decarboxylase 1 (GAD67)—an essential gene of GABAergic neurons. In *Tbr1* deletion mice, the expression of *Gad1* is noticeably upregulated (Chuang et al., 2015). Because Tbr1 is specifically expressed in glutamatergic projection neurons, it is possible to speculate that increased *Gad1* expression is indirectly linked to a reduction in the population of glutamatergic neurons.

Consistent with the function of TBR1 in the regulation of axonal projection, TBR1 also regulates eight membrane proteins (CNTN2, CDH8, GPC6, CD44, FLRT3, CNTN6, NTNG1, and KIAA0319) that are involved in cell adhesion; although it is still unclear whether these genes are directly or indirectly regulated by TBR1 (Chuang et al., 2015). Interestingly, seven of these eight membrane proteins are upregulated in *Tbr1*$^{-/-}$ brains (**Table 5**). Because these genes control cell adhesion and axonal growth, the impairment of axonal projection in *Tbr1* deficient neurons is likely due to imbalanced cell–cell and cell–matrix interactions. Alteration of the strength of these interactions may preclude neurite growth and extension (Chuang et al., 2015).

In conclusion, TBR1 controls the expression of a series of genes that regulate cell-cell adhesion, axonal growth, neurotransmission and gene expression.

TBR1 INTERACTING PROTEINS

To date, only two proteins, CASK and FOXP2, have been identified as interacting partners with TBR1. Both CASK and FOXP2 are associated with ASDs (Samuels et al., 2007; O'roak et al., 2011). TBR1 was identified as a binding partner for CASK from a yeast two-hybrid screen using the guanylate kinase domain of CASK as bait (Hsueh et al., 2000). The C-terminal region of TBR1 is required for the interaction with CASK (Hsueh et al., 2000). CASK—a multidomain adaptor protein—is widely distributed in various subcellular compartments and interacts with more than two dozen cellular proteins (Hsueh, 2006). The interaction with CASK increases the transcriptional activity of TBR1 (Hsueh et al., 2000) by recruiting a nucleosome assembly protein CINAP (CASK interacting nucleosome assembly protein,

TABLE 3 | TBR1 influences the expression (upregulation or downregulation) of genes associated with autism or dyslexia.

Gene symbol	Gene name	Molecular function	$Tbr1^{-/-}$ vs. WT	Disease
Cd44	CD44 molecule (Indian blood group)	Cell adhesion	Up	Autism
Cdh8	Cadherin 8, type 2	Cell adhesion	Up	Autism
Cntn6	Contactin 6	Cell adhesion	Up	Autism
Gpc6	Glypican 6	Cell adhesion	Up	Autism
Ntng1	Netrin G1	Cell adhesion	Up	Autism
Kiaa0319	Hypothetical protein D130043K22	Cell adhesion	Up	Dyslexia
Nefl	Neurofilament, light polypeptide	Cytoskeleton	Up	Autism
Gpd2	Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	Enzyme	Up	Autism
Drd1	Dopamine receptor D1A	Neurotransmission	Down	Autism
Gad1	Glutamate decarboxylase 1 (brain, 67 kDa)	Neurotransmission	Up	Autism
Grin2b	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	Neurotransmission	Down	Autism
Baiap2	BAI1-associated protein 2	Signaling	Down	Autism
Lasp1	LIM and SH3 protein 1	Signaling	Down	Autism
Lypd6	LY6/PLAUR domain containing 6	Signaling	Down	Autism
Ppp1r1b	Protein phosphatase 1, regulatory (inhibitor) subunit 1B	Signaling	Down	Autism
Ptprk	Protein tyrosine phosphatase, receptor type, K	Signaling	Down	Autism
Reln	Reelin	Signaling	Down	Autism
Auts2	Autism susceptibility candidate 2	Transcription factor	Down	Autism
Nfia	Nuclear factor I/A	Transcription factor	Down	Autism
Nr4a2	Nuclear receptor subfamily 4, group A, member 2	Transcription factor	Down	Autism
Sox5	SRY (sex determining region Y)-box 5	Transcription factor	Down	Autism
Slc4a10	Solute carrier family 4, sodium bicarbonate transporter, member 10	Transporter	Up	Autism
Stxbp6	Syntaxin binding protein 6 (amisyn)	Vesicle trafficking	Up	Autism
Sv2b	Synaptic vesicle glycoprotein 2B	Vesicle trafficking	Down	Autism

also known as testis specific protein Y-encoded like 2, TSPYL2) to the promoter region containing the T-box DNA binding motif (Wang et al., 2004a). CINAP also interacts with the guanylate kinase domain of CASK. However, it does not compete with TBR1 for CASK binding. Instead, TBR1, CASK and CINAP form a tripartite complex to regulate *Grin2b* expression (Wang et al., 2004a,c). *CASK* is well-known as a causative gene in X-linked mental retardation (Najm et al., 2008). The interaction of CASK and TBR1 and the consequent effect on the regulation of *Grin2b* expression and neural development has been suggested to contribute to the phenotype of patients with *CASK* mutations (Hsueh, 2009). It can also be speculated that *Grin2b* expression, as controlled by the TBR1–CASK complex, might also be involved in ASDs due to *TBR1* or *CASK* mutations.

FOXP2 is a critical transcription factor that controls speech (Lai et al., 2001; Enard et al., 2002) and is also associated with ASDs (Gong et al., 2004; Li et al., 2005). In contrast to the interaction between CASK and TBR1, the interaction between TBR1 and FOXP2 is less clear. Research suggests that both the T-box and C-terminal regions of TBR1 are involved in the interaction with FOXP2 (Deriziotis et al., 2014). For FOXP2, both its N- and C-terminal regions contribute to the interaction between FOXP2 and TBR1 (Deriziotis et al., 2014). Although it has been speculated that the interaction of FOXP2 and TBR1 is likely relevant to the verbal deficits in ASD patients, the molecular function of the TBR1-FOXP2 interaction is unclear. Furthermore, FOXP2 and TBR1 are only coexpressed in layer 6 of the cerebral cortex and not in other layers of the cerebral cortex and amygdala. Thus, the interaction with FOXP2 can only partly account for the function of TBR1.

TBR1, its binding partners CASK and FOXP2 and its direct downstream target GRIN2B, are all associated with ASDs, reinforcing the role of TBR1 in ASDs.

TBR1 MUTATIONS ASSOCIATE WITH NEUROLOGICAL DISORDERS

Genetic analyses of patients have identified *TBR1* as a high-confidence risk factor for ASDs (https://gene.sfari.org/autdb/GSGeneList.do?c=1). Identified mutations in TBR1 genes are summarized in **Figure 1**. Both *de novo* and inherited mutations in *TBR1* have been found in patients with ASDs (**Figure 1**). Two of the mutations, p.A136PfsX80 and p.S351X, result in early termination and generate truncated proteins that lack a functional DNA-binding T-box domain (O'roak et al., 2012a,b; De Rubeis et al., 2014). These two truncated mutants can no longer function in transcription or in interactions with CASK and FOXP2. The remaining three *de novo* mutations are p.K228E, p.W271C, and p.N374H (**Figure 1**). Based on simulations with the T-box DNA binding domain of Brachury (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=250216), the K228 residue is predicted to directly contribute to DNA binding (**Figure 1**). Thus, the p.K228E mutation is expected to disrupt

TABLE 4 | Deletion of *Tbr1* influences the expression of a panel of transcription factors in neurons.

Gene Symbol	Tbr1$^{-/-}$ vs. WT	Gene name
Bcl6	0.3623	B-cell leukemia/lymphoma 6
Nfe2l3	0.4017	Nuclear factor, erythroid derived 2, like 3
Nhlh2	0.4219	Nescient helix loop helix 2
Sox5	0.4875	BB018032 RIKEN full-length enriched, adult male testis (DH10B) *Mus musculus* cDNA clone 4930572C18 3′ similar to AJ010604 *Mus musculus* mRNA for transcription factor L-Sox5, mRNA sequence.
Nr4a2	0.5179	BB703394 RIKEN full-length enriched, *in vitro* fertilized eggs *Mus musculus* cDNA clone 7420451N07 3′, mRNA sequence.
Rorb	0.529	RAR-related orphan receptor beta
Tp73	0.5355	Transformation related protein 73
Btbd11	0.5719	BTB (POZ) domain containing 11
Tle4	0.5821	Transducin-like enhancer of split 4, homolog of Drosophila E(spl)
Rbm14	0.59	RNA binding motif protein 14
Foxf2	0.5983	Forkhead box F2
Tfap2c	0.6348	Transcription factor AP-2, gamma
Zswim4	0.639	Zinc finger, SWIM domain containing 4
Nfia	0.643	RIKEN cDNA 9430022M17 gene
Neurod6	0.6644	Neurogenic differentiation 6
Runx1t1	1.5064	CBFA2T1 identified gene homolog (human)
Ascl1	1.6328	Achaete–scute complex homolog-like 1 (Drosophila)
Pou3f1	1.6528	POU domain, class 3, transcription factor 1

TABLE 5 | *Tbr1* deletion mainly upregulates expression of cell adhesion molecules in neurons.

Gene symbol	Tbr1$^{-/-}$ vs. WT	Gene name
Cdh8	1.5013	Cadherin 8
Gpc6	1.5874	Glypican 6
Cd44	1.7384	CD44 antigen
Flrt3	1.8118	Fibronectin leucine rich transmembrane protein 3
Cntn6	2.0967	Contactin 6
Ntng1	2.3218	Netrin G1
Kiaa0319	2.8456	Hypothetical protein D130043K22
Cntn2	0.6331	Contactin 2

the DNA binding ability of TBR1. The residues W271 and N374 are adjacent to the DNA binding residues (**Figure 1**). Thus, the p.W271C and p.N374H mutations could alter protein conformation and indirectly influence DNA binding. The p.V356M inherited mutation is localized in the T-box, but it is relatively far from the DNA binding and dimerization motifs. The remaining inherited mutations, including p.E31Q, p.P38T, p.Q178E, p.Q418R, p.V437L, and p.P542R, are localized to the N- and C-terminal regions and are not known to influence DNA binding. The impact of these inherited mutations is unclear.

To date, only two studies have analyzed the effects of these ASD mutations on TBR1 function. We contributed to the first functional study, which examined axonal growth in the amygdalar neurons of the TBR1 N374H mutant (Huang et al., 2014). An experiment comparing *Tbr1*$^{+/-}$ and wild-type amygdalar neurons showed that the deletion of one *Tbr1* gene results in multiple and shorter axons in amygdalar neurons (Huang et al., 2014). The reintroduction of wild-type *Tbr1* into *Tbr1*$^{+/-}$ amygdalar neurons effectively promotes axon growth and reduces the percentage of neurons carrying multiple axons to the levels seen in wild-type neurons. However, the N374H mutant fails to rescue the axonal defects of *Tbr1*$^{+/-}$ amygdalar neurons, suggesting that the p.N374H mutation identified in patients with ASD results in a loss of function (Huang et al., 2014).

The second study focused on the effect of ASD mutations on the subcellular distribution, transcriptional activity, dimerization and protein-protein interaction of TBR1 using heterologous HEK293 cells as a model (Deriziotis et al., 2014). De novo mutations, including p.K228E and p.N374H, change the subcellular distribution of TBR1 in HEK293 cells. The mutant proteins tend to form large aggregates in the nuclei. The impact of these two mutations on the transcriptional activity of TBR1 is unclear because the luciferase reporter assay did not show a difference between the wild-type TBR1 and K228E and N374H mutants. However, similar to the truncated mutants, the K228E and N374H mutants no longer interact with FOXP2, which is consistent with the observation that the T-box domain is also involved in FOXP2 interactions, as described above. The mechanisms by which rare inherited mutations impair the function of TBR1 remain largely unclear, except for the p.Q418R mutation, which is known to reduce the interaction between TBR1 and FOXP2. Because TBR1 is a projection neuron-specific transcription factor, the relevance of the interaction between TBR1 and FOXP2 must be investigated in neurons instead of HEK293 cells.

In addition to ASDs, *TBR1* is also associated with intellectual disability. The *TBR1* locus is at chromosome 2q24.2. Both a microdeletion of the chromosome region that contains 2q24.2 and *de novo* mutations of the *TBR1* gene have been found in

patients with intellectual disabilities (Traylor et al., 2012; Burrage et al., 2013; Hamdan et al., 2014; Palumbo et al., 2014). Moreover, the expression levels of TBR1 are increased in patients that suffer from schizophrenia (Molnar et al., 2003). Taken together, TBR1 is closely associated with ASDs, schizophrenia and intellectual disability.

Tbr1 HAPLOINSUFFICIENCY RESULTS IN NEURONAL DEFECTS

In ASD patients, only one of the two *TBR1* alleles is mutated (Neale et al., 2012; O'roak et al., 2012a,b; De Rubeis et al., 2014; Deriziotis et al., 2014). Several possibilities may explain the effect of *TBR1* heterozygosity on brain function: haploinsufficiency or a dominant negative or gain-of-function effect of the mutated allele. Because two of the *de novo* mutations of the *TBR1* gene, p.A136PfsX80, and p.S351X, result in early termination and generate truncated proteins that lack a full length T-box DNA binding domain and dimerization domain (**Figure 1**), the mutants are not expected to exert a dominant negative effect on the activity of TBR1 based on the known molecular function of TBR1. Instead, the defects are likely caused by haploinsufficiency. $Tbr1^{+/-}$ neurons are characterized by shorter and multiple axons (Huang et al., 2014), indicating that loss of a copy of the *Tbr1* gene results in abnormal neuronal differentiation. Thus, *TBR1* deficits in patients are likely due to haploinsufficiency.

$Tbr1^{+/-}$ MICE SERVE AS A MOUSE MODEL FOR ASDS

Because only one of two *TBR1* alleles is mutated in patients with ASDs and *Tbr1* heterozygosity does not influence survival and the general health of mice, $Tbr1^{+/-}$ mice serve as a good animal model to elucidate the role of *Tbr1* in ASDs. The core symptoms of patients with ASDs are both verbal and non-verbal communication defects, impaired social interaction and cognitive inflexibility. ASDs are also frequently associated with learning disability. A series of behavior paradigms have been applied to characterize the behavioral defects of *Tbr1* (**Table 6**). Compared with wild-type littermates, the locomotor and exploratory activities, the level of anxiety and the hippocampus-dependent memory of $Tbr1^{+/-}$ mice are normal (Huang et al., 2014). However, the amygdala-dependent behaviors of $Tbr1^{+/-}$ mice are noticeably affected. Conditioned taste aversion and auditory fear conditioning—two amygdala-dependent learning and memory paradigms—are both impaired in $Tbr1^{+/-}$ mice. Cognitive flexibility, as examined by appetitive-motivated T-maze and two-choice digging tests, is also noticeably reduced in $Tbr1^{+/-}$ mice. The three-chamber test, reciprocal social interactions and social transmission of food preferences have also been applied to characterize the social interactions of $Tbr1^{+/-}$ mice. These paradigms all indicate that the social interactions of $Tbr1^{+/-}$ mice are impaired. Moreover, the frequency of ultrasonic vocalization is significantly lower in isolated $Tbr1^{+/-}$ pups. Thus, these behavioral analyses strongly support that $Tbr1^{+/-}$ mice exhibit autism-like behaviors (Huang et al., 2014).

TABLE 6 | *Tbr1* haploinsufficiency results in autism-like behaviors in mice.

Behavior paradigm	Assay for	Phenotype in $Tbr1^{+/-}$ mice
Conditioned taste aversion	Learning and memory	Learning defect
Auditory fear conditioning	Learning and memory	Learning defect
T-maze test	Reverse learning	Cognition inflexibility
Two-choice digging task	Reverse learning	Cognition inflexibility
Three-chamber test	Social interaction	Poor social interaction
Reciprocal social interactions	Social interaction	Poor social interaction
Social transmission of food preference	Social interaction	Poor social interaction
Ultrasonic vocalizations	Communication	Poor communication

DEFECTS IN AMYGDALAR CIRCUITS AND ACTIVATION ARE CRITICAL FOR ASDS CAUSED BY *Tbr1* DEFICIENCY

When *Tbr1* is completely deleted from mice, the most prominent phenotypes are observed in the neuronal migration and axonal projection of the embryonic cerebral cortex (Bulfone et al., 1998; Hevner et al., 2001). However, none of these defects are found in the $Tbr1^{+/-}$ brain (Huang et al., 2014). Cortical lamination, contralateral cortical projection, corticothalamal projection and the size of the cerebral cortex of $Tbr1^{+/-}$ brains are comparable to those of wild-type brains (Huang et al., 2014). Unexpectedly, the posterior part of the anterior commissure is either missing or dramatically reduced in $Tbr1^{+/-}$ mice (Huang et al., 2014). This defect is 100% penetrant in all $Tbr1^{+/-}$ mice. Thus, the posterior part of the anterior commissure is the structure most sensitive to *Tbr1* haploinsufficiency. Consequently, defects of the posterior part of the anterior commissure are more relevant to the pathogenesis of *TBR1*-dependent ASDs.

The posterior part of the anterior commissure serves to connect the contralateral amygdalae (interamygdalar projections). The amygdala contains three major nuclei, namely the lateral, basal and central amygdala (**Figure 2**). Interamygdalar projections emerge from the lateral and basal amygdala. These two nuclei also project to the ipsilateral central amygdala (intraamygdalar projections). The lateral and basal amygdalae are the nuclei that receive inputs from the cortex, thalamus and hippocampus. To induce a freezing response, the lateral and basal amygdalae deliver the signals to the central amygdala, and the central amygdala further projects to the brainstem and hypothalamus. In addition to the central amygdala, the lateral and basal amygdalae also project back to the cortex, hippocampus, and thalamus, which are believed to regulate memory and social behavior (Lee et al., 2013; Janak and Tye, 2015). Because the amygdala is the pivotal brain structure for social intelligence, the amygdala is an obvious target for the etiology of *TBR1*-dependent ASDs.

The results of DiI tracing and retrograde red bead labeling show that both inter- and intra-amygdalar axonal projections

are noticeably impaired in *Tbr1*$^{+/-}$ mice (**Figure 3**; Huang et al., 2014). The lateral and basal amygdala neurons are the major target of Tbr1 haploinsufficiency. Consistent with the axonal projection defects in the brain, *Tbr1*$^{+/-}$ amygdala neurons possess shorter and multiple axons, which suggests that *Tbr1* haploinsufficiency results in a cell-autonomous effect that restricts axonal extension and differentiation (Huang et al., 2014).

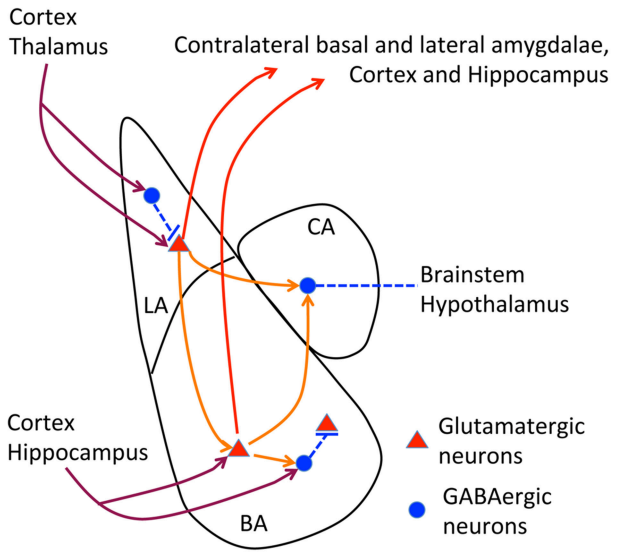

FIGURE 2 | Neural circuits of amygdala. The relative position of lateral, basal and central amygdalae and the known axonal connections of amygdala are summarized. In lateral and basal amygdalae, glutamatergic neurons comprise the majority of cells. In contrast, GABAergic neurons are the major population in the central amygdala. Dark red lines, afferent pathways from cortex, thalamus and hippocampus; red lines, efferent pathways to contralateral amygdala, cortex and hippocampus; orange lines, intraamygdalar pathways. The central amygdala also delivers signals to the brainstem and hypothalamus to regulate freezing and emotion responses.

To investigate how TBR1 controls axonal growth, we examined the TBR1 downstream target genes in a gene list from our microarray data. Specifically, we examined *Cntn2*, *Cdh8*, and *Ntng1* because these three genes encode adhesion proteins that regulate neurite outgrowth and fasciculation (Furley et al., 1990; Stoeckli and Landmesser, 1995; Kunz et al., 1998; Nakashiba et al., 2002; Bekirov et al., 2008). Restoring the expression levels of *Cntn2*, *Cdh8*, and *Ntng1* in *Tbr1*$^{+/-}$ amygdalar neurons effectively ameliorates axonal growth and differentiation in cultures and promotes axonal projection to form the posterior part of the anterior commissure *in vivo*. Thus, TBR1 controls the expression of a panel of genes that regulate amygdalar axonal projections. Note that although the axonal projections of lateral and basal amygdalae are significantly impaired in *Tbr1*$^{+/-}$ brains, the size and cell density of the lateral and basal amygdalae do not differ between *Tbr1*$^{+/-}$ mice and wild-type littermates. It is unclear whether *Tbr1*$^{+/-}$ amygdalar neurons mistarget to other brain regions. More investigations are needed to characterize this regulation in detail.

Although the significance of interamygdalar connections is unclear, a reduction of intraamygdalar axonal projections implies that the amygdala is functionally impaired in *Tbr1*$^{+/-}$ mice. Indeed, neuronal activation of the amygdala is impaired in *Tbr1*$^{+/-}$ mice (Huang et al., 2014). Two amygdala-dependent learning/memory paradigms—conditioned taste aversion and auditory fear conditioning—have been used to investigate amygdalar responses. Both c-FOS and NMDAR2B protein levels were used to monitor neuronal activation in amygdalae. These experiments showed that the induction of both c-FOS and NMDAR2B are either much lower or completely absent in the lateral and basal amygdalae of *Tbr1*$^{+/-}$ mice after conditioned taste aversion training and auditory fear conditioning (Huang et al., 2014; Chuang et al., 2015). Thus, both axonal projection and neuronal activation are defective in *Tbr1*$^{+/-}$ amygdalae.

Possibly due to an impairment of NMDAR induction in the *Tbr1*$^{+/-}$ amygdala, electrophysiological recording data showed

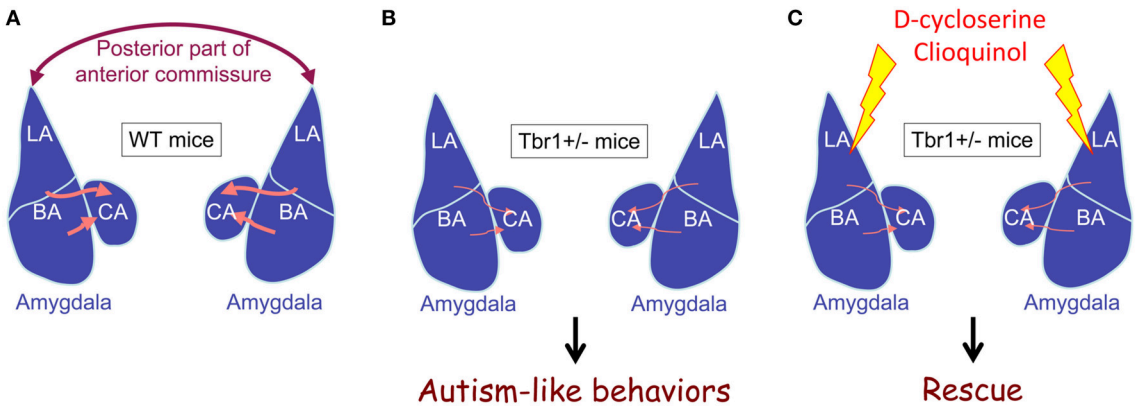

FIGURE 3 | *Tbr1* haploinsufficiency results in defects of intra- and inter-amygdalar axonal projections. (A) Wild-type mice. **(B)** *Tbr1*$^{+/-}$ mice. **(C)** *Tbr1*$^{+/-}$ mice with D-cycloserine or clioquinol injection. The interamygdalar projection via the posterior part of the anterior commissure is significantly impaired in *Tbr1*$^{+/-}$ mice. Intraamygdalar connections between basolateral and central amygdalae are also noticeably reduced in *Tbr1*$^{+/-}$ mice. *Tbr1*$^{+/-}$ mice are characterized by autism-like behaviors. Increased neuronal activity in the amygdala using either D-cycloserine or clioquinol restores behavioral defects to normal levels, although axonal projection defects are not rescued.

that the NMDA/AMPA ratio is noticeably lower in the thalamic-lateral amygdala synapses of *Tbr1*$^{+/-}$ brains compared with those of wild-type brains (Lee et al., 2015b). Consistent with amygdala-specific defects in *Tbr1*$^{+/-}$ mice, the hippocampal Schaffer collateral-CA1 pyramidal synapses do not exhibit this abnormal NMDA/AMPA ratio (Lee et al., 2015b). These electrophysiological studies clearly demonstrate a functional NMDAR deficiency in *Tbr1*$^{+/-}$ amygdalae.

If impairment of amygdala activation, and particularly reduced NMDAR activity, is critical for autism-like behaviors in *Tbr1*$^{+/-}$ mice, the activation of amygdalar neurons should ameliorate the behavioral defects of *Tbr1*$^{+/-}$ mice. Indeed, a bilateral local infusion of D-cycloserine (a coagonist of NMDAR) into the amygdala clearly ameliorates the reciprocal social interaction and conditioned taste aversion defects seen in *Tbr1*$^{+/-}$ mice (Huang et al., 2014). D-cycloserine applied 30 min before the behavioral assay does not influence the expression of TBR1 target genes, suggesting the behavioral effects are mediated by an acute enhancement of NMDAR transmission. Because administration of D-cycloserine to the amygdala is sufficient to ameliorate the behavioral defects of *Tbr1*$^{+/-}$ mice, the etiology of autism-like behaviors in *Tbr1*$^{+/-}$ mice very likely involves amygdala defects (Huang et al., 2014).

In addition to local infusion, systemic administration of D-cycloserine via an intraperitoneal injection also effectively restores neuronal activation of the *Tbr1*$^{+/-}$ amygdala and improves social interaction, cognitive inflexibility and associative memory of *Tbr1*$^{+/-}$ mice (Huang et al., 2014). These results indicate a potential therapeutic avenue for ASD patients possessing *TBR1* gene mutations. *Tbr1*$^{+/-}$ mice are not the only mouse model to have been used to demonstrate the beneficial effect of D-cycloserine. The behavioral deficits of *Shank2*$^{-/-}$, *Nlgn1*$^{-/-}$, and *Grid1*$^{-/-}$ mice can also be ameliorated by systemic administration of D-cycloserine. Specifically, D-cycloserine improves social interactions in both *Shank2*$^{-/-}$ and *Grid1*$^{-/-}$ mice in a three-chamber test (Won et al., 2012; Yadav et al., 2012). Furthermore, D-cycloserine reduces the repetitive grooming behavior of *Nlgn1*$^{-/-}$ mice (Blundell et al., 2010). These three mutant mice all show NMDAR defects, which is consistent with the idea that NMDAR deficits are critical to the etiology of ASDs (Lee et al., 2015a). Improving NMDAR activity can ameliorate the behavioral defects of these mutant mice.

To further support the NMDAR deficit hypothesis in ASDs, we recently showed that by improving NMDAR activity via the administration of clioquinol, the social defects of *Tbr1*$^{+/-}$ mice are rescued (Lee et al., 2015b). Clioquinol is a zinc chelator and ionophore that promotes the mobilization of zinc from presynaptic vesicles to the postsynaptic site. The postsynaptic elevation of zinc activates the protein tyrosine kinase SRC and consequently enhances NMDAR activity. Systemic administration of clioquinol noticeably improves the sociability of the mutant mice in the three-chamber test. Consistent with the behavioral rescue, the defective electrophysiological responses of mutant brains are also ameliorated by clioquinol treatment. In *Tbr1*$^{+/-}$ brains, clioquinol can restore the reduced NMDA/AMPA ratio of the thalamic-lateral amygdala synapses. Clioquinol treatment also shows a beneficial effect on *Shank2*$^{-/-}$ mice. It enhances the NMDAR activity of hippocampal Schaffer collateral-CA1 pyramidal synapses in *Shank2*$^{-/-}$ mice (Lee et al., 2015b). Even though the molecular mechanisms responsible for the NMDAR deficit differ between *Tbr1*$^{+/-}$ and *Shank2*$^{-/-}$ mice, increasing NMDAR activity via D-cycloserine or clioquinol efficiently ameliorates the behavioral defects of these two mutant mice. These data support that an NMDAR deficit is likely to be a common pathogenic mechanism of ASDs. Moreover, studies using D-cycloserine and clioquinol suggest that activation of amygdalar neurons using suitable pharmacological treatments can ameliorate the behavioral defects caused by *Tbr1* haploinsufficiency, even though the axonal projection defects of the *Tbr1*$^{+/-}$ amygdala cannot be rescued in adult animals.

Tbr1 SERVES AS IMMEDIATE EARLY GENE TO CONTROL NEURONAL ACTIVATION IN MATURE NEURONS

Although the expression levels of *Tbr1* gradually decline after birth, the protein levels of TBR1 remain detectable in adult mouse brains (Hsueh et al., 2000; Hong and Hsueh, 2007). Based on the following scenario, TBR1 may also play a role in the adult brain. TBR1 regulates *Grin2b* expression (Wang et al., 2004a), and CASK phosphorylation by protein kinase A (PKA) enhances this regulation. CASK phosphorylation increases the interaction between TBR1 and CASK and thus upregulates *Grin2b* promoter activity (Huang et al., 2010). Therefore, PKA phosphorylation may increase the ability of TBR1 to influence *Grin2b* expression, even though the expression levels of *Tbr1* are lower in adult brains.

A study of *in vitro* cultured neurons unexpectedly showed that glutamate and bicuculline treatments noticeably upregulates *Tbr1* expression (Chuang et al., 2014). Two to six hours after glutamate or bicuculline treatment, both the RNA and protein levels of *Tbr1* are obviously increased. This induction is transient. The RNA levels of *Tbr1* are decreased to basal levels 12 h after stimulation. This feature is shared among the cortical, hippocampal, and amygdalar neurons, although the induction of *Tbr1* expression is much less pronounced in hippocampal neurons (Chuang et al., 2014). In addition to cultured neurons, behavioral stimulation also changes *Tbr1* expression levels in mouse brains. When conditioned taste aversion is applied to stimulate neuronal activation, similar to *c-Fos* induction, *Tbr1* RNA levels in the lateral amygdala, the insular cortex and the ventral hippocampus are also transiently increased 2 h after training (Chuang et al., 2014). Neuronal activation also induces *Grin2b* expression *in vitro* and *in vivo*, but this induction occurs several hours after that of *Tbr1*. Moreover, deletion of *Tbr1* completely blocks *Grin2b* induction in culture (Chuang et al., 2014). Both NMDAR and CaMKII are required to induce *Tbr1* expression (**Figure 4**). Thus, in addition to regulating axonal differentiation and neuronal migration during the early developmental stage, *Tbr1* also acts as an immediate early gene in response to synaptic stimulation in mature neurons, which might contribute to the etiology of *TBR1*-related ASDs. In particular,

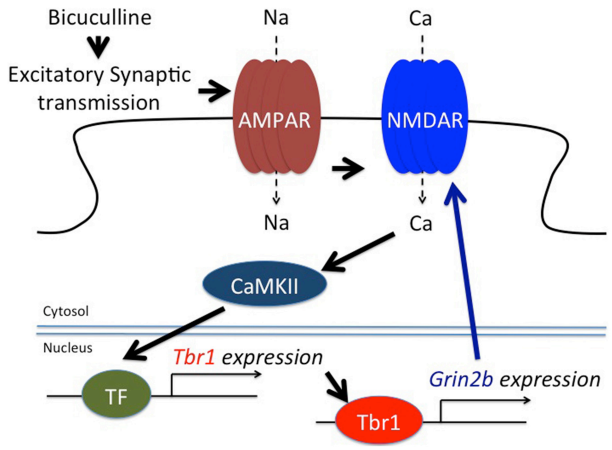

FIGURE 4 | TBR1 acts as an immediate early gene to induce *Grin2b* expression during neuronal activation. Both bicuculline, an antagonist of $GABA_A$ receptors, and glutamate treatment induce *Tbr1* expression. Although the transcription factor (TF) required for *Tbr1* expression is unknown, evidence indicates that CaMKII is involved in *Tbr1* upregulation. TBR1 can then induce *Grin2b* expression to enhance the synaptic responses (adapted from Chuang et al., 2014).

the cerebral cortex of $Tbr1^{+/-}$ mice likely exhibits defective electrophysiological responses and thus influences behaviors, even though anatomic defects of the cerebral cortex have not been identified in $Tbr1^{+/-}$ mice. More investigations need to be conducted to address this possibility.

ABNORMAL BRAIN WIRING AND EXCITATION/INHIBITION IMBALANCE—TWO PROMINENT MODELS FOR THE PATHOGENIC MECHANISM OF ASDs

Although the etiology of ASDs is heterogeneous, the two most prominent models for autism pathogenesis in the literature are abnormal brain wiring and an imbalance of neuronal activity (excitatory/inhibitory imbalance; Rubenstein and Merzenich, 2003; Walsh et al., 2008; Bernardinelli et al., 2014; Cellot and Cherubini, 2014; Deidda et al., 2014). These two defects lead to abnormal information processing and result in autism-like behaviors. These two models are interconnected to a certain extent. During neurodevelopment, neurons must extend their axons and form synapses with their target neurons, which allows the activity of the downstream target neurons to be regulated. In the absence of correct excitatory or inhibitory inputs, the activity of target neurons will be either too low or too high. These inappropriate levels of neuronal activity result in abnormal information processing, which leads to aberrant behaviors. Moreover, the imbalanced activity of neurons also influences (either strengthens or attenuates) their connections to other neurons. When the connection is too weak, it may be eliminated, which may alter brain wiring. In the $Tbr1^{+/-}$ mouse model, amygdalar axonal projections are defective. Both inter- and intra-amygdalar connections are noticeably impaired. Moreover, the NMDAR activity of amygdalar neurons is also much lower in $Tbr1^{+/-}$ brains. Thus, $Tbr1^{+/-}$ brains are characterized by both abnormal brain wiring and defective neuronal activation. Further investigation is required to see whether one deficit contributes more substantially to ASD pathology.

CONCLUDING REMARKS

$Tbr1^{+/-}$ mice constitute the first genetic mouse model to show that defects in amygdalar circuits and activity result in autism-like behaviors. TBR1 controls the expression of a panel of genes that is associated with ASDs. TBR1 regulates axonal growth and the neuronal activation of amygdalar neurons by regulating downstream genes. Enhancing NMDAR activation with D-cycloserine and clioquinol to increase neuronal activity can ameliorate the behavioral defects of $Tbr1^{+/-}$ mice, although the anatomic defects caused by *Tbr1* haploinsufficiency are not rescued. $Tbr1^{+/-}$ mice thereby serve as a model to elucidate how mutation of an autism causative gene influences brain wiring and impairs neuronal activity and consequently results in autism-like behaviors. Nevertheless, several issues remain unresolved. First, why is the amygdala the brain structure most sensitive to *Tbr1* haploinsufficiency? Second, since $Tbr1^{+/-}$ amygdalar neurons do not correctly form inter- and intra-amygdalar connections, a study of the mistargeting of $Tbr1^{+/-}$ amygdalar axons might further illustrate the neural circuit defects caused by *Tbr1* haploinsufficiency. Third, although the anatomical features of the cerebral cortex of $Tbr1^{+/-}$ mice are normal, the electrophysiological responses of the $Tbr1^{+/-}$ cerebral cortex remain to be measured. Fourth, the TBR1 downstream genes have not been annotated in detail. Only four TBR1 direct target genes have so far been identified. Further work is necessary to understand the individual actions of other TBR1 downstream target genes and how their dysfunction could generate autism-like behaviors. Finally, it is unclear how TBR1 functions as a transcriptional activator in some cases but acts as a repressor in others. This phenomenon also deserves further investigation. Addressing these questions will further elucidate the roles of TBR1 in brains and potentially impact on autism research.

AUTHOR CONTRIBUTIONS

Both YH and TH wrote the manuscript and prepared the tables and figures.

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REVIEW
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Mutations and Modeling of the Chromatin Remodeler *CHD8* Define an Emerging Autism Etiology

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Autism Spectrum Disorder (ASD) is a common neurodevelopmental disorder with a strong but complex genetic component. Recent family based exome-sequencing strategies have identified recurrent *de novo* mutations at specific genes, providing strong evidence for ASD risk, but also highlighting the extreme genetic heterogeneity of the disorder. However, disruptions in these genes converge on key molecular pathways early in development. In particular, functional enrichment analyses have found that there is a bias toward genes involved in transcriptional regulation, such as chromatin modifiers. Here we review recent genetic, animal model, co-expression network, and functional genomics studies relating to the high confidence ASD risk gene, *CHD8*. *CHD8*, a chromatin remodeling factor, may serve as a "master regulator" of a common ASD etiology. Individuals with a *CHD8* mutation show an ASD subtype that includes similar physical characteristics, such as macrocephaly and prolonged GI problems including recurrent constipation. Similarly, animal models of *CHD8* disruption exhibit enlarged head circumference and reduced gut motility phenotypes. Systems biology approaches suggest *CHD8* and other candidate ASD risk genes are enriched during mid-fetal development, which may represent a critical time window in ASD etiology. Transcription and CHD8 binding site profiles from cell and primary tissue models of early development indicate that *CHD8* may also positively regulate other candidate ASD risk genes through both direct and indirect means. However, continued study is needed to elucidate the mechanism of regulation as well as identify which CHD8 targets are most relevant to ASD risk. Overall, these initial studies suggest the potential for common ASD etiologies and the development of personalized treatments in the future.

Keywords: autism, autism spectrum disorder (ASD), CHD8, systems biology, co-expression networks, functional genomics, subtype, *de novo* mutations

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INTRODUCTION

Autism spectrum disorder (ASD) is a lifelong neurodevelopmental disorder characterized by restricted, repetitive behaviors and impaired communication and social interactions (American Psychaitric Association, 2013). ASD can have a considerable impact on quality of life as many people with ASD experience difficulties communicating, developing relationships, and managing restrictive behaviors (American Psychaitric Association, 2013). Additionally, the prevalence of ASD appears to have steadily increased over the last few decades, from <0.5% of American

school aged children in the 1970s to 2% in 2012 (Blumberg et al., 2013). The most recent estimates for the median worldwide prevalence of autism and pervasive developmental disorders is about 1 in 160 children (Elsabbagh et al., 2012) and 1 in 68 children in the US (Centers for Disease Control, 2014). The apparent rise in ASD has prompted a great deal of interest and research into identifying the underlying causes of the disorder.

Many studies have indicated a strong genetic contribution for ASD risk (Geschwind and State, 2015). Early twin and familial studies demonstrated compelling evidence for the heritability of ASD due to the high rate of concordance for ASD between monozygotic twins (0.62–0.94) as opposed to dizygotic twins (0.05–0.62) and increased relative recurrence risk for siblings of affected individuals (10.1%; Abrahams and Geschwind, 2008; Risch et al., 2014; Colvert et al., 2015). However, the overall ASD genetic architecture is complex with (1) risk being conferred by *many* independent genomic loci and contributions from common and rare variants, as well as new or *de novo* mutations, (2) factors that range in size, from single base changes to large chromosomal deletions/duplications or other rearrangements, (3) impacts that include necessary and sufficient single variants, to small (oligogenic) and large (polygenic) sets of factors, (4) the relative contributions of these factors are likely different in subpopulations, such as simplex (single sporadic presentation in an individual, with no previous family history) vs. multiplex (multiple affected individuals in a family), or other demographics yet to be elucidated. These realities have hampered traditional gene discovery methods.

Despite these challenges, recent advances in sequencing technology and novel approaches have begun to unlock the genetics of idiopathic ASD and *high confidence* risk genes are beginning to emerge that shed new light on the underlying biology of this disorder. Here, we highlight one such gene, *Chromodomain helicase binding protein 8 (CHD8)*. We review the discovery of *CHD8* as one of the most mutated genes in simplex ASD, its molecular function, associated ASD subtype, and its potential role as a master regulator of other candidate ASD risk genes. Although *CHD8* is still just one of many ASD risk genes, these data point toward at least one converging neuromolecular mechanism in ASD etiology and the potential stratification of ASD into distinct genetic/biologic subtypes. This promises to have major implications for the future as we strive to develop personalized therapies and precision treatments for individuals with ASD.

GENOMIC DISCOVERY REVOLUTION

Earlier comparative genomic hybridization and genotyping array studies advanced the genomewide discovery of large regions of chromosomal deletions or duplications, termed copy number variants (CNVs), as substantial contributors to the risk of developing neurodevelopmental disorders (Rosenfeld et al., 2010). CNVs typically involve gains or losses of many genes and can show diversity in penetrance and expressivity. While these CNVs play a critical role in the architecture of neurodevelopmental disorders, with few expectations, it has been difficult to get at the underlying locus or loci that are responsible for this risk. The advent of whole-exome (the entire protein coding exons of the genome) sequencing allowed for mutation detection at single-base resolution and was initially applied to Mendelian disorders (Ng et al., 2010). A number of groups began applying these methods to small cohorts of sporadic cases of a variety of conditions, including idiopathic intellectual disability (ID), ASD, and schizophrenia, focusing on parent-child trios or families (Vissers et al., 2010; Girard et al., 2011; O'Roak et al., 2011; Xu et al., 2011). It was hypothesized that such families would likely be enriched for *de novo* mutations related to the condition, thereby allowing the identification of novel genetic events with major biologic effect in an unbiased genomwide fashion. These initial studies demonstrated the feasibility of obtaining exome data of sufficient quality across the trios (>90% jointly covered) and filtering strategies to identify the ∼1 true *de novo* event expected per generation (Veltman and Brunner, 2012). Moreover, they appeared to yield a large number of *possible* candidate genes for these conditions.

For simplex or sporadic ASD, these efforts were greatly expanded in 2012 with four groups publishing the results of hundreds of families independently (Iossifov et al., 2012; Neale et al., 2012; O'Roak et al., 2012b; Sanders et al., 2012). Mutations occurred in genes with very diverse functions and in >900 ASD probands (affected individuals) only six genes exhibited recurrent likely loss-of-function (LoF) mutations, defined as nonsense (premature stop codon resulting in gene truncation), canonical splice-site (altered donor or acceptor splice sites leading to improperly spliced mRNA) or frameshifting nucleotide insertion/deletion (indel), suggesting extreme locus heterogeneity. One of the genes identified with two LoF mutations in 209 ASD affected children from the Simons Simplex Collection (SSC) by O'Roak and colleagues was *CHD8*, an ATP-dependent chromatin-remodeling factor (O'Roak et al., 2012b; **Table 1**). CHD8 protein was also part of a large protein-protein interaction network emerging from the LoF and most severe missense mutations. This network was ranked highly for similarity to previously identified ASD risk genes using a network walking approach. Furthermore, the locus specific mutation rate for *CHD8* suggests that it was highly unlikely to find two independent mutations at random (in the single study), providing additional evidence for *CHD8* as an ASD risk factor (O'Roak et al., 2012b). Mutation of *CHD8* had not been previously implicated with ASD, except in one concurrent study examining balanced chromosomal abnormalities in ASD and other neurodevelopmental disorders (Talkowski et al., 2012). Talkowski and colleagues mapped a balanced translocation involving 3q25.31 and 14q11.1 [46 XX, $t_{(3;14)}$ (q25.31;q11.2)dn], which directly disrupted only one gene, *CHD8* at the 14q11.1 breakpoint. The proband was diagnosed with ASD, ID, and had dysmorphic facial features. Large *de novo* deletions of 14q11.2 were previously observed in three other subjects that all exhibited developmental delay (DD) and cognitive impairment and similar dysmorphic features including widely spaced eyes, short nose with broad nasal tip, and unusual helical root formation of the ear (Zahir et al., 2007). The presence or absence of ASD was not noted. The overlapping regions lost in these three

TABLE 1 | *De Novo CHD8* Mutations.

Chr 14 Position	Ref[a] Allele	Alt[a] Allele	Mutation Location (HGVS)[b]	Mutation[c] Type	Diagnosis[d]	VIQ[e]	NVIQ[f]	FSIQ[g]	Database /Cohort[h]	Author, Year
2,189,9618	G	C	p.Ser62X	Ns	ASD	75	78	74	SSC	O'Roak et al., 2012a
21,899,168	C	T	p.Arg212Gln	Ms	ASD		72		TASC	O'Roak et al., 2014
21,895,989	del(47)†	A	c.1593_1601+38del†	Ssv	ASD				SSC	Iossifov et al., 2014
21,882,516	G	T	p.Gln696Lys	Ms	ASD	88	125		TASC	O'Roak et al., 2014
21,878,133	G	GT	p.Tyr747X	Fs	ASD	25	38	32	SSC	O'Roak et al., 2012a
21,876,700	A	G	p.Leu834Pro	Ms	ASD				ASC	De Rubeis et al., 2014
21,876,489	C	T	p.Met904Ile	Ms	ASD/ID		63		TASC	O'Roak et al., 2014
21,871,373	T	C	c.3519-2A>G	Sp	ASD	37	47	43	SSC	O'Roak et al., 2012a
21,871,790	C	A	p.Glu1114X	Ns	ASD	27	41	34	APP	Bernier et al., 2014
21,871,178	G	A	p.Gln1238X	Ns	ASD	20	34	27	SSC	O'Roak et al., 2012b
21,870,652	C	T	p.Arg1242Gln	Ms	ASD				ASC	De Rubeis et al., 2014
21,870,169	G	A	p.Arg1337X	Ns	ASD	85	86	84	SSC	O'Roak et al., 2012a
21,868,219	G	A	p.Arg1580Trp	Ms	ASD	97	74		TASC	O'Roak et al., 2014
21,867,866	T	G	p.Tyr1642LeufsX25	Ssv-Fs	ASD				ASC	De Rubeis et al., 2014
21,867,866	T	G	c.4818-2A>C	Ssv	ASD		93		TASC	O'Roak et al., 2014
21,865,980	A	T	p.Ser1606ArgfsX8	Ssv-Fs	ASD				ASC	De Rubeis et al., 2014
21,865,980	A	T	c.5051 + 2T>A	Ssv	ASD	96	103		TASC	O'Roak et al., 2014
21,862,642	C	T	p.Gly1602ValfsX15	Ssv-Fs	ASD				ASC	De Rubeis et al., 2014
21,862,535	G	A	p.Arg1834X	Ns	ASD				TASC	O'Roak et al., 2014
21,862,159	CC	C	p.Glu1932SerfsX3	Fs	DD/ID/ASD			46	Troina	Bernier et al., 2014
21,861,643	TCTTC	T	p.Glu2103ArgfsX3	Fs	ASD	44	67	59	SSC	O'Roak et al., 2012a
21,861,376	ACT	A	p.Leu2120ProfsX13	Fs	ASD	90	93	91	SSC	O'Roak et al., 2012b
21,860,919	C	A	p.Ser2173X	Ns	ASD/SHZ					Mccarthy et al., 2014
21,861,328	T	TC	p.Glu2136ArgfsX6	Fs	ID			<40	Troina	Bernier et al., 2014
21,854,022	GGGT	G	p.His2498del	Aa	ASD	84	98	92	SSC	O'Roak et al., 2012a
			t.3;14,q25.31;q11.2	Tr	ASD/ID				AGRE	Talkowski et al., 2012

[a] *VCF format, hg19 coordinates.*
[b] *Accession number: NP_001164100.1; Ref Seq number: NM_001170629.1.*
[c] *Fs, Frameshift; Ns, Nonsense; Tr, Translocation; Aa, Single amino acid deletion; Sp, Splice; Mns, Missense near splice site; Ms, Missense; Ssv, Splice site variant.*
[d] *ASD, Autism Spectrum Disorder; ID, Intellectual Disability; SHZ, Schizophrenia.*
[e] *VIQ, Verbal I.Q.*
[f] *NVIQ, Non Verbal I.Q.*
[g] *FSIQ, Full Scale I.Q.*
[h] *SSC, Simons Simplex Collection; AGRE, Autism Research Genome Exchange; ASC, Autism Sequencing Consortium; TASC, The Autism Simplex Collection.*
† *CAAGCTCAAGTGAGTACTCCTTGCTACTGTGATGGGACGT.*

cases included *CHD8* and *SUPT16H*. *CHD8* was suggested as a possible candidate gene for these abnormalities (Zahir et al., 2007).

In an effort to identify additional mutations and firmly implicate *CHD8* and other strong candidates, targeted re-sequencing studies utilizing cost-effective modified molecular inversion probes (MIPs) were applied to larger cohorts of ASD and DD probands. In the first such study, the protein coding regions of 44 genes were successfully sequenced in 2446 ASD probands from the SSC (O'Roak et al., 2012a). Seven additional *de novo CHD8* mutations were identified: six LoF variants and a single amino acid deletion, including a mutation missed by exome sequencing (**Table 1**). This finding was highly significant as determined by a model of recurrent gene mutation, firmly implicating *de novo* LoF mutations in *CHD8* with ASD risk ($p < 2 \times 10^{-9}$). Including exome data available at the time, 9/2573 (0.35%) SSC probands carried a *CHD8* mutation, making this locus one of the most frequently mutated in sporadic/simplex ASD.

These resequencing studies were further extended in a broader neurodevelopmental cohort of 3730 children with ASD or DD (Bernier et al., 2014), 898 ASD confirmed probands from The Simplex Autism Collection (TASC; O'Roak et al., 2014), as well as ∼2500 unaffected children from SSC or TASC. These studies identified six additional *de novo* LoF variants, four *de novo* missense variants, and one inherited LoF variant. LoF mutations were not seen in unaffected siblings or in an additional 6503 general population control (Bernier et al., 2014; O'Roak et al., 2014). In total, 16 *de novo CHD8* mutations (0.46%, Poisson 95% CI: 0.26–0.75%) were identified in the SSC and TASC cohorts, both of which required probands to meet ASD criteria on the Autism Diagnostic Interview-Revised (ADI-R) and Autism Diagnostic Observation Schedule (ADOS; Lord et al., 1994,

1999). Combined with the more broadly defined Bernier et al. cohort, the observed *de novo* rate is 0.3%. These findings further strengthen the association of *de novo* mutations in *CHD8* with ASD risk by identifying mutations in independent cohorts.

In late 2014, two large-scale exome sequence studies were also published, including ~2500 families from the SSC (Iossifov et al., 2014) and 1478 families and 1673 case-only by the Autism Sequencing Consortium (De Rubeis et al., 2014). In the SSC one *new* additional *CHD8* mutation was identified (nine previously identified including MIP and exome data). The ASC identified two *CHD8 de novo* missense mutations from the family and three splice site variants from case only exome data (through genotyping selected parents; De Rubeis et al., 2014). Risk genes were identified using a statistical approach called TADA (Transmission and *De Novo* Association), that integrates family-based (de novo and transmitted) and case-control data (He et al., 2013). In this analysis, *CHD8* was also identified as one of the top 13 ASD risk genes with a false discovery rate (FDR) of <0.01. In summation, both exome and targeted resequening data have firmly demonstrated that *de novo CHD8* mutations play an important role in ASD risk.

CHD8 MUTATIONS DEFINE A SUBTYPE OF ASD

CHD8 is not only one of the most recurrently mutated genes in sporadic ASD, but also appears to give rise to a distinct ASD phenotype. The first two probands with *CHD8* mutations identified from the early sequencing efforts of O'Roak and colleagues, interestingly, had unusually large head circumferences (macrocephaly; O'Roak et al., 2012b). Similarly, the index case from Talkwoski et al. carrying the *CHD8* disrupting balanced translocation also presented with macrocephaly and had dysmorphic facial features including prominent forehead and eyes and posteriorly rotated ears (Talkowski et al., 2012). Noting this, head circumference of the the eight probands with LoF mutations in *CHD8* identified from targeted re-sequencing of the SSC (O'Roak et al., 2012a) was examined. They found that head size was significantly larger in the individuals with *CHD8* mutations (greater than two standard deviations) as opposed to those without. Additionally, about 2% of SSC probands with macrocephay had *CHD8* mutations. Macrocephaly has been recognized in other genetic etiologies of ASD, mainly loss of *PTEN* function and deletions in 16p11.2 (Butler et al., 2005; Shinawi et al., 2010).

To expand the group of individuals with known *CHD8* mutations and assess the potential for a *CHD8* related subphenotype, patients identified through the exome and targeted sequencing ASD and DD cohorts were invited to participate in a comprehensive structured evaluation using a battery of standard cognitive, adaptive, and language tests ($n = 8$; Bernier et al., 2014). Including previous clinical reports on other subjects ($n = 7$), phenotypic data on 15 total patients with disruptive *CHD8* variants were included in the evaluation (13 *de novo*, one inherited, and one of unknown origin; **Figure 1A** and **Table 2**). ASD was the most common diagnosis with 13 of 15 meeting a strict diagnosis on both "gold standard approaches," ADI-R and ADOS. Macrocephaly, defined as orbitofrontal circumference greater than two standard deviations of age and sex matched means, was exhibited in 12 of

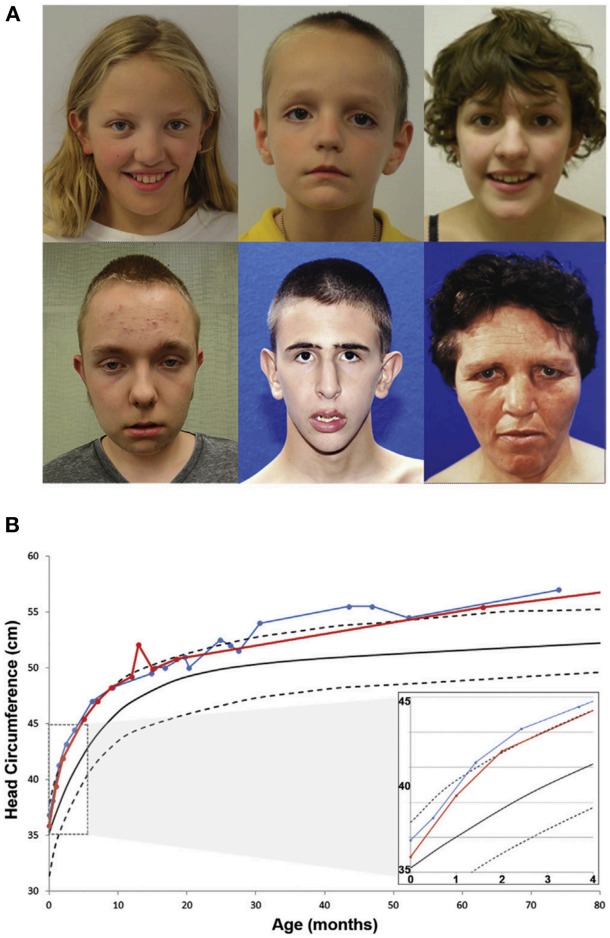

FIGURE 1 | Phenotypic characteristics of patients with *CHD8* Mutations. (A) Common facial features of patients with *CHD8* mutations include macrocephaly, hypertelorism, down-slanted palpebral fissures, broad nose, pointed chin, and prominent supra-orbital ridge. **(B)** Longitudinal head circumference data for two patients (red and blue). At 2 months after birth, orbital frontal head growth is pronounced. Head growth continues to be in the 97th percentile throughout childhood. Figure originally published in Bernier et al. (2014) used with permission.

TABLE 2 | Phenotypic Summary of Patients with CHD8 Mutations.

Patient characteristics	Number (%)
ASD	13/15 (87%)
Tall stature	12/14 (86%)
Macrocephaly*	12/15 (80%)
GI problems*	12/15 (80%)
Sleep problems	10/15 (67%)
Intellectual disability	9/15 (60%)

**Indicates significantly different from the typical ASD population.*

15 patients (**Table 2**). Head circumference velocity data ($n = 2$) showed an initial orbital overgrowth within the first 2 months post birth and a continued trajectory of large head growth at or above the 97th percentile throughout early childhood (**Figure 1B**). The proportion of *CHD8* mutation carriers with macrocephaly is significantly greater than that seen in the typical ASD population ($p = 2.1 \times 10^{-21}$). There were also many similarities in facial features among the group including: prominent forehead, wide set eyes, broad nose with full nasal tip, and pointed chin (**Figure 1A**). A majority also reported gastrointestinal (GI) problems, including recurrent constipation, and sleep problems, particularly with falling asleep (**Table 2**). Two patients described suffering from an inability to sleep for two-three straight days. Cognitive impairment was also pervasive, but intelligence spanned a wide range with some in normal range. Moreover, there is indication that additional symptoms may manifest as the children age. Three of the three female patients (including the translocation case) experienced precocious puberty (Talkowski et al., 2012; Bernier et al., 2014).

The implications of a *CHD8* specific phenotype suggest ASD could be stratified into different subtypes based on genetic etiology. Identification of these subtypes would provide insight into ASD presentation and pathology in genetically homogenous populations and aid in the development and evaluation of treatment approaches (Bernier et al., 2014). Other high confidence ASD risk genes that exhibit recurrent loss of function mutations, such as *ADNP* and *DYRK1A* also appear to be associated with emerging subphenotypes (Helsmoortel et al., 2014; Bronicki et al., 2015; Ji et al., 2015). *CHD8* appears to have a great deal of specificity for the ASD phenotype as almost all individuals identified from the screen met the criteria for ASD even though the cohort was not strictly defined for ASD individuals. This however may often not be the case as other strong ASD risk genes show more variable expressivity. For example, *SYNGAP1* and *CHD2* have been independently implicated in other neurological disorders such as epileptic encephalopathies (Carvill et al., 2013). Future studies examining how these similar genetic mutations span our current diagnostic and clinical boundaries may provide novel insights into the development of common neuropathologies.

A CHROMATIN MODIFIER WITH DIVERSE FUNCTIONS

CHD8, previously called Duplin, was first identified in a screen for novel interactors within the canonical Wnt/β-catenin pathway using a yeast two-hybrid assay with a cDNA library generated from rat brain (Sakamoto et al., 2000). *CHD8* is a member of the chromodomain-helicase-DNA binding protein family. The CHD family is characterized by a SNF-2 like ATPase and two chromo (chromatin organization modifier) domains (Marfella and Imbalzano, 2007). The first CHD gene, *CHD1*, was identified in a search for Ig promoter binding proteins (Delmas et al., 1993; Woodage et al., 1997) At least nine other members have been characterized to date with varying patterns of expression in timing and localization. The CHD family is a structurally diverse group comprised of three main subfamilies categorized by the presence of certain functional domains (Marfella and Imbalzano, 2007). CHD1 and CHD2 proteins contain DNA binding domains near the C-terminus that are thought to recognize AT rich motifs. CHD3 and CHD4 lack DNA binding domains and instead have PHD (plant homeo domain) Zinc finger like domains that may recognize methylated histones and potentially have repressive functions. CHD5-CHD9 also have DNA binding domains, along with additional functional domains located at the C-terminus. CHD8, as well as CHD7 and CHD9, contain a BRK domain, which is often seen in other SWI/SNF like proteins, and a DNA binding domain. Other SWI/SNF like complexes, such as BAF, regulate neural tube closure and neuron progenitor identity and differentiation (Ronan et al., 2013). Based on its ATP-dependent chromatin remodeling capabilities and SWI/SNF like domains, a potential role for CHD8 may lie in the regulation of neuronal progenitor cells.

Studies of CHD8 protein in the context of human cellular function show that it is involved in the regulation of transcription factor activity. CHD8 is able to activate the transcription of genes driven by U6 promoters through interactions with the transcription factor hStaf (Yuan et al., 2007). CHD8 is also required for CTCF, a major transcriptional repressor, to function as an inhibitor and insulator (Ishihara et al., 2006). CHD8 can also regulate RNA synthesis as well due to interactions with RNA polymerase II (Rodríguez-Paredes et al., 2009). CHD8 also promotes cellular proliferation in multiple human-derived cell types. Cells with *CHD8* knockdown were found to have highly reduced S-phase cell populations and increased populations of cells arresting in G1 (Rodríguez-Paredes et al., 2009). This effect was later found to be a result of CHD8 binding to S-phase-dependent promoters and recruiting E2F, a transcription factor which controls cell cycle regulation (Subtil-Rodríguez et al., 2014). CHD8's role in cell cycle regulation could be a contributing factor in the observed macrocephaly exhibited by individuals with *CHD8* LoF mutations. While macrocephaly may suggest overproliferation, in mouse models deficient in PTEN, macrocephaly appears to be attributed to increased cell body or soma size. Similarly, if cells are unable to progress through the cell cycle due to CHD8 loss, they may continue to grow in size (Kwon et al., 2001; Luikart et al., 2011). However, both CHD8 upregulation and downregulation has been observed in cancers suggesting CHD8 cell cycle regulation is likely complex and further study will be needed to determine how it impacts neurodevelopment (Lawrence et al., 2014; Subtil-Rodríguez et al., 2014).

What is also still unclear is the mechanism by which CHD8 controls transcription factor activity. Many studies have demonstrated that CHD8 has affinity for histone H3 lysine 4 (H3K4me3), a marker for active transcription, and is present at active promoters (Yuan et al., 2007; Rodríguez-Paredes et al., 2009; Sugathan et al., 2014; Cotney et al., 2015). Additionally the loss of CHD8 protein was associated with CpG hypermethylation and histone hypoacetylation near CTCF binding sites. Though CHD8 did not affect the ability of CTCF to bind DNA, potentially the CTCF-CHD8 complex may

interact with methyltransferases and histone acetyltransferases to influence the state of nearby chromatin (Ishihara et al., 2006). Thus, CHD8 could promote transcription factor binding by making chromatin more accessible or acting as a scaffold between H3K4me3 labeled histones and the transcription factor.

CHD8 DISRUPTION IN ANIMAL MODELS MIMICS HUMAN PHENOTYPES

In the *in vivo* setting, *CHD8* was first examined in the context of β-catenin signaling due to its nuclear localization and ability to repress β-catenin dependent transcription (Sakamoto et al., 2000). In zebrafish, β-catenin is necessary for axis formation. Dorsal injections of *Chd8* mRNA, causing overexpression of the protein, resulted in loss of the head. *CHD8* expression in the zebrafish is enriched in the brain and spinal cord at 1 day post fertilization, and becomes progressively restricted to head and gut after 3–4 days post fertilization. Expression appears strongest in the mid-gastrula stage suggesting an important role for *CHD8* in embryonic development regulating Wnt/β-catenin activity (Sakamoto et al., 2000).

The association of macrocephaly with a reduction in *Chd8* expression is also observed in zebrafish. An increase in the interorbital distance, a surrogate measurement for head circumference, is seen in fish treated with *Chd8* targeting morpholinos (Bernier et al., 2014; Sugathan et al., 2014). The distance increases in a morpholino dose dependent fashion. The reduction in *CHD8* also coincides with increases in chordin, required in forebrain development, otx2, early marker of midbrain/forebrain neural progenitor cells, and HuC/D, marker for newborn neurons, indicating elevated proliferation, particularly in the mid and forebrain (Bernier et al., 2014). The zebrafish studies also suggest a role for *CHD8* in GI development. Many of the human patients with *CHD8* mutations suffer from severe GI issues. Morpholino treated zebrafish have decreased gut motility as evidenced by delayed progression of fluorescent microspheres (Bernier et al., 2014). There is also an overall reduction in enteric neurons in the hindgut.

Similarly in mice, *Chd8* expression is at its highest embryonically at E8.5–E12.5 but reduced by E16.5 and low in newborns. Therefore, function is likely restricted to early-middle stage embryonic development (Nishiyama et al., 2004). Expression of *Chd8* is most prominent in the brain, face, branchial arches, limb buds, and tail. Complete knockout of *Chd8* is embryonic lethal. Embryos are resorbed by E9.5 but still recoverable by E8.5. Growth begins to delay as soon as E5.5 and is completely arrested at E6.5 with pronounced apoptosis occurring at E7.5. Heterozygotes though did not have gross abnormalities and were fertile. Interestingly, Wnt/β-catenin targets are not increased suggesting that aberrant signaling is likely not the cause for lethality. Rather, Chd8 protein regulates p53 activity by recruiting histone H1, forming a p53-Chd8-H1 complex that represses p53 target gene expression (Nishiyama et al., 2009). Unrestrained p53 activity appears to be the cause for the massive apoptosis seen in embryos. Deletion of p53 in $Chd8^{-/-}$ mice delayed arrest to E10.5 at which time embryos died from a heart defect. $Chd8^{-/-}$ $p53^{+/-}$ mice did not recover as well. Taken together, these studies demonstrate a conserved role for *CHD8* as a regulator of early formative developmental pathways.

A *CHD8*-ASD DEVELOPMENTAL WINDOW

With the great functional diversity observed in the *de novo*, rare variant, and other candidate ASD risk genes, it seems difficult to reconcile their role in an overarching mechanism for ASD biology. However, a number of biologic network approaches suggest that there may be a converging biology at particular developmental windows and brain regions, which includes *CHD8* as a central player. These approaches are leveraging new spatial and temporal genomics data from developing human and non-human primates, such as the BrainSpan Atlas, a freely available resource that includes gene expression (microarray and RNA-seq) and *in-situ* hybridization data sets (Shen et al., 2012). As in other organisms, *CHD8* expression seems to be most significant early in human development. While *CHD8* is widely expressed in the adult brain, expression is highest at 9–16 post conception weeks (PCW) in both progenitor and post mitotic neocortical layers and then gradually declines (Bernier et al., 2014).

To discover time points of development and brain regions for which candidate and high-confidence ASD risk genes, such as *CHD8*, may converge, two recent concurrent studies made use of BrainSpan's gene expression data to develop co-expression networks or modules representing genes that share similar expression patterns during development (Parikshak et al., 2013; Willsey et al., 2013). Each of these networks or modules corresponded to different brain regions along progressing stages of development. Remarkably, while the groups took different approaches to develop these networks and assess the potential for enrichment of candidate ASD risk genes they arrived at similar results implicating the mid-fetal time periods as a possible critical window for ASD etiology.

Parikshak and colleagues used BrainSpan RNA-seq data from human neocortex 8 PCW to 12 months after birth to construct co-expression modules by Weighted Gene Coexpression Network Analysis (WGCNA), an unbiased, genomewide method for constructing networks based on pairwise correlations of gene expression (Langfelder and Horvath, 2008; Parikshak et al., 2013).

They identified 17 modules and mapped sets of candidate ASD risk genes onto proteins within the modules. These gene sets included: "asdM12" which was derived from a WGCNA expression module from ASD patient postmortem cerebral cortex and "SFARI ASD" which was a subset of 155 genes from the larger AutDB gene list filtered by gene evidence score (Voineagu et al., 2011; see **Table 3** for detailed gene list descriptions). *De novo* variants identified from 2012 whole-exome sequencing studies performed by O'Roak et al., Sanders et al., and Neale et al. were also mapped onto networks. They then assessed enrichment for candidate ASD risk gene within specific co-expression modules, cortical layers and cell types. The *de novo* mutation candidate ASD risk genes were more concentrated in modules designated M2 ($p = 0.006$) and M3 ($p = 0.0011$). M2 peaks at 12–22 PCW and M3 is highly upregulated until PCW 12. The overall set

TABLE 3 | Referenced Candidate ASD Risk Gene Lists.

Gene list name	Compilation method	Type	Number of genes	Author, Year
AutDB	"PubMed" database search for "gene" + "autism" or "autistic" in the titles and abstracts. Genes are divided into genetic subcategories and assigned evidence scores. Licensed to SFARI as SFARI Gene by MindSPec.	Broad	667	Basu et al., 2009
asdM12	Set of genes within a co-expression module ("M12") highly correlated with ASD status. Module was constructed using WGCNA on gene expression from ASD (19) and control (17) postmortem cortex tissue. Samples from Autism Tissue Bank and Harvard Brain Bank.	Co-expression	88	Voineagu et al., 2011
SFARI ASD	Subset of genes from AutDB filtered for gene category syndromic (S) (associated with syndromes in which a significant percentage of individuals develop autistic symptoms) and evidence score 1–4 (high confidence—minimal evidence).	Broad	155	Parikshak et al., 2013
Willsey set	Genes identified with *de novo* LoF mutations from whole-exome sequencing studies (Iossifov et al., 2012; Kong et al., 2012; Neale et al., 2012; O'Roak et al., 2012a) and an additional 56 quartets from the SSC. Genes are grouped as, high confident (hcASD) genes (2 or more *de novo* LoF mutations), or probable (pASD) genes (one *de novo* LoF mutation). The genes in the pASD group were estimated to have a >50% chance of being "true ASD" genes based on TADA analysis.	Simplex dnLoF	131	Willsey et al., 2013
Liu set	Genes identified by the DAWN algorithm as being implicated in ASD risk using data from the PFC-MSC co-expression module developed in Willsey et al. and from whole-exome sequencing studies in family trios (Iossifov et al., 2012; Kong et al., 2012; Neale et al., 2012; O'Roak et al., 2012a; Sanders et al., 2012), quartets (Willsey et al., 2013) and case-controls (ARRA Autism Sequencing Consortium, De Rubeis et al., 2014)	dnLoF Rare transmitted variants Co-expression	127	Liu et al., 2014

of genes in these modules, including non-ASD risk genes, were highly enriched for chromatin modifiers, DNA binding proteins, and transcriptional regulators ($p < 1 \times 10^{-4}$). Modules M2 and M3 seemed specific to the superficial cortical layers L2–L4 and cells expressing glutamatergic neurons.

In contrast, the "SFARI ASD" and "asdM12" gene sets were enriched in modules M13 (SFARI ASD, $p = 0.059$, asdM12, $p = 3 \times 10^{-15}$), M16 (SFARI ASD, $p = 0.0024$, asdM12, $p = 3.5 \times 10^{-15}$), and M17 (SFARI ASD, $p = 0.033$, asdM12, $p = 1.0 \times 10^{-7}$). The overall set of genes in these modules had a concentration of synaptic proteins ($p < 1 \times 10^{-4}$) and peaks late in fetal development, starting at 16 PCW and into birth. An enrichment for fragile-X mental retardation protein (FMRP) targets was seen in the M2 module, suggesting a common mechanism between ASD and fragile-X syndrome, in line with the enrichment of FMRP targets first observed by Iossifov et al. (2012). On the other hand, 401 genes implicated in monogenic forms of ID compiled from four publications, showed little enrichment for any one module suggesting the mid-fetal time period may be specific to ASD.

Willsey and colleagues took an alternative approach by focusing on nine high-confidence ASD risk genes, defined as having two or more LoF mutations, and determining their co-expression networks (Willsey et al., 2013; **Table 3**). These nine genes were used as seeds to generate networks composed of the top positively correlated genes. Gene expression data was also derived from BrainSpan, but based on the Affymetrix GeneChip Human Exon 1.0 ST Array rather than RNA-seq. In addition, all brain regions were considered and 15 periods of development, 5.7 PCW–82 years were examined. Developmental periods were condensed into three time windows resulting in 52 networks based on time and brain region with transcriptional similarity as determined by hierarchical clustering. To see if additional candidate ASD risk genes were enriched in these networks, a list of 122 candidate ASD genes (termed "Willsey set") with at least one LoF mutation identified from whole-exome sequencing studies was generated and mapped onto the networks (**Table 3**). Permutation testing of enriched networks was used to correct for gene size and GC content. They also looked for enrichment of this gene set within specific cortical layers and cell types. Immunostaining and *in-situ* hybridization was performed on frontal cortex sections to determine protein expression for five of the high-confidence ASD genes. Similar to Parikshak et al., they found enrichment in networks exhibiting peak expression between 10–19 ($p = 0.003$) and 13–24 ($p = 0.05$) PCW (mid-fetal time period). These networks were specific to the prefrontal and primary motor-somatosensory cortex, but in contrast to Parikshak et al., the networks were specific to the deep layers, L5-6. Immunostaining revealed that *CHD8* and other ASD genes like *SCN2A*, *DYRK1A*, and *TBR1* were highly expressed in cortical projection neurons, particularly glutamatergic cell types.

In summary, both groups found a convergence of candidate ASD genes with *de novo* mutations within the mid-fetal stage of development, 10–24 PCW. The resulting networks appeared specific for glutamaterigic cortical projection neurons, but differed in their implication of specific cortical layers. The cortical projection neurons form synaptic connections early in development and may then be highly sensitive to changes in gene regulation (Willsey et al., 2013). Therefore, *de novo* mutations in genes like *CHD8* may largely impact the midfetal stage of development particularly prefrontal and motorsensory cortex development.

CHD8 DISRUPTION ALTERS MANY TARGETS INCLUDING OTHER CANDIDATE ASD RISK GENES

The heterozygous LoF *CHD8* mutations seen in ASD likely result in insufficient levels of CHD8 protein and the disregulation of CHD8 targets. The regulatory landscape and widespread reach of *CHD8* regulation has been examined in three recent studies using genomic approaches and a number of different neural cellular models that attempt to mimic early development (**Figure 2A**). Sugathan and colleagues performed knockdown of *CHD8* in induced pluripotent stem cell (iPSC) derived neuron progenitor cells (NPCs) using lentiviral delivery of six independent shRNA achieving a range of 38–69% reduction in *CHD8* mRNA expression (Sugathan et al., 2014). They then assessed changes in gene expression using RNA-seq in these knockdown lines and also performed chromatin immunoprecipitation-sequencing (ChIP-seq) in control NPCs to identify CHD8 binding sites. By contrast, Cotney and colleagues performed ChIP-seq to locate CHD8 binding sites (under non-perturbed conditions) in three different systems: human midfetal cortical tissue 16–19 PCW, H9 derived human neural stem cells (hNSCs), and embryonic mouse cortex (Cotney et al., 2015). Using primary tissue allowed for representation of mid-fetal stages critical in CHD8-ASD development and to evaluate potential differences between CHD8 binding sites identified in *in vivo* tissue and *in vitro* cells. They also performed *CHD8* knockdown in hNSCs using two lentiviral shRNA, followed with RNA-seq, to assess changes in gene expression. Finally in the third study, Wilkinson and colleagues performed knockdown of *CHD8*, achieving about a 50% reduction, using siRNA in human SK-N-SH neural progenitor cells followed by RNA-seq (Wilkinson et al., 2015). These studies reveal a complex role for *CHD8* in ASD development as it appears to regulate expression of additional candidate ASD risk genes by both indirect and direct means.

Sugathan and colleagues found that knockdown of *CHD8* lead to differential expression of some 1756 genes (at nominal $P < 0.05$; 369 genes at $q < 0.05$ Benhamini-Hochberg), most of which were upregulated ($n = 1140$, $p < 0.05$; 286, $q < 0.05$; Sugathan et al., 2014). The set of down regulated genes ($n = 616$) were enriched for roles in neuronal development. Intriguingly, this same set of downregulated genes was also enriched for candidate ASD risk genes from a large set of 628 genes from AutDB ($p = 3.25 \times 10^{-8}$; **Table 3**). Though no enrichment for any candidate ASD risk gene sets were observed in the set of up-regulated genes, cancer associated genes, defined by The Cancer Genome Atlas, were enriched in this group. Loss of *CHD8* function has been observed in a broad range of tumor types and other chromatin regulators have been implicated in cancer development (Lawrence et al., 2014). This suggests *CHD8* may have other important roles extending past development and may be a point of commonality between some cancers and ASD.

In the ChIP-seq data, 7324 reproducible binding sites were identified (Benjamini-Hochberg $q < 0.05$). CHD8 preferentially bound to regions that in ES cell-derived neuroprogenitor lines from the Roadmap Epigenomics are marked with histone H3 trimethyl Lysine4 (H3K4me3), an indicator of active TSSs (Bernstein et al., 2010). These TSSs were strongly enriched in the CHD8 set, 83%, relative to the genome background, 1%. A small number of binding sites, 4%, overlap enhancer marked sites (H3K4me1), representing a two-fold enrichment. Sequence motifs at CHD8 binding sites were enriched for CTCF and YY1 transcription factor motifs. Genes with CHD8 sites were enriched for p53, hedgehog, and cell cycle pathways.

Combining the differentially expressed (DE) set of genes with CHD8 binding data, Sugathan and colleagues further explored a number of enrichments using functional classifications, candidate ASD risk gene sets, and other disease gene sets. Overall, only 9.2% of genes with CHD8 binding sites were DE in the nominal P gene set (29.7% of all the DE genes). Candidate ASD risk genes from AutDB were enriched in the set of nominally downregulated genes, but not targeted by CHD8 ($p = 1.09 \times 10^{-9}$). This set of candidate ASD risk genes was enriched for pathways involved in neurodevelopment such as axon guidance and neurotransmitter regulation. Enrichment of the more restricted set of AutDB genes from Parikshak et al., "SFARI ASD" was also observed in the downregulated, non-CHD8 bound genes ($p = 2.26 \times 10^{-2}$). However, genes from Willsey et al., "Willsey set" were enriched as CHD8 bound genes ($p = 4.34 \times 10^{-3}$), but were not associated with any changes in gene expression. CHD8 bound genes in the "Willsey set" were enriched for chromatin and transcription. Though these data sets are not independent, they do have different gene representations, with the "Willsey set" more biased toward rare *de novo* variants. CHD8 binding sites with or without differential expression were explored in the modules described in Parikshak et al. The downregulated genes with no CHD8 binding site were enriched in modules M13, M16, and M17 which correspond to mid-late fetal develop whereas CHD8 bound genes were enriched in modules M2 and M3 corresponding to early-midfetal development. CHD8 bound genes were also highly enriched in TGCA cancer associated genes and also showed no specific enrichment in either up or downregulated genes. No other disease gene sets from the 184 lists for complex disease and traits available from the National Human Genome Research Institute were as significantly enriched as the ASD or cancer gene sets for CHD8 targets or genes affected by *CHD8* knockdown. These data suggest both direct and indirect roles of CHD8 in gene regulation, particularly in the development of ASD and cancer.

Cotney et al. found 9414 reproducible CHD8 binding sites in hNSCs and 4428 in human midfetal brain. Between these two human sets, 2777 sites were overlapping. Chromatin state of hNSC CHD8 binding sites was determined from data generated on hNSCs from their own lab and the ENCODE/Roadmap Epigenomics (Bernstein et al., 2010). Similarly to Sugathan and colleagues, they found that CHD8 had strong affinity for TSSs labeled with H3K4me3 or H3K27ac and 99% of promoters bound by CHD8, 8056, were active (Cotney et al., 2015). Additionally, they observed that a small fraction of CHD8 binding sites, 1028, were indicative of enhancer functions. Moreover, CHD8 binding negatively correlated with H3K27me3, indicative of repression. Enriched sequence motifs for transcription factors included CTCF, E2F, YY1, and Sp/Kruppel-like family.

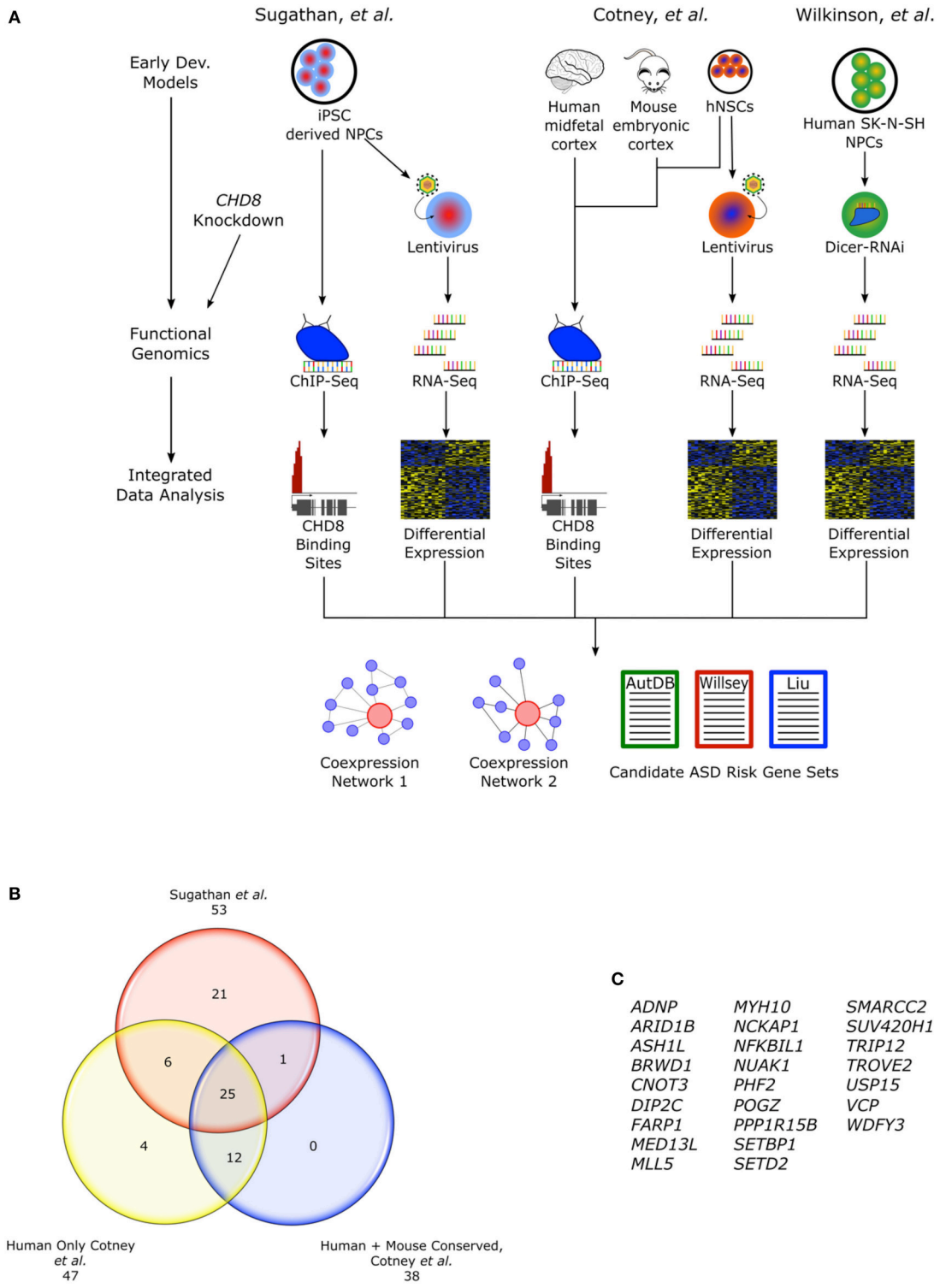

FIGURE 2 | *CHD8* functional genomics studies. (A) Flow chart describing functional genomics studies, Sugathan et al., Cotney et al., and Wilkinson et al. including *CHD8* knockdown in cellular models of early neural development and ChIP-seq. Cotney et al. also incorporated primary fetal human cortical and embryonic mouse brain tissue into the ChIP-seq analysis. Integrating the gene expression and CHD8 binding profiles, networks of CHD8 regulated genes were constructed and analyzed for enrichment of candidate ASD risk genes. **(B)** CHD8 targeted candidate ASD risk genes found in the "Willsey set" observed between studies. Genes found in Cotney et al. specifically have CHD8 bound promoters. Human sites in Cotney et al. are shared between hNSCs and brain tissue. Though not included as part of the "Willsey set" in Cotney et al., *POGZ* is included here. *POGZ* was noted as having a CHD8 bound promoter and it is one of the high confidence "Willsey set" genes. **(C)** List of the shared candidate ASD risk genes from the "Willsey Set" bound by CHD8 between both studies and conserved between human and mouse.

Genes that had CHD8 binding sites identified in both midfetal brain and hNSCs were highly enriched for candidate ASD risk genes from two gene sets as defined by and Liu et al., "Liu set" ($p < 0.0001$) and the "Willsey set" ($p < 0.0001$; Willsey et al., 2013; Liu et al., 2014), while genes with binding sites only in hNSCs were not enriched. Candidate ASD risk genes with CHD8 binding sites were enriched for chromatin regulation and modification. CHD8 targets supported by both human datasets were largely evolutionarily conserved. Among mouse (E17.5 cortex), human midfetal brain, and hNSC, 1910 CHD8 binding sites were shared and also enriched for candidate ASD risk genes from both aforementioned gene sets ($p < 0.0001$), further suggesting a conserved mechanism for CHD8 regulation in development. Of the candidate ASD risk genes from the "Willsey set" found to contain CHD8 binding sites, 25 were shared between Cotney et al. and Sugathan et al. and were also conserved in mouse (**Figures 2B,C**). Enrichment of CHD8 bound proteins was also observed in networks reconstructed from the Willsey et al. 10–19 and 13–24 PCW networks using genes with active promoters (Willsey et al., 2013).

In their knockdown of *CHD8* in hNSC, genes with conserved CHD8 binding sites had the greatest fraction of DE genes (56%) as compared to genes with binding sites only found in hNSC (46%) or hNSC and human brain (54%). Cell cycle, Hippo, and p53 pathways were among the pathways most affected. Candidate ASD risk genes actually exhibited the most significant dysregulation compared to any other set of CHD8 targeted genes (∼60% for both gene sets). Additionally, those candidate ASD risk genes most DE, tended to be downregulated. While Sugathan et al. did find that candidate ASD risk genes from the Willsey set were enriched as CHD8 targets, they were not significantly DE in either direction. In contrast, Cotney et al.'s data suggest that CHD8 directly *activates* this same set of candidate ASD risk genes.

In line with the other studies, Wilkinson et al. found *CHD8* reduction had a global impact on gene expression. Interestingly, the top DE genes in this study were noncoding RNAs, the majority of which were upregulated. Noncoding RNAs have only recently been appreciated as having a potential role in neurodevelopment and may be yet another layer in transcriptional control. Enrichment of candidate ASD risk genes Parikshak et al. "SFARI ASD" was only seen in the set of downregulated genes, similar to Cotney et al. as were processes involved in regulation of neuron projection, differentiation and neurogenesis (Parikshak et al., 2013; Wilkinson et al., 2015).

In summation, *CHD8* appears to act as a master regulator in the foundational pathways of the developing brain, particularly those that may also be implicated in ASD development. Yet the mechanism for regulation remains unclear. Many of the candidate ASD risk genes are direct targets of CHD8 protein and are also involved in chromatin regulation (**Figure 3A**). Cotney et al. and Wilkinson et al. indicate that CHD8 directly activates expression of these genes but Sugathan et al. suggest that the presence or absence of CHD8 may not influence expression, despite the genes having a binding site. Other candidate ASD risk genes lack a CHD8 binding site, but still appear to be affected by CHD8 expression indicating an indirect mechanism of regulation. How CHD8 may be able to influence gene activation or transcription factor activity without directly binding to DNA is not clear, but may involve binding to other co-regulators or chromatin marks (**Figure 3B**). Continued study will be needed to fully elucidate the mechanism for CHD8 regulation in ASD.

CONCLUSIONS AND FUTURE DIRECTIONS

From exome sequencing studies, a number of high-confident ASD risk genes have been identified with *CHD8* emerging as particularly strong. Both human case studies and animal models suggest a common phenotype for *CHD8* mutation including a significant association with ASD diagnosis and behavior, macrocephaly, and dysfunction in the enteric nervous system. Though ASD is highly heterogeneous, *CHD8* systems biology and functional genomics studies give some hint as to the complex nature of the disorder. The CHD8 protein and those of many of the other candidate genes serve as master regulators, influencing the expression of a large network of genes and pathways that control neuron formation, proliferation, and differentiation. The timing of proper neural development is a highly regulated phenomenon, requiring a concerted effort by many different genes. While it's clear that CHD8 controls expression of genes involved in these developmental processes, the manner in which CHD8 regulates them is still unclear. Initial studies suggest that CHD8 may act as a direct repressor by modifying chromatin to make it less accessible. However, the loss of function studies point toward a role for CHD8 as an activator in the emerging ASD gene network. One of the consistent findings from these and previous studies is that CHD8 is associated with actively transcribed genes and has affinity for H3K4me3 labeled promoters suggesting an influential role in global gene expression. CHD8 may function to make promoters more accessible through chromatin remodeling, promote transcription factor binding, or indirectly enhance expression through as of yet, unknown mechanisms that may involve interactions with H3K4me3 itself. The mechanism of CHD8 regulation is likely to be gene dependent as well.

It's also still uncertain which CHD8 targets may be relevant for ASD. Though the studies indicate a number of candidate ASD risk genes contain CHD8 binding sites, further functional studies will be required to demonstrate if these genes interact in a physiologic setting. Additionally, although enrichment for candidate ASD risk genes may be seen in specific neuron types, it is still unclear how the alterations in these neurons ultimately relate to behavioral phenotypes. It will be important to determine how *CHD8* affects neural circuitry and connect these affects to autistic associated behaviors. Lastly, development of co-expression modules have proved useful in identifying relevant ASD developmental time periods and interactions between candidate ASD risk genes, including *CHD8*. However, developing methods that integrate multiple types of data such

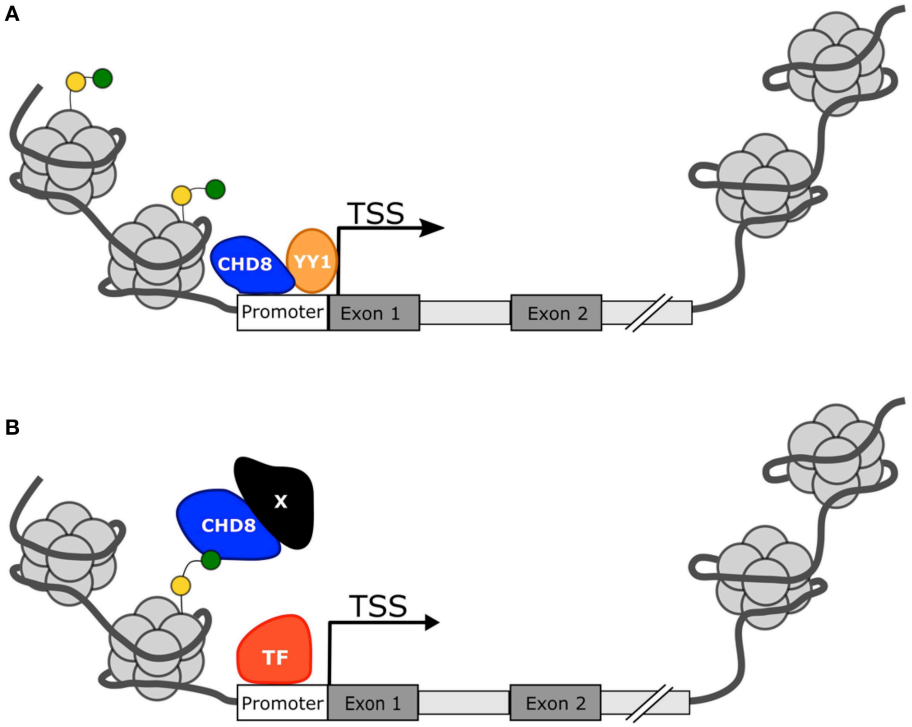

FIGURE 3 | Proposed mechanisms for CHD8 transcriptional activation. (A) CHD8 is most commonly found near active transcription start sites with histone modifications H3K4me3 (green circle) or H3K27ac (yellow circle). CHD8 may directly activate genes by directly binding near the transcriptional start site and promote transcription factor activity or recruitment. **(B)** CHD8 may indirectly activate genes through interactions between modified histone sites and other co-regulators to make chromatin more assessable.

as co-expression and protein-protein interactions may improve identification of ASD and other neurodevelopmental disorder specific networks, as proposed recently (Hormozdiari et al., 2015). Through the combination of rapidly advancing genetics and the development of relevant neural models, ASD biology is slowly beginning to resolve, and providing the potential for individualized approaches to therapy.

AUTHOR CONTRIBUTIONS

RAB: writing, editing, and figure conception, MBP: writing and figure design, BJO: writing, editing, and figure conception.

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Brian J. O'Roak is an inventor on patent PCT/US2009/30620: mutations in contactin associated protein 2 are associated with increased risk for idiopathic autism.

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