Chapter 1
ENGRAILED2 and Cerebellar Development in the Pathogenesis of Autism Spectrum Disorders

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Abstract Autism and autism spectrum disorders (ASD) are complex neurodevelopmental diseases with strong genetic etiologies. While many brain regions are implicated in ASD pathogenesis, many studies demonstrate that the cerebellum is consistently abnormal in ASD patients, both neuroanatomically and functionally. Recently, the human gene *ENGRAILED2* (*EN2*), an important regulator of cerebellar development, was identified as an ASD-susceptibility gene, a finding replicated in three data sets. We review much of the literature implicating an abnormal cerebellum in ASD, as well as the molecular and cellular development of the cerebellum with respect to possible pathogenetic mechanisms that contribute to the ASD phenotype. In addition, we explore the role of *EN2* in normal cerebellar development as well as animal models in which abnormal *En2* expression produces ASD-like behavior and neuropathology. We also share preliminary data from our laboratory that suggest that *En2* promotes postnatal cerebellar development via cell cycle regulation and interactions with extracellular growth signals. After reviewing these data from different disciplines, it is our hope that the reader will better understand how abnormal cerebellar development contributes to ASD pathophysiology and pathogenesis. Further, we underscore the importance of multidisciplinary approaches to identifying ASD-associated genes and their functions during development of brain structures known to be abnormal in ASD patients.

Keywords Autism · cerebellum · genes · *engrailed2* · patterning · neurodevelopment

Introduction

Autism spectrum disorders (ASD) are highly heritable, neurodevelopmental disorders that affect 1:150 children in the United States [1]. Public and scientific interest in ASD has exploded over the last two decades, spurring intense

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investigations into specific genetic and environmental factors that contribute to ASD susceptibility. The result of these efforts is a broadened clinical definition that better describes the heterogeneity of ASD phenotypes and a profounder understanding of the molecular and neuropathologic complexities that underlie the disease. For the first time in history, we can assay the human genome at the level of the individual nucleotide to look for molecular patterns in ASD patients and families. At the same time, neuroimaging provides windows into the living brain of autistic children and adults, allowing comparisons that were once only possible postmortem. To put together all these data from genetics, biochemistry, neuroimaging, and epidemiology is daunting and, given the numerous brain regions and molecular pathways implicated in ASD pathogenesis, is beyond the scope of this text. Thus, our goal in this chapter is to focus on one brain structure, the cerebellum, and the evidence supporting its role as the most consistently abnormal region of the ASD brain. To do so, we will review the evidence from neuropathology and neuroimaging studies demonstrating reproducible deficits in the cerebella of ASD patients. Additionally, we will examine cerebellar development and pay attention to specific genes that coordinate cerebellar growth and cellular organization, while considering the possible consequences of disrupting these ontogenetic processes. Finally, we will demonstrate that one cerebellar gene, *ENGRAILED2*, is not only vital for normal cerebellar development but is indeed an ASD-susceptibility gene that functions to regulate postnatal cerebellar growth during critical periods at which ASD symptoms may first become noticeable. Thus, it is our goal that the reader will come away with a better appreciation for the role of the cerebellum in normal brain function, as well as ASD pathogenesis, and an understanding of how molecular genetics and neurodevelopmental biology can be utilized together to investigate a complex disease such as ASD.

History of Autism

Autism was first described by Leo Kanner in 1943 [2] as a developmental disease, present from birth, in which reciprocal social behavior, language, and communication are impaired and patients display restricted interests and repetitive behaviors. Today, these deficits make up the core DSM-IV criteria of ASD, which includes diagnoses of classical autism, Asperger syndrome, and pervasive developmental disorder-not otherwise specified (PDD-NOS). Though no closer to a cure for ASD than Dr. Kanner was in the 1940s, our clinical and basic science understanding of ASD symptoms and disease pathogenesis has improved dramatically, fueled by ever-advancing genetic and biomedical technologies. Further, increases in prevalence and public awareness of ASD have stimulated financial and intellectual movements to make ASD research a priority at all academic and government levels. As a result of these efforts, we now appreciate ASD as a complex genetic disorder and have begun to identify ASD-associated

genes that regulate the development of brain structures known to be abnormal in humans with ASD. Uncovering these relationships between genes and normal neurodevelopmental processes provides insight into the mechanisms underlying abnormal brain development, and this information will bring us closer to fully understanding, and hopefully preventing, ASD.

ASD Neuropathology and Pathogenesis

Though extensively characterized clinically, ASD pathogenesis remains a mystery. Thus, pathologic abnormalities in brain structure and function may provide clues about the etiologies of this disease process. Postmortem and imaging studies have demonstrated a wide range of underlying neuropathology, involving a multiplicity of cortical and subcortical regions (reviewed by Palmen et al. 2004 [3]). For example, abnormal brain growth, and overgrowth, has been demonstrated by several neuroimaging studies comparing autistic children to normal age-matched controls [4, 5, 6, 7]. Further, increased brain size and weight have been described in postmortem analyses of autistic brains [3, 8, 9, 10, 11]. These studies suggest overall brain development is abnormal in ASD; however, it is currently unknown whether these pathologic abnormalities are causes or symptoms of ASD. More focused analyses of specific brain regions have found developmental abnormalities grossly, as well as microscopically. Several studies demonstrated cytoarchitectural abnormalities within cortical minicolumns, suggesting that ASD symptomology may result from abnormal connectivity within and between functional domains [12, 13, 14]. Additionally, several studies found reduced corpus callosal size in children with ASD, as well as abnormal communication between cortical structures during cognitive tasks, further suggesting that underconnectivity contributes to behavioral and cognitive abnormalities of autism ([15, 16, 17, 18, 19], see Minshew Chapter 18). Cortical dysgenesis, including cortical thickening, ectopic gray matter, disorganized lamina, and poor differentiation of the gray-white matter boundary, was described in six case studies by Bailey et al. (1998) [11]. In another study of subcortical brain structures, abnormally dense cell packing was described in hippocampi, amygdalae, and entorhinal cortices from 9 of 14 autistic brains [3, 9, 20]. A more recent study found amygdalar overgrowth during early postnatal development in ASD patients [21]. While compelling, based on the role of limbic structures, these studies await replication by other researchers and may not be generalizable to the ASD population at large. Collectively, though, these data describe the ASD brain as abnormal in structure, and accordingly in function, thus highlighting the importance of normal brain development. It is likely that multiple brain abnormalities exist within and between ASD patients and that the heterogeneity of clinical presentation is mediated by these brain differences. Therefore, the reader is encouraged to consider how hindbrain and cerebellar development and dysfunction, discussed below, could interact with these brain abnormalities in ASD.

Cerebellar Development, Function, and ASD

The cerebellum remains the most consistently abnormal brain region studied in humans with ASD. Cerebellar size, morphology, and function have all been found to be abnormal, and irregular patterns of cerebellar growth were found to begin from early infancy and continue into adolescence. In fact, 21 of 29 postmortem studies have found reduced numbers of Purkinje neurons, the primary cerebellar efferent neurons, without evidence of gliosis, suggesting these deficits may have occurred early in development [3, 9, 22, 23]. Purkinje neurons develop prenatally and are necessary for coordinating late gestation and postnatal cerebellar development via mechanical and neurochemical support of another cell population, the cerebellar granule neurons. Therefore, genetic or biochemical insults affecting early Purkinje neuron development could have sustained effects that disrupt cerebellar growth and function, contributing to ASD pathogenesis. Before discussing the body of evidence implicating cerebellar dysfunction in ASD, it is important to review how the cerebellum develops, keeping in mind that ASD pathogenesis may stem from perturbations at the genetic, biochemical, or cell biological level. The developing mouse brain serves as one of the best models to understand these processes.

Cerebellar Development

Following neural tube closure in early development, the anterior region is subdivided into three vesicles: the prosencephalon, which gives rise to the telencephalon and diencephalon of the forebrain; the mesencephalon, which develops into the midbrain; and the rhombencephalon, which gives rise to the metencephalon (rostral) and myencephalon (caudal) of the hindbrain [24]. The anterior portion of the rhombencephalic metencephalon abuts the mesencephalon, at a genetically distinct boundary known as the mid–hindbrain junction. The identification and assignment of cells to this region (a process known as patterning) is achieved through induction of transcription factors in spatially and temporally restricted domains, which will be discussed later (Fig. 1.1 and Color Plate 1, following p. XX).

At this early stage (Embryonic day (E)8–9 in the mouse), the cerebellar anlage is delineated just caudal to the mid–hindbrain junction, along the dorsolateral aspect of the first rhombomere. As development progresses, morphogenetic bending of the neural tube creates the pontine flexure ventrally, causing a wide gap to develop dorsally and producing the distinctive diamond shape of the fourth ventricle [25]. Neuroepithelial proliferation in the cerebellar primordium occurs along the ventricular zone (VZ), similar to the forebrain, allowing the cerebellar primordium to thicken and take shape along the fourth ventricle. Between E10 and 14 in the mouse, cerebellar neuron progenitors begin to exit the cell cycle, migrate out of the proliferative VZ, and differentiate;

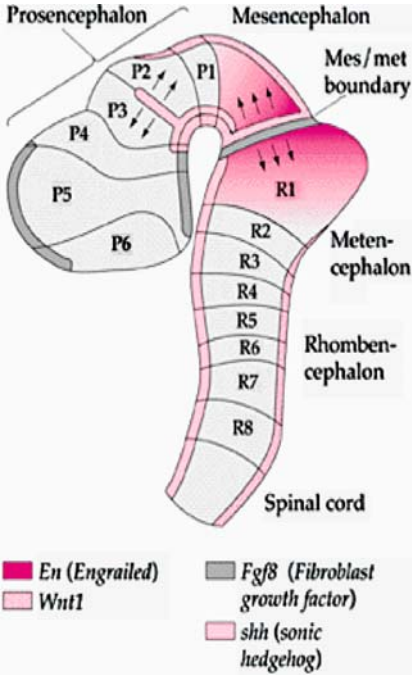

Fig. 1.1 Shortly after neural tube closure, patterning genes become expressed along the anterior-posterior, dorsal-ventral, and medial-lateral axes. These genes help compartmentalize the neural tube, giving rise to the forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). Depicted here is the neuromeric structure of the brain at approximately E8.5 in the mouse, when diffusible morphogens Wnt1, Fgf8, and *En* patterning genes are delineating the mid–hindbrain junction, and Shh, expressed by the floorplate, is involved in dorsal-ventral patterning. Note: *italicized* print denotes genes, whereas non-italicized print denotes gene products (i.e., proteins). From: Gilbert, S. F. 1997, http://8e.devbio.com/about.php, April 18, 2003

the first cells to do so between E10 and 11 will become neurons of the deep cerebellar nuclei [25, 26]. Between E12 and 14, cells that exit the cell cycle migrate out of the VZ and form a layer under the presumptive deep nuclei cells, giving rise to Purkinje neuron precursors, one of the two major types of cerebellar cortical neurons. Over the next several days of development, these Purkinje cell precursors continue to migrate out through deep nuclei precursors, forming a primitive Purkinje cell layer (PCL) that is several cells thick. A secondary site of proliferation develops as early as E10–12 at the posterior (or dorsal) edges of the cerebellar primordial neuroepithelium, the rhombic lip. These rhombic lip cells migrate out rostrally to form a superficial layer over the differentiated deep nuclei and Purkinje cell precursors; this layer

is known as the external germinal layer (EGL) and will remain a secondary site of proliferation into postnatal development [25, 26, 27]. To achieve these early cytoarchitectural arrangements, cerebellar progenitor cells rely on radial glial- and extracellular matrix-supported migration away from the VZ [28]. In fact, mutations in mice that disrupt Purkinje precursor migration, such as *reeler*, disrupt the growth and trilaminar organization of the adult cerebellar cortex. Thus, early in cerebellar development, three major neuronal precursor populations develop: deep cerebellar nuclei, Purkinje, and rhombic lip precursors; the production and organization of these cells form a blueprint for later cerebellar patterning and histogenesis.

Postnatal Cerebellar Development

In mammals, following prenatal hindbrain development, the cerebellum undergoes a major postnatal expansion during which neurogenesis continues through infancy into early childhood. In humans, this period encompasses the ages (1–3 years) when ASD symptoms first appear, long after forebrain development is completed. Cerebellar granule neurons develop from a genetically distinct population of cells from the dorsal rhombic lip as early as E10.5 [29]. These cells express the bHLH transcription factor *Math1*, deletion of which eliminates the entire population of granule neuron precursors (GNPs) [27]. Throughout embryogenesis, these *Math1*-expressing cells migrate rostrally along the subpial surface of the developing cerebellum to form the EGL, a strongly proliferative niche overlying the Purkinje cells, as described above. Postnatal GNP development is spatially restricted, such that proliferating and differentiating cells are compartmentalized, even within domains. In the EGL, for example, proliferation occurs superficially in the outer EGL while cell cycle exit and inward migration along Bergmann glia begin in the inner EGL. Differentiation begins as GNPs migrate from the inner EGL into the molecular layer, where granule neuron parallel fibers are extended, and form contacts with Purkinje cell dendrites. While parallel fibers are left in the molecular layer, differentiating granule neuron cell bodies continue to migrate inwardly, extending their axons behind them to form the inner granule layer (IGL), below the PCL. Having arrived in the IGL, fully differentiated granule neurons terminate their migration and become integrated into cerebellar circuitry as glutamatergic interneurons (Fig. 1.2).

Proliferating GNPs in the EGL rely on the underlying neurons of the PCL to secrete extracellular growth factors, such as Sonic hedgehog (Shh) [30, 31, 32], pituitary adenylate cyclase activating peptide (PACAP) [33, 34, 35, 36], and insulin-like growth factor-1 (IGF-1) [37, 38, 39]; these growth factors, each acting through their own receptor(s), provide trophic support and survival of the developing EGL cells, as well as mitogenic (cell cycle promoting) and anti-mitogenic (cell cycle inhibiting) regulation. While homotypic cell–cell contacts

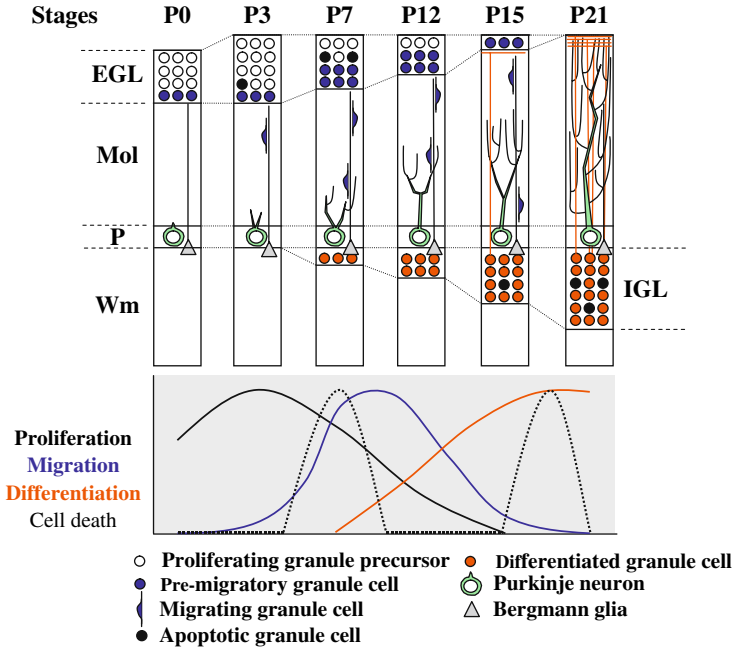

Fig. 1.2 Granule neuron precursors proliferate superficially in the external germinal layer (EGL), then migrate inwardly along Bergmann glia. During migration, the granule neuron precursors (GNPs) differentiate, producing an axon with parallel fibers that communicate with Purkinje cell dendrites, thereby forming the molecular layer. GNP migration terminates below the Purkinje cells, forming the IGL, where they are functionally integrated into the cerebellar circuitry. EGL, external granule cell layer; Mol, molecular layer; P, Purkinje cell layer; IGL, internal granule cell layer; Wm, white matter. Illustrated by Anthony Falluel-Morel, PhD (falluean@umdnj.edu) (*see* Color Plate 1)

between GNPs has been found to promote proliferation in vitro [40], in vivo proliferation is severely compromised by Purkinje cell loss [41]. Indeed, Shh, IGF-1, and PACAP mRNA and proteins have all been localized to Purkinje cells during development [30, 31, 33, 36, 38]. Further underscoring the developmental relationship between Purkinje cells and GNPs, adult granule neuron numbers were found to correlate to the number of Purkinje cells [42, 43, 44], and reciprocally, EGL integrity is required for normal Purkinje cell migration [45]. Genetic deletions targeted to Purkinje neurons that disrupt their development are deleterious to the future production of granule neurons, underlying the importance of the Purkinje-granule neuron relationship during development [32, 41].

Cerebellar Patterning Genes

Constructing regionally distinct boundaries in the neural tube so that future structures develop properly requires spatially and temporally regulated patterning

gene expression. The neuroanatomical and functional abnormalities described in ASD may result from aberrant genetic events during crucial developmental timepoints. Further, many developmental patterning genes are evolutionarily conserved orthologs of *Drosophila* genes that encode positional information in developing fly larvae; *En1* and *En2*, for example, are two mammalian orthologs of the *Drosophila* gene *en*. These genes belong to a family of homeodomain containing transcription factors that bind to DNA to regulate the expression of other genes. The mammalian *En* genes operate similarly in mammals to specify and pattern the mid–hindbrain junction and the developing cerebellum. While mutations in essential patterning genes are often incompatible with life, variations in non-coding sequences may consequently alter the regulation of that gene's expression. These subtle changes in genetic code (as well as functional mutations) are responsible for the evolution of species but may also contribute to neurodevelopmental disease pathogenesis by altering brain development. As alluded to earlier, the mid–hindbrain junction is a genetically distinct boundary in which transcription factors and secreted signals induce and cross-repress one another to delineate the cerebellar anlage from the midbrain, rostrally, and the hindbrain caudally [25] (see Fig. 1.1). The strategy of cross-repression and induction between transcription factors is not limited to the mid–hindbrain but is a common phenomenon at anatomical boundaries during development. In preventing ectopic expression of a gene within a neighboring population of neural precursors, specialized populations of neurons can develop side by side, a strategy well characterized in motor neuron development of the ventral spinal cord [46, 47, 48, 49, 50]. These epistatic relationships between patterning genes are essential, and abnormalities in their spatiotemporal expression patterns may be pathogenetic events in developmental disorders such as ASD.

As early as E8 in the mouse, the first murine ortholog of *Drosophila en*, *En1* is expressed across the mid–hindbrain junction, and its expression extends caudally demarcating the entire cerebellar anlage [51, 52]. *En2* is expressed 6–12 h later just caudal to the mid–hindbrain junction and overlaps with *En1* to demarcate the cerebellar anlage [51, 53]. The *En* genes are highly homologous [52] and functionally redundant [54, 55]; however, their expression patterns are spatially distinct, therefore they demonstrate divergent roles during development, as demonstrated by phenotypic differences between *En* mutants. Thus, normal brain development demands gene expression to occur in the right places, but also at the right times, to avoid aberrant gene interactions; given the dynamic interplay between patterning genes, it is not hard to imagine that dysfunction in one signal could have a "ripple effect" by altering expression in otherwise normal systems.

Highlighting the importance of interacting molecular systems in brain development, *En* signaling at the mid–hindbrain border was found to depend on the secreted factor *Wnt1*. Though *Wnt1* demarcates the mid–hindbrain border rostrally, and is not expressed in the cerebellar anlage, it was found to induce *En* expression. In fact, deletion of *Wnt1* results in loss of the entire cerebellum, presumably through lost induction of *En* [56]. Further, En proteins were found

to work in concert with Fgf8 to repress *Pax6.1*, an anterior forebrain patterning gene, to maintain midbrain integrity [57]. Consistent with this, ectopic *Fgf8b* expression in either the forebrain or other hindbrain regions was found to induce *En1*, *En2*, *Pax5*, and *Gbx2* expression, conferring an anterior hindbrain phenotype to these ectopic sites [58]. Thus, the power of a gene to alter a tissue's fate is not limited to a particular group of cells but may be manifested in any neural population in which its protein can be biochemically active (e.g., in the case of FGF8b to bind to its receptor). Additionally, this group also demonstrated that *En2* and *Gbx2* are the first genes induced by Fgf8b in E9.5 mice, and that while *Fgf8*, *Wnt1*, and *Pax5* can initially be expressed independent of *En*, intact *En* expression is required for the maintenance of these genes' expression [59]. Thus, the cerebellar anlage arises from complex gene–gene, and gene–growth factor interactions in which induction and cross-repression are utilized to specify boundaries and confer distinct mesencephalic and metencephalic identities.

Patterning Gene Expression

As cerebellar development progresses and populations of cerebellar neurons emerge, patterning gene expression remains integral, and as the reader will see, unique combinations of genes are utilized to direct neural precursors to become a specific type of cerebellar neuron. Recently, Morales and Hatten [60] identified different populations of cells in the developing cerebellar VZ at E12.5. These populations were identified as either proliferating progenitor cells or postmitotic deep nuclear precursor cells via their combinatorial patterns of transcription factor expression. Both cell groups expressed three amino acid loop (TALE) family transcription factors *MEIS1* and *IRX3;* however, only postmitotic deep cerebellar nuclei precursors that had migrated superficially were found to express *MEIS2* and the LIM homeodomain containing transcription factors *LHX2/9* [60]. These transcription factors remain expressed in deep cerebellar nuclear precursors through mid-gestation as they migrate rostrally and inwardly below developing Purkinje cell precursors. Thus, gene expression allows identification of emerging cell populations but may also allow their tracking from birth through functional integration into neuronal circuitry.

A similar approach to that of Morales and Hatten (2006) [60] was used to demarcate deep cerebellar nuclear precursors originating from the rhombic lip. Fink et al. (2006) [61] found sequential expression of transcription factors *Pax6*, *Tbr1*, and *Tbr2* identified rhombic lip cells that migrated from the subpial stream through a nuclear transitory zone to join their VZ-derived counterparts. Interestingly, these rhombic lip cells gave rise exclusively to glutamatergic projection neurons, whereas VZ-derived deep cerebellar nuclear precursors expressed *Pax2* and were fated to become GABAergic interneurons [61]. Further, subpopulations of deep cerebellar nuclear cells found to be molecularly distinct in development

remained so into adulthood and would later be found to occupy predictable medial-lateral positions within the deep cerebellar nuclei [61]. These data suggest that early gene expression functionally segregates some cerebellar neurons as progenitors; therefore, developmental abnormalities that disrupt gene expression could have predictable consequences on cerebellar function.

Combinatorial Gene Expression Confers Cell-Specific Identity

Given that combinatorial gene expression can specify multiple neuronal subtypes from a population of neural precursors, it follows that combinatorial stimuli (intrinsic and extracellular) can have a similar effect on a seemingly homogeneous population of differentiating cells: Purkinje neuron precursors. These cells give rise to a heterogeneous population of mature neurons that express positionally defined combinations of genes and proteins during development and adulthood. Given that Purkinje neuron deficits are the most consistent neuropathologic finding in ASD, these subtle differences in Purkinje cell identities (and possibly also functions?) may be very important in ASD pathogenesis and heterogeneity. To understand these differences in Purkinje neuron identity, we can examine developmental timepoints at which Purkinje neuron precursors genetically diverge from other neural precursors and from themselves. Beginning around E10.5, *En2* is expressed in all proliferating cells of the midbrain and cerebellar primordia [53]. By E12.5, though, some Purkinje neuron precursors exit the cell cycle, migrate out of the VZ, and begin to express the transcription factors *LHX1/5*. Thus, at this early stage, postmitotic Purkinje neuron precursors are homogeneously *LHX1/5* positive and can be differentiated from both proliferating (*En2*-expressing) VZ cells as well as the postmitotic deep nuclei precursors [60]. Interestingly, from E12.5 to 17.5, Purkinje cell development is regulated by *En2*, which works antagonistically with other homeodomain proteins, *Hoxa5* and *Hoxb7*, by repressing a Purkinje cell-specific gene, *Pcp2* [62]. However, by E16.5, the majority of Purkinje neurons express both *LHX1/5* and the differentiation marker calbindin, irrespective of individual cell *En2*, *Hox*, or *Pcp2* expression, suggesting that different genetic mechanisms are employed simultaneously to achieve a Purkinje cell fate. Thus, heterogeneity within the Purkinje neuron population is further achievable by augmenting the level of activity of each of these components and by other genetic and extracellular signals expressed later during development.

In addition to promoting cerebellar neuronal fates and subpopulation specialization, patterning genes, including *En2*, influence the rate of cerebellar growth as well as the organization of the organ along several spatial axes. To achieve these patterning events, *En2* expression during late embryogenesis (mouse E17.5-P0) becomes restricted to longitudinally oriented stripes, in which all cell types within the anterior-posterior/dorsal-ventral stripe express the gene. This striped pattern of *En2* expression sets up a cytoarchitectural map across the developing cerebellum, providing positional information to

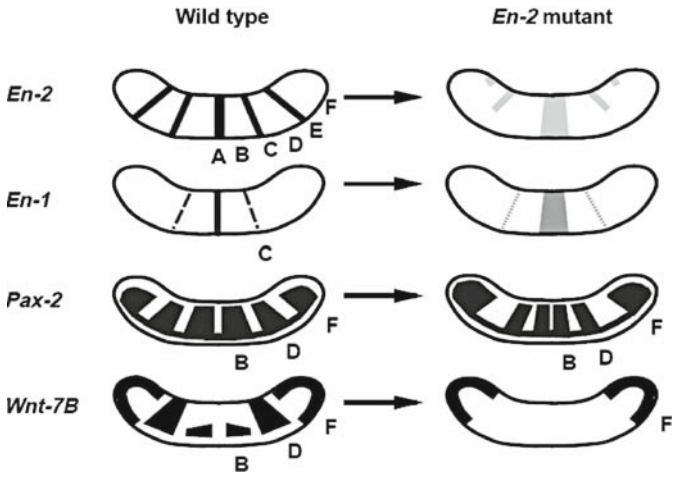

Fig. 1.3 Several patterning genes are expressed complementary to *En2* expression. While expression of cerebellar patterning genes is preserved in the *En2* knock-out (KO) mouse mutant, discussed below, their spatial expression patterns are disrupted. This suggests that positional information necessary for developing functional compartments and neuronal subpopulations may be lost, contributing to abnormal cerebellar morphology and function. WT, wild type. Adapted from Millen et al. (1995) [111]

innervating fibers and migrating neurons to assist finding their targets and regulates where other important patterning genes are expressed [63, 64, 65]. These other genes include *En1*, *Wnt7b*, and *Pax2*, which are also expressed in spatially restricted patterns of stripes, complementary to those of *En2* expression (Fig. 1.3). These patterning gene expression profiles are reminiscent of their phylogenetically older *Drosophila* orthologs, suggesting that mammalian compartmentalization may be the evolutionarily conserved process of larval segmentation in the fly [66].

Biochemical and Molecular Signals in Cerebellar Development

As cerebellar development progresses, the organ becomes less homogeneous, forming a recognizable ultrastructure composed of the hemispheres, the vermis, and the deep cerebellar nuclei. Within these structures, biochemical and molecular signals are expressed in parasagittal banding patterns that compartmentalize the developing cerebellum into reproducible topographies and functional domains [63, 67, 68, 69, 70, 71, 72]. This process begins mid-gestation and may be important for conferring identities to subpopulations of otherwise homogeneous cell types,

such as Purkinje neurons. Though the functional consequence of having biochemically distinct Purkinje neuron subpopulations is not fully understood, patterns of Purkinje cell loss in ASD may reflect differential susceptibility between these neurons to genetic or biochemical insults. Characterizing the biochemical differences between surviving Purkinje neurons in postmortem samples from ASD patients may provide insight into the mechanisms of cell loss in these patients. For example, Leclerc et al. [71] demonstrated that Zebrin II (aldolase-C) and P-path (9-*O*-acetylated glycolipids) immunopositivity marked parasagittal bands of complementary compartments across the cerebellum with medial-lateral symmetry. Further, these markers identified three distinct populations of Purkinje neurons: Zebrin+, P-path−; Zebrin−, P-path+; and Zebrin+, P-path+. Additionally, olivocerebellar and mossy fiber afferents were found to largely respect these compartments [71]. Further, expression of the previously mentioned Purkinje cell gene *L7*/*Pcp2* similarly follows parasagittal banding during late embryogenesis, and olivocerebellar afferents again project to specific clusters of immunopositive and negative Purkinje cells [73, 74]. Thus the cerebellum is functionally and biochemically, as well as anatomically, compartmentalized during embryogenesis by genetic signals [72], suggesting that genetic perturbations could disrupt biochemical expression patterns and functional compartments.

Cerebellar Abnormalities in ASD

Given that almost all social or cognitive behaviors that are abnormal in ASD require some degree of attention regulation, it is possible that several functionally abnormal brain regions communicate with a common dysfunctional brain structure: the cerebellum. Though once believed to be solely involved in motor coordination, balance, and motor memory, the human cerebellum participates in higher order functions, including speech and attention regulation. In fact, shifting attention between stimuli requires an intact cerebellum, because even slight cerebellar abnormalities have been found to disrupt rapid shifting of attention. Interestingly, cerebellar activation patterns in ASD patients have been found to be abnormal, with reduced cerebellar activation in tasks requiring attentional shifting and increased activation in motor tasks [75]. In fact, ASD patients were found to perform similarly to patients who sustained cerebellar injury, with both groups showing reduced accuracy in shifted attention as a function of reaction time, compared to age-matched controls [76]. Cerebellar dysfunction at the behavioral and imaging level may have correlates at the molecular level. Purkinje neurons are activated by glutamatergic (i.e., excitatory) inputs from granule neurons and olivary climbing fibers but are themselves GABAergic (i.e., inhibitory). The rate-limiting enzyme glutamic acid decarboxylase (GAD67) that converts glutamate to GABA is highly expressed in adult Purkinje neurons. Recently, Yip et al. (2007) [77] found GAD67 expression by Purkinje neurons in postmortem cerebella of humans with ASD was reduced by 40% compared to normal controls.

This same group previously reported an abnormal increase in GAD67 expression by cerebellar basket cells [78], which function to inhibit Purkinje neuron firing by modulating action potential propagation at the axon hillock. These findings together suggest that surviving Purkinje neuron function is depressed in ASD by reduced neurotransmitter production and increased synaptic inhibition [77]. That deficits in attention regulation correlate with abnormal cerebellar activation, together with consistent cerebellar neuropathological findings and recent evidence of molecular dysregulation of Purkinje neurons suggests that ASD pathogenesis may be linked to abnormal cerebellar development in many patients.

Effects of Cerebellar Abnormalities in ASD

With even a basic understanding of how the cerebellum develops, one can appreciate how ASD-associated cerebellar abnormalities may arise. Given their prenatal development, the Purkinje neuron deficits described in ASD suggest that a prenatal insult may disrupt survival or maturation of these cells. If so, then cerebellar growth rates could be altered from birth, with growth retardation depending upon the severity of Purkinje cell loss or dysfunction, whereas overgrowth may be due to compensatory growth factor production by olivocerebellar afferent fibers; additionally, cerebellar size may appear grossly normal though underlying cellular losses have begun. However, Purkinje neuron maturation continues concurrently with GNP proliferation and the formation of functional circuitry; therefore, later Purkinje cell loss could also occur with minimal effects on cerebellar size, while greatly altering its function. Neuroimaging studies of cerebellar grey and white matter growth from early infancy through adolescence have indeed demonstrated dysregulated cerebellar growth in ASD patients, with overgrowth in early childhood correlating with increased head circumference [4, 5]. At 2–3 years old, cerebellar grey matter volumes were identical between ASD and normal children; however, by age 6–9 normal children's grey matter volumes increased whereas ASD children's did not; grey matter volumes remained reduced in ASD into adulthood. White matter volumes were 40% larger in ASD infants than normal children at age 2–3, normal children experienced a 50% increase in volume from ages 2–3 to 12–16 years, whereas ASD children's white matter volume growth was almost flat (7%). Naturally, these disparate grey and white matter growth patterns translate to vastly different developmental gray : white matter ratios, a possible indication of gross cerebellar dysfunction. While compelling, these data reflect changes in a subpopulation of children with ASD and may not be generalizable to the ASD community at large.

Numerous studies have measured overall cerebellar volume in children, adolescents and adults with ASD, with differing results. While initial studies suggested posterior cerebellar vermi were smaller in ASD than normal adults and exhibited abnormal foliation, these findings have not been replicated

[79, 80]; in fact, methodological irregularities, such as failure to control for IQ, may have accounted for these changes [81]. That said, when matched for IQ and age and corrected for total brain volume, the majority of neuroimaging and postmortem studies have reported an increase in cerebellar volume in ASD [4, 5, 81, 82, 83, 84, 85, 86]. Deep cerebellar nuclei neurons were also found to undergo changes in size and appearance, beginning large in childhood and becoming small and pale later in life. Though these changes remain to be replicated, similar changes were noted in the presynaptic neurons of the inferior olive, suggesting that olivocerebellar circuitry may be functionally abnormal in ASD. Similar to Purkinje-granule neuron numbers, there exists a linear relationship between brainstem olive and cerebellar Purkinje neuron numbers [68, 87]. Indeed, olivocerebellar projections begin early in brain development, and the olivary climbing fibers are known to provide trophic support for developing Purkinje neurons [88, 89, 90, 91]. Purkinje cell losses described in ASD ought to correlate with similar losses in correlating brainstem nuclei; however, only sporadic ASD case studies have found inferior olive cell losses [11, 92]. Thus, the period of ASD pathogenesis appears to begin prenatally, beginning early enough for neuronal populations to compensate for abnormalities in their target cells, though the exact timing and causes remain unknown.

Extracerebellar Functional Deficits in ASD

The maxim "structure dictates function" is often repeated in biology classes; therefore, structural brain abnormalities observed in ASD brains may underscore the behavioral deficits observed in humans with ASD. Functional neuroimaging in live patients suggests that functionality or connectivity within and between specific brain loci is abnormal [93]. For example, social cognition and emotion recognition deficits correlated with reduced amygdalar activation in patients with ASD [94, 95, 96, 97]. In several other studies, ASD patients showed hypoactivation and abnormal lateralization of the posterior superior temporal sulcus in tasks that infer mental intent by tracking the eye movements of others, as well as reduced activation in response to human voices [98, 99, 100, 101]. Further, abnormal processing of faces by patients with ASD is well characterized and correlates with reduced activation of the fusiform gyrus and inappropriate activation of adjacent cortical structures not associated with processing face stimuli [102, 103, 104, 105]. However, abnormal face perception and fusiform activation in ASD may be a byproduct of inappropriate focus, as these patients attended away from eyes and toward other facial features; in fact two studies found a correlation between time spent focusing on eye regions and magnitude of fusiform gyrus activation in ASD [104, 106]. Together, these studies suggest abnormal socialization and behavior in ASD may be due to processing of stimuli by inappropriate brain regions. Further, abnormal function in one structure may disrupt global processes in which that abnormal region communicates with other

brain loci to code and respond to environmental cues. Thus, abnormal amygdalar coding of emotional information in facial expressions may disrupt higher order face recognition, leading to disrupted social behaviors associated with ASD.

En2 Transgenic Mice Phenocopy ASD Cerebellar Neuropathology

Animal models are useful tools for studying complex human diseases such as ASD. However, finding an appropriate animal model for ASD is difficult because it is a uniquely human disease characterized by behavior deficits in modalities absent from rodents (i.e. language, emotional reciprocity, etc.). However, several mouse mutants have been generated over the last several decades demonstrating a range of neuropathological deficits that appear similar to (or phenocopy) human disease states. Specifically, transgenic animal models in which *En2* expression has been altered, either by knock out (KO) or by overexpression, exhibit cerebellar neuropathology similar to that of ASD [63, 64, 107, 108, 109, 110, 111]. *En2* KO cerebella are approximately 30% smaller than the wild type (WT), resulting from a 30% reduction in numbers of all cell types, including Purkinje and granule neurons [63, 64, 110, 111]. Differences between WT and *En2*-deficient cerebellar development are evident as early as E15–16, when mutant cerebella fail to fuse medially and overall cerebellar size is diminished [64]. By E17.5, though, these differences have disappeared, suggesting that the absence of *En2* expression imparts a delay in cerebellar maturation during embryogenesis. In addition to gross morphological changes during late embryogenesis, *En2* mutant cerebella maintain expression of patterning genes although abnormalities are found [111]. Defects in hemispheric and vermal foliation are apparent in *En2* KO mice early in the postnatal period; while overall morphology is similar, there is an apparent delay in foliation such that mutant cerebella resemble 1–2 days younger WT cerebella. In addition to retarded growth and foliation, the formation of fissures that separate lobules is also delayed, and often incomplete, leaving normally separated lobules to appear fused in the KO hemispheres and vermis. These morphological deficits are more severe in the vermis where *En2* expression is greatest and mostly limited to the posterior lobules; in fact, early postnatal development of the anterior cerebellum appears morphologically normal though significantly delayed [64]. As a consequence of abnormal fissure formation, posterior lobes of the vermis are abnormally positioned, such that lobe VIII becomes a branch of IX and the sizes of lobes VII and VIII are reduced; interestingly, specific hypomorphic vermal phenotypes have also been described in groups of ASD patients [5, 64, 79, 80, 111].

Molecular Abnormalities in En2 Mutant Mice

The gross morphological abnormalities observed in postnatal cerebella of the *En2* KO mouse are punctuated by changes in the normal biochemical bands

discussed above, and at the molecular level, these posterior lobules appear transformed so that they express characteristics of more anterior cerebellar molecular phenotypes [63, 111]. These patterns of molecular expression create compartments that are critical for establishing proper pre- and postsynaptic connections. Thus, an improper molecular environment may lead to abnormal cerebellar circuitry in these mice, similar to the functionally abnormal cerebella of human ASD patients. As demonstrated embryonically, postnatal expression of patterning genes are maintained in the absence of *En2* although their spatial boundaries are abnormal; for example, *Wnt7b* expression extends further posteriorly in *En2* KO cerebella, suggesting that these lobules have taken on an anterior genetic phenotype. Further, Zebrin II- and P-path banding patterns are overall maintained in the KO cerebella though there are several regions displaying abnormal or absent banding patterns [63]. Though gross abnormalities are largely absent from the anterior lobes, KO cerebella display abnormal Zebrin II banding anteriorly in lobule III and dorsomedially in lobule V, as well as posteriorly in lobules VIII and IX; in the lateral hemisphere, there is precocious banding along the fused crus II-paramedian lobule at P2 that gives way to an abnormally small band in the adult. In addition to abnormal biochemical markers of compartmentalization, the *En2* KO mouse cerebellum exhibits absent bands of expression of the Purkinje cell-specific *L7*/*Pcp*, most notably in the hypomorphic lobes of the posterior cerebellum. These disordered and lost compartmental markers suggest that subpopulations of Purkinje cells may be more susceptible to abnormal *En2* expression; these subpopulation differences may provide insight into patterns of Purkinje cell loss in humans with ASD [3]. By extension, this would suggest that functionally distinct cerebellar domains may be more susceptible to genetic perturbation and thus contribute to an ASD phenotype. In support of this idea, spinocerebellar afferents were found to form abnormal terminal fields in *En2* KO mouse posterior vermal lobules VIII and IX [65]. The KO afferent pattern was simpler than observed for the WT and formed a similar pattern of absent banding found by Zebrin II and *L7* expression though lobule identity was maintained. Several studies of humans with ASD have found abnormal functional circuitry by neuroimaging, as well as deficits in cerebellar-mediated attentional regulation [76, 79, 96, 98, 106, 112, 113, 114], thus the abnormal connectivity of the posterior cerebellar vermis observed in *En2* KO may have clinical correlates in humans with ASD.

Overexpression of En2 also Results in ASD-Like Cerebellar Pathology

In addition to the complete deletion of *En2* function in the KO, abnormal expression of the gene (specifically, ectopic overexpression in postmitotic Purkinje neurons) has been found to alter cerebellar development, suggesting

that proper levels as well as spatiotemporal regulation are required to achieve cerebellar patterning. Similar to the KO, cerebellar development is retarded, overall size is reduced in the rostrocaudal and dorsoventral axes but relatively normal mediolaterally, and Purkinje cell numbers are reduced by 30% when *En2* is ectopically overexpressed in differentiating Purkinje cells [107]. Purkinje cell losses are consistent across all regions of the cerebellum; however, there is a selective loss of Purkinje cells underlying the reduced, or absent, fissures that normally separate sublobules. Further, *En2* overexpression in Purkinje cells delays their expression of parvalbumin and dendritic arborization, causes Purkinje cell soma to be smaller, and increases Purkinje cell ectopia outside the normal monolayer [115]. Accompanying Purkinje cell loss in fissures is a selective reduction in size of the EGL, once more demonstrating the functional relationship between Purkinje cells and EGL development. Given these disruptions in Purkinje and granule neuron development, as well as morphological abnormalities, it follows that biochemical compartmentalization and spinocerebellar afferent inputs also are disrupted by *En2* overexpression. In contrast to the *En2* KO mouse, early banding patterns corresponding to *L7* or cadherin-8 expression are normal; however, Zebrin II and P-path sagittal bands are disrupted in all lobules and severely fractured in posterior lobules VIII and IX. Whereas banding borders are sharply delineated in the WT, borders are diffuse in the transgenic, with mottling in the normally unmarked interband regions. Further, sagittal banding of a granule neuron marker, NADPH-diaphorase, is disrupted anteriorly and more severely disrupted in posterior lobules VI and VII [116]. As sagittal banding is thought to specify distinct Purkinje cell subpopulations for the purpose of functional compartmentalization [71], abnormal *En2* expression in transgenic mice disrupts this specification process, thereby disrupting topographical mapping of the cerebellum during development. In light of these patterning disruptions, spinocerebellar afferents are found to innervate transgenic cerebella in similar patterns to WT cerebella. However, like the biochemical banding patterns, spinocerebellar afferents are more diffusely spread with less distinct banding borders. Thus, two different genetic perturbations of *En2*, complete absence and ectopic overexpression, disrupt normal cerebellar development, resulting in similar morphologic, biochemical, cytological, and patterning abnormalities; further, these transgenic mouse cerebella phenocopy some of the most consistently cited neuropathologies reported in humans with ASD [3, 108, 109].

Functional Deficits in En2 *Mutant Mice*

Given the relatively severe cerebellar deficits exhibited by *En2* KO mice, it is remarkable that they are not ataxic although they performed worse than WT on rotorod tests, suggesting that motor learning is impaired in the absence of *En2* [117]; these findings are in contrast to other cerebellar mouse mutants, including

reeler, staggerer, and weaver, that display severe ataxia [118]. Humans with ASD, however, are also not ataxic even though they display cerebellar deficits and perform similarly to patients with cerebellar lesions on tests of attention regulation [76]. A recent study characterized social and neurocognitive behaviors in *En2* KO mice and found a reduction in social interactions with normal mice across developmental ages, as well as impaired learning and memory, in the absence of gross locomotor abnormalities [119]. In addition, this study found a cerebellum-specific increase in serotonin and its metabolites, a neurotransmitter system often found to be dysregulated in ASD [119]. A previous genetic analysis in humans found association between specific haplotypes of the serotonin transporter gene (*5HTT*) and autism in a homogeneous Irish population [120]. These haplotypes were later found to alter mRNA transcription of *5HTT*, which could alter brain serotonin levels [121]; thus in addition to neuropathology, *En2* KO mice behaviorally and neurochemically phenocopy ASD. These similarities between humans with ASD and *En2* transgenic mouse phenotypes were the impetus for exploring human *EN2* as a possible autism-susceptibility gene.

ASD Genetic and Environmental Etiology

The etiology of the ASD, in the absence of known cytogenetic abnormalities such as Fragile X, tuberous sclerosis, or duplication of 15q, remains unknown. Although environmental factors, including neurotoxicants such as pesticides and heavy metals, have been raised as possible disease-causing agents, presently they remain unsubstantiated [122]. However, several teratogens, including the anti-emetic thalidomide, anti-convulsant/mood stabilizer valproic acid (VPA), and the abortificant misoprostol, are associated with ASD; therefore, understanding their period of teratogenicity may provide insight into the timing, if not the mechanisms, of ASD pathogenesis. On the contrary, there is strong evidence for the heritability of ASD, including twin studies, duplex and multiplex families, and recent genetic association studies of specific genes. Further, there is an approximate 4:1 male : female ratio in ASD, and autistic females are more likely than autistic males to have very low IQs [1, 123]; there is currently no explanation for this sex ratio inequality.

Thalidomide and VPA

Prenatal exposure to thalidomide and VPA produce very similar dysmorphic features including neural tube defects, congenital heart defects, craniofacial abnormalities, abnormally shaped or posteriorly rotated ears, genital abnormalities, and limb defects [124], consistent with an early period of teratogenicity during the first 3–4 weeks postconception. Some of these dysmorphic features

have been reported in idiopathic ASD, further suggesting that early embryonic insults increase ASD susceptibility. While thalidomide is not teratogenic in animal models, in utero-VPA administration in rodents has duplicated many of the skeletal and organ abnormalities (brain, heart), and most importantly has resulted in ASD-like behavioral abnormalities. Varying the embryonic day (E10–12.5) of VPA administration to pregnant rats produced different brainstem abnormalities in their offspring, suggesting that neurogenesis was disrupted at the time of exposure; importantly, Purkinje neuron deficits and reduced deep cerebellar nuclei volumes were found following VPA administration on and after E12.5 [125]. Behaviorally, both humans with ASD and rats exposed to VPA during early embryogenesis show enhanced eyeblink conditioning [126, 127]. Compared to late gestational or postnatal destruction of Purkinje neurons in which eyeblink conditioning is disrupted, early exposure has been shown to enhance this learning, suggesting that compensatory reorganization of brainstem–cerebellar circuitry may occur following early insults. Additionally, brain and systemic hyperserotonemia has also been described in both VPA animal models and humans with ASD; in fact, brain serotonin synthesis, which decreases after age 5, remains higher in autistic children as they age than their non-autistic siblings [128]. Further, both autistic children and adults demonstrate functionally asymmetric serotonin synthesis in pathways important for language and integration of sensory stimuli that correlate with specific language deficits [129, 130, 131]. The mechanism of VPA teratogenicity is not fully characterized though it has been shown to activate the retinoic acid response element (RARE), an important regulator of HOX gene expression [132]; in fact, VPA was found to up-regulate *Hoxa1* expression before and after its critical period of expression during hindbrain development, possibly via inhibition of histone deacetylase [133]. While mutations in HOXA1 have been found to cause syndromes of craniofacial abnormalities associated with mental retardation and ASD [134], it has failed to be associated with ASD in several populations [135, 136, 137, 138, 139]. Finally, rates of ASD among *in utero* thalidomide- and VPA-exposed children is approximately 4 and 11% [124, 140], respectively, suggesting that environmental exposures may increase ASD risk in genetically susceptible individuals but are unlikely to be causative of the developmental disease itself.

Twin Studies in ASD

The first ASD twin studies were published in 1977, and though they included a relatively small number of twins (21 pairs), they demonstrated a large imbalance in concordance between monozygotic (MZ) and dizygotic (DZ) twins [141, 142]. Further, these studies raised the possibility that broader phenotypes beyond classically defined autism may exist and that there would be strong genetic liability between MZ twins for these phenotypes as well. These results

and assertions were validated by further twin and family studies published during the 1980s and 1990s, demonstrating that MZ concordance rates were 60–90%, whereas DZ concordance rates were 0–10%; a quantitative assessment of concordance inequality between MZ and DZ twins estimated greater than 90% heritability for autism [143, 144, 145, 146]. Unexpectedly, these studies also found that clinical heterogeneity extended to MZ twins, suggesting that shared gene expression does not necessarily dictate similar behavioral phenotypes [147]. Additionally, family studies conducted during this period extended analysis to non-twin siblings and other first- and second-degree relatives of ASD patients to better ascertain the genetic liability of the disease [143, 148, 149]. These studies found 2–6% autism rates among siblings of autistic patients compared to no autism found in siblings of Down syndrome patients; when a broader phenotype was considered (what is now considered ASD), the rate increased to 10–20% ASD incidence in autism siblings compared to 2–3% in Down syndrome siblings. The pattern of increased risk for first-degree versus second-degree relatives, in conjunction with the MZ–DZ concordance inequality led researchers to believe that autism was a complex heritable disease that resulted from epistatic interactions between approximately 2–10 genes [143]. It is currently believed that between 3 and 15 genes may confer susceptibility to ASD, with complex epistatic interactions contributing to the heterogeneity of clinical presentations [109, 150]. Further, epigenetic regulation of gene expression and interactions is now recognized to be essential to normal brain development, and several neurodevelopmental disorders are linked to abnormal epigenetics.

Rett Syndrome and Epigenetic Regulation

One such disease is Rett Syndrome, a progressive developmental disorder similar to ASD but almost exclusively affecting females. Unlike ASD in which several abnormal genes may be inherited, Rett syndrome results from mutations of methyl CpG-binding protein 2 (*MECP2*), an epigenetic effector molecule that represses gene expression by binding to methylated CpG islands (151; see Chapter 4 by Kaufmann et al.). Mutations that alter function of Mecp2 in mice, as well as humans with Rett, ASD, and Angelman syndromes, were recently demonstrated to disrupt expression of both imprinted and non-imprinted genes in the Angelman/Prader-Willi region of 15q11–q13 [152]. Thus, disruptions of complex epigenetic interactions may contribute to developmental disease pathogenesis, making individual genetic contributions difficult to ascertain. Further, novel mutations and polymorphic copy number variants within *MECP2* exons, introns, and the 3′-untranslated region (3′-UTR) have been identified in both males and females in several autistic populations [153, 154, 155]. This suggests that slight alterations in *MECP2* that are non-fatal can still disrupt brain function, possibly contributing to ASD pathogenesis. Interestingly, a recent study found

that restoration of *Mecp2* expression fully reversed the disease phenotype in null mice exhibiting neurological impairments similar to humans with Rett syndrome [156]. These results suggest that PDD phenotypes may be reversible in humans and that future therapies aimed at correcting abnormal genetic and epigenetic interactions may prevent or attenuate the severity of developmental diseases such as ASD.

Genes of Interest in ASD

Once a genetic etiology of ASD was established, the search for ASD-associated genes began, first by identifying regions in the human genome containing marker variants [157] and then by identifying candidate genes within these regions [147, 158]. Ideal candidate genes were not only found in or near ASD-susceptibility loci but were expressed during brain development and/or brain function. Given the clinical and genetic heterogeneity of ASD, candidate gene association has been difficult to establish, and replication across populations nearly impossible. For example, a functional variant in the promoter of the tyrosine kinase receptor *MET* (7q31) was recently found to be associated with ASD [159] though this finding is yet to be replicated. *MET* signaling is found in various peripheral organs, as well as the developing brain, thus a functional variant that possibly alters receptor activity may contribute to abnormal growth and ASD pathophysiology. Several other genes involved in brain growth and function have been found to be associated with ASD in selected populations. Mutations in postsynaptic cell adhesion molecules neuroligins (NGLN) 3 and 4 on the X-chromosome [160, 161, 162] and a member of their receptor family, neurexin-beta [163] have been identified in ASD families, suggesting that disrupted synaptogenesis may contribute to ASD dysfunction. Further, the hormone oxytocin is implicated in regulating social behaviors including fear and pair bonding and is commonly administered during labor to stimulate parturition; recently, several allelic polymorphisms in the oxytocin receptor (*OXTR*) were found to be associated with ASD in Chinese and Caucasian populations though different alleles at the same locus were differentially associated between the two studies [164, 165]. Other genetic associations have been identified in case studies or subpopulations, some demonstrating a particular phenotype, such as macrocephaly and the tumor suppressor gene *PTEN* on chromosome 10q23 [166, 167]; the cerebellar Ca^{2+}-dependent secretion protein CAPS2 on human chromosome 7q that promotes neurotrophin release [168–170]; and the extracellular glycoprotein Reelin (*RELN*) on distal chromosome 7q, which is required for normal neural migration and cytoarchitecture during development [171, 172, 173]. Each of these genes alone may confer risk or may interact with other genetic or environmental factors that increase an individual's susceptibility to ASD. Our understanding of these genes' roles in ASD will be improved by replication across large populations; however, the heterogeneity of ASD makes replication very difficult.

Endophenotypes and ASD Genes

As our understanding of the underlying causes of ASD improves, we may be able to subdivide the disease into endophenotypes, which focus on select features of the disorder, such as language impairment or repetitive movements. Such features by themselves are not unique to the disorder but may exhibit greater genetic heritability than the entire spectrum of signs and symptoms comprising ASD. For example, *PTEN* abnormalities may segregate with a macrocephalic subpopulation of ASD but may not be associated with ASD in the general population. Recently, a quantitative trait loci (QTL) analysis of non-verbal communication in ASD families suggested that there are chromosomal regions that harbor ASD-susceptibility genes segregating selectively with severe language impairment but not other endophenotypes [174]. Further, despite lack of association between ASD and *HOXA1* in numerous studies, one polymorphism was found to be associated with increased head circumference in ASD and was reported to explain 5% of the variance of head size in the data set studied [175]. Thus, links between candidate genes and symptomology may help simplify the genetic and clinical heterogeneity of ASD into parsable domains and may also be better prognostic indicators of behavioral and pharmacological interventions.

ENGRAILED-2 is an Autism-Associated Gene

The pervasiveness of ASD across races, ethnicities, continents, and socioeconomic status may seem at odds with its heterogeneity; however, our current diagnostic criteria are inclusive and symptom-based, thus multiple modes of inheritance may all give rise to a similar clinical label [82, 109, 150, 176, 177, 178, 179, 180]. Thus, it is increasingly difficult to identify ASD-associated genes and replicate these associations across large, heterogeneous populations. That said, we have recently identified human *ENGRAILED-2* (*EN2*) as an ASD-susceptibility locus [108, 109]. As stated earlier, genome-wide analyses have identified chromosomal regions linked to ASD, thus candidate genes in these regions can be explored further for association with the disease. One region of interest has been the long arm of human chromosome 7 [157, 181]. The human *EN2* gene is found distally on the long arm of human chromosome 7, just outside an ASD-linkage region [182, 183, 184]. As described above, *EN2* is expressed during brain development and has been found to be essential for normal cerebellar function; further, *En2* mutant mice exhibit cerebellar neuropathology, as well as neurochemical and behavioral abnormalities that phenocopy humans with ASD. Thus, *EN2* was selected as a possible candidate gene to be screened for association with ASD.

The human *EN2* gene is relatively simple, spanning approximately 8.0 Kb and containing only two exons separated by a 3.3 Kb intron; four single nucleotide

polymorphisms (SNPs) that span most of the *EN2* gene were chosen for analysis. SNPs are common (occur in at least 1% of the population) heritable variations found across the entire genome and can be used to track parental transmission of disease alleles to their children. Gharani et al. (2004) [109] genotyped four SNPs that span the *EN2* gene in 138 parent–child triads and a total of 167 extended pedigrees (including other affected and/or unaffected siblings) from the autism genetic research exchange (AGRE). They assessed allelic transmission from heterozygous parents to children with ASD and their unaffected siblings, using the transmission-disequilibrium test (TDT) [185]. Two intronic SNP alleles were each found to be overtransmitted to children diagnosed narrowly with autism as well as broadly with any ASD diagnosis and undertransmitted to their unaffected siblings. This suggests that inheritance of each allele is associated with autism and more broadly with ASD. Further, these two alleles were found to be overtransmitted together as a haplotype to both narrow and broadly diagnosed children; the exonic SNPs were not found to be associated with ASD, alone or even as haplotypes with the intronic SNPs, suggesting that the disease locus is either located within the *EN2* intron or is located elsewhere but is in tight linkage disequilibrium (LD) with the intronic SNPs [109].

These findings were replicated in two more non-overlapping, unrelated data sets of 222 additional AGRE pedigrees and 129 families from the National Institute of Mental Health (NIMH) [108]. All three data sets were combined to give a total of 518 families, and again the individual intronic SNPs, as well as the SNP haplotypes, were significantly associated with both the narrow diagnosis of classical autism and the broader spectrum of ASD; importantly, these associations were found to increase in statistical significance in the large, combined data set. This study also expanded analysis to 14 more SNPs spanning the entire *EN2* gene; however, no other SNPs individually, or in a haplotype with the two associated intronic SNPs, were found to be associated with ASD; in fact no other markers within two megabases 5′ or 3′ of *EN2* were found to be associated with ASD, suggesting that the intronic SNPs 972 and 973 are the ASD-susceptibility locus. Furthermore, *EN2* was found to confer disease risk in approximately 40% of the ASD cases studied [108], supporting *EN2* as an ASD-susceptibility locus. To our knowledge, this is the first time an ASD association has withstood replication across heterogeneous populations. Further, preliminary evidence suggests that the ASD-associated intron alters *En2* promoter-driven luciferase expression compared to the non-ASD-associated intron, suggesting that abnormal *cis*-regulation of *EN2* expression during development contributes to ASD pathogenesis (Dr. J. H. Millonig, personal communication).

En2 Function During Cerebellar Development

Despite its well characterized expression, the function of *En2* during cerebellar development remains largely unknown. As mentioned previously, *En2* expression prenatally plays an important part in specifying Purkinje neuron subpopulations

and creating a topographical map of the embryonic cerebellum. Within the last decade, studies of Engrailed proteins have revealed expanded developmental roles for these presumptive transcription factors. Although traditionally viewed as DNA-binding proteins, the *Engrailed* homeoproteins have been empirically shown to participate in non-transcriptional activities, including translational regulation [186, 187, 188]. Amino acid sequence analysis has demonstrated that an 11-residue sequence in the Engrailed homeodomain is necessary and sufficient to function as a putative nuclear export signal (NES) [189, 190]. Indeed, Engrailed1 and 2 proteins were found to localize outside of the nucleus, in subplasmalemmal and intracellular vesicles of primary midbrain neurons [191]. These data suggested that homeoproteins are made available for secretion and correlated with previous findings that other homeodomain proteins, including Emx1 and HoxA7, localize to axons [191, 192]. In fact, Engrailed proteins were found to contain an unconventional secretion sequence that allowed their export from the cell, as well as internalization by other cells, through non-endocytotic mechanisms [190]. This secretion was blocked by the serine-threonine kinase CK2-phosphorylation of specific serine residues within the Engrailed secretion sequence [193]. Engrailed protein is also phosphorylated on a serine in its homeodomain by protein kinase A (PKA) that reduces its DNA-binding affinity [194]. Therefore, beyond *cis*- and *trans*-regulation of *Engrailed* by other developmental transcription factors, it is important to consider posttranslational modifications as important regulators of Engrailed functions and localization during development. Very recent work by Brunet et al. (2005) [186] found that Engrailed2 could differentially regulate retinal axon guidance in the *Xenopus* optic tectum, simultaneously attracting nasal growth cones yet repelling temporal growth cones. These functions were independent of transcription but rather involved internalized En2 binding to eukaryotic initiation factor 4E (eIF4E) and promoting the phosphorylation of eIF4E and its binding protein (eIF4EB), leading to local axonal protein translation [186]. Engrailed1 protein, however, has been found to be increased in an activity-dependent manner in the dendrites of adult midbrain neurons; further, hemizygous *En1* leads to midbrain neuron death in adult rodents [188, 195]. Thus, Engrailed proteins play many roles in the development and homeostasis of brain structures beyond their traditional roles as homeodomain transcription factors.

Preliminary Data

Our laboratory's ongoing research is aimed at defining *En2*'s cell biological function in cerebellar neural precursors, with particular emphasis on GNPs and postnatal cerebellar development. We demonstrated previously that ectopic overexpression of *En2* in embryonic cortical neuron precursors alters neurogenesis [108]. This is important given that ASD-associated SNPs within *EN2* may

alter the timing or localization of its own expression, and ectopic expression has been shown to produce ASD-like neuropathology [107, 115]. Thus, in its native population (cerebellar GNPs), we hypothesized that *En2* could modulate cell cycle, thereby participating in granule neurogenesis. Postnatal *En2* expression begins in postmitotic, premigratory GNPs of the inner EGL [64]; therefore, we further hypothesized that *En2* promotes GNP cell cycle exit and differentiation. By comparing *En2* KO and WT mouse GNPs in vitro as well in vivo, we have preliminary evidence that in the absence of *En2*, GNPs remain more proliferative and fail to differentiate. This difference in proliferation was greatest in KO GNPs from the central vermis, the region of greatest *En2* expression; in fact, there were no statistical differences in DNA synthesis (a measure of proliferation) between KO and WT GNPs isolated from the lateral hemispheres, a region of very low or absent *En2* expression [64]. These regional differences in *En2*-regulated DNA synthesis further underscore the heterogeneity of cerebellar neuronal populations. Additionally, these data suggest that abnormal *En2* regulation during development may differentially disrupt medial cerebellar pathways required for attention regulation and speech production, requiring compensation from lateral cerebellar circuitry involved in higher order cognitive functions [24], thereby contributing to ASD phenotypes.

En2 and IGF-1

Although there appears to be increased GNP proliferation in the KO, we found that even in the absence of *En2,* overall cell cycle regulation by extracellular growth factors remains intact; however, *En2* is found to modulate signaling through one developmentally relevant growth factor and its receptor, IGF1 [196]. IGF-1 is produced in high concentrations by Purkinje neurons, which are supplied by anterograde transport from the inferior olive afferent fibers, and is released into the EGL during postnatal GNP proliferation [37, 41, 88, 91] (Riikonen, Chapter 10). IGF-1, acting through its tyrosine-kinase receptor, IGF-1R, stimulates proliferation, survival, and/or differentiation, depending upon which of several possible downstream messengers are activated. In the absence of *En2*, IGF-1 stimulates a greater increase in DNA synthesis compared to WT cells; this effect was demonstrable in cultured GNPs, as well as in vivo through peripheral IGF-1 injection. Further, in the absence of *En2*, GNPs demonstrated reduced neurite outgrowth (a marker of neuronal differentiation) in response to several differentiation signals (including IGF-1). Taken together, these data support a role for *En2* in regulating granule neurogenesis by promoting cell cycle exit and differentiation, possibly via modulation of IGF-1 signaling. Although preliminary, these data provide a model in which ASD-associated changes in *EN2* expression would have predictable consequences on postnatal cerebellar growth. Abnormalities in cerebellar size, gray : white matter ratios, and growth rates are well characterized in ASD; therefore, understanding postnatal

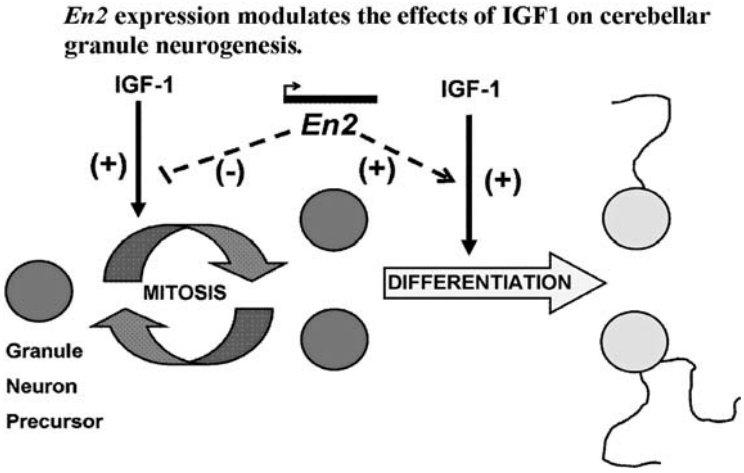

Fig. 1.4 This cartoon depicts our current hypothesis regarding the modulation of insulin-like growth factor-1 (IGF-1) signaling by *En2* expression. En2 interacts with undefined downstream signals to attenuate IGF-1-stimulated granule neuron precursor (GNP) proliferation while promoting GNP differentiation

En2 functions may shed light into the molecular pathways underlying these developmental abnormalities. On-going and future experiments will aim to modulate *En2* expression levels in cultured GNPs via cDNA overexpression and siRNA knockdown. Based upon our existing model (Fig. 1.4), we expect reduced *En2* expression to result in increased proliferation with a concomitant reduction in differentiation while overexpression will facilitate cell cycle exit and granule neuron differentiation. Through these experiments, we aim to functionally characterize *En2* to better understand the molecular and cellular complexities of normal cerebellar development. Further, it is our hope that these experiments will aid our understanding of ASD pathogenesis with respect to *En2*, which, at the time of writing, is the only ASD-associated gene to withstand replication across several data sets.

Conclusion

ASD are complex neurodevelopmental diseases with strong genetic etiologies and are highly variable among individuals. This clinical heterogeneity is symbolic of the complex genetic etiology and neuropathology observed across the ASD population. Despite this heterogeneity, many studies demonstrate that the cerebellum is consistently abnormal in ASD patients, both neuroanatomically and functionally. For example, humans with ASD demonstrate abnormal brain growth, with some patients exhibiting cerebellar gray : white matter ratios that deviate from age-matched controls throughout childhood and into adolescence.

Further, cerebellar-dependent cognitive tasks, including attention regulation and speech, are disrupted in humans with ASD while motor coordination is preserved, suggesting that the cerebellum is grossly intact in these patients. Recently, the human gene *ENGRAILED2*, an important regulator of cerebellar development, was identified as an ASD-susceptibility gene, a finding replicated in three data sets. Together, these data suggest that abnormal brain, and in particular, abnormal cerebellar development, underlie ASD pathogenesis. The role of *EN2* in normal cerebellar development is still under investigation; however, disruption of this gene in animal models produces ASD-like behavior and neuropathology. Thus, we aim to better understand normal functions of *EN2* as a possible window into the pathogenesis of ASD. Our preliminary data suggest that *En2* promotes postnatal GNP cell cycle exit and differentiation. Further, *En2* interacts with a developmentally important extracellular growth factor, IGF-1, to regulate GNP proliferation and differentiation in the postnatal period. Future studies will further explore the biochemical function of *En2*, as well as compare possible differences between *cis*-regulation of *EN2* expression by normal and ASD-associated introns.

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