

Autism

CURRENT CLINICAL NEUROLOGY

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Autism

Current Theories and Evidence

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Editor

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Preface

Andrew W. Zimmerman

Moving Beyond Hypotheses: A Call for Theories and Evidence

Creative thinking and collaborative scientific research have advanced our understanding of autism, and we are now beginning to synthesize the data into evidence and theories. Our lack of knowledge about the causes and pathogenesis of autism has been associated with widely divergent approaches to diagnosis and management, most of which have not been subjected to rigorous scientific scrutiny. The consequence for affected families is that while they recognize the importance of early intervention for their children, they are often left with indecision and confusion as to how to proceed. This book presents current theories about autism and the evidence that supports them. The goal is to show how the scientific method is revealing the biological bases of this spectrum of disorders, thereby leading the way to their treatment and prevention using evidence-based medicine.

Quotations from Leo Kanner

In 1943: We must, then, assume that these children have come into the world with innate inability to form the usual, *biologically provided* affective contact with people, just as other children come into the world with innate physical or intellectual handicaps [1].

In 1971: At long last, there is reason to believe that some answers to these questions (about autism) seem to be around the corner. *Biochemical explorations*, pursued vigorously in the very recent past, may open a new vista about the fundamental nature of the autistic syndrome [2].

Since Leo Kanner's 1943 description of autism as a biological disorder and follow-up of his original 11 patients in 1971, we have amassed large amounts of descriptive data. However, as in many areas of neuroscience, we are "data rich and theory poor" [3]. Furthermore, despite Kanner's optimism in 1971, substantive neurobiological answers were not "around the corner."

A plethora of hypotheses has been proposed to explain the various manifestations of autism, and many untested medical treatments being utilized are inspired by the needs of affected persons, yet are based on limited empirical

data. Psychodynamic approaches to autism prevailed in the past, but in the last decade, there has been explosive growth in autism science as new researchers have been willing to discard or revise earlier concepts. Armed with an understanding of brain development from a neuroscience perspective and with the tools of molecular genetics, imaging, and environmental science, they are providing novel insights, energy, and fresh approaches, thereby ushering in a new era that would have amazed Kanner and his contemporaries.

Autism research is now a fast-moving field in which terminology changes frequently and challenges the classical definitions of the phenotypes used in experimental designs and patient care. Not surprisingly, scientific interest in autism has increased in parallel with similar increases in its prevalence, along with public awareness and concern. Thus, the annual numbers of original publications as well as review articles in the field have increased fourfold during the past decade (Fig. 1).

Now is the time to examine how we approach scientific discovery in autism. The clinical heterogeneity of this disorder, together with the inherent dynamic, changes during children's growth and development, confound static, linear models and simplistic, unilateral approaches. Global thinking and collaborative approaches are needed. *Dedicated efforts using logic and the scientific method are leading many researchers and funding agencies to move beyond the descriptive phase of observations, to form hypotheses, collect data systematically, define the evidence, and establish testable theories.*

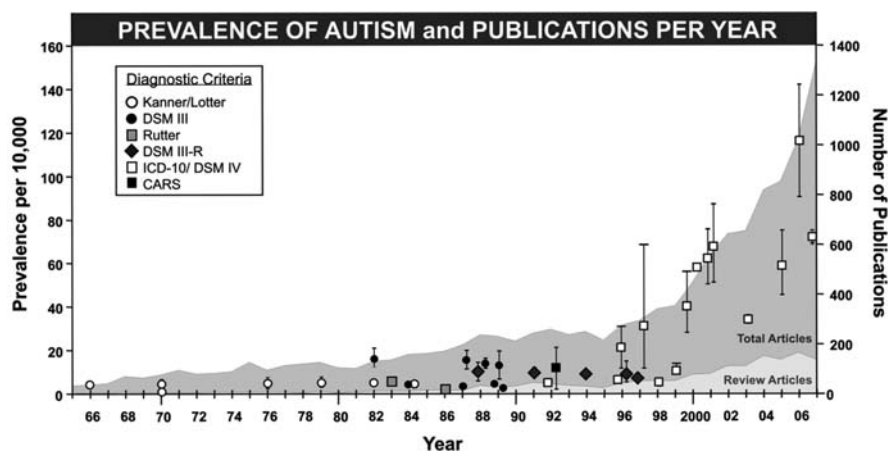


Fig. 1 Prevalence of autism per 10,000 (left) and autism publications (right) by year, 1966–2007. Prevalence figures are from separate publications, according to different diagnostic criteria used for autism assessment (inset), with confidence intervals (Courtesy of Lisa A. Croen, PhD). Total original research and review articles with key word “autism” are from PubMed [4]. Diagnostic criteria: Kanner [1]/Lotter [5]; DSM, Diagnostic and Statistical Manual III, III-R, IV [6]; ICD, International Classification of Diseases-10 [7]; CARS, Childhood Autism Rating Scale [8]

Definition

Hypothesis: a proposition that attempts to explain facts in a unified way and forms the basis of experiments designed to establish its plausibility.

Theory: a set of statements, including hypotheses, that explains observations in terms of those hypotheses [9].

In the scientific sense, a theory is a more advanced form of knowledge that emerges from hypotheses, empirical data, and evidence. The process of forming theories leads to discoveries, fitting Kuhn's description of how researchers observe "anomalies" that lead to a "paradigm shift" and eventually, "scientific revolution" [10]. The drive to understand autism and related neurodevelopmental disorders has motivated observations of clinical anomalies in autism for decades, but in recent years, important new advances in neuroscience, molecular biology, and related fields have offered the means to move beyond observations, develop hypotheses, and acquire experimental evidence. The application of scientific methodologies allows for translation of findings from basic science to the development of evidence-based clinical medicine in autism. This process was slow to start due to autism's inherent clinical heterogeneity and a lack of adequate funding for research. Now, however, consensus has been developing on the description of phenotypes, funding for research has increased, and investigators are actively communicating and validating various theories.

Theories result from observations of phenomena that are the basis for hypotheses, which lead to experimentation and empirical data, and with analysis, evidence. In turn, theories generate new and more refined observations and hypotheses, repeating the cycle and thereby confirming or modifying the original theory. This process represents a natural cycle in the development of new knowledge, which is especially relevant to autism research today (Fig.2).

Fig. 2 Cycle of progress in autism science. Observations lead to testable hypotheses; through collection of data and analysis of evidence, investigators develop theories. In turn, the cycle is repeated, informing new hypotheses and expanding generalizable knowledge



In developing theories, it is important, first and foremost, to distinguish science from “pseudo-science” or scientific from nonscientific theories [11]. Real progress will require that we engage the scientific method to develop theories. Valid theories emerge from hypotheses that can be refuted by empirical evidence. Einstein’s work was an inspiring example of genuine science, in which theories have been validated and improved over time, because they are open to “falsification”. In 1952, he reflected on the development of theories, noting that “The deeper we penetrate and the more extensive our theories become, the less empirical knowledge is needed to determine those theories”[12].

Increasingly focused investigation in autism research is now leading to theories that are maturing due to new evidence being generated by investigators to support them. This process is still in its infancy in autism but is maturing well, nourished by families affected by autism and private and public funding for science, with researchers working in a “balance between competition and cooperation, and between criticism and trust” [13].

The authors of *Autism: Current Theories and Evidence* have been instrumental in developing important biological theories about autism. All are contributing to autism research by constantly refining their theories through rigorous interdisciplinary collaboration and writing. They have generously accepted the invitation to describe the theories they have been developing and the evidence that supports them.

Autism: Current Theories and Evidence has 20 chapters divided into six sections: Molecular and Clinical Genetics; Neurotransmitters and Cell Signaling; Endocrinology, Growth, and Metabolism; Immunology, Maternal-Fetal Effects, and Neuroinflammation; Neuroanatomy, Imaging, and Neural networks; and Environmental Mechanisms and Models. The subjects cover a wide range of current scientific work in the field of autism, with strong and growing evidence to support them and demonstrate both the breadth and the depth of current autism research.

These theories support Kanner’s optimism that we will find the biological bases for autism, resulting in improved treatment and prevention. Although it is clear that there is still much to be done, the work presented here demonstrates substantive progress in autism research. It is likely that no unifying theory will explain the myriad processes and forms of autism; rather, interrelated theories will weave together interacting causal pathways to explain its diverse manifestations.

This volume is by no means complete, as there are many other researchers who are developing valid theories in the field of autism. This is a challenging yet rewarding field that is engaging talented investigators. The reader is encouraged to consider how theories and the scientific method, in the hands of these and other dedicated researchers, are leading to greater knowledge and continued progress in autism research.

Acknowledgments I thank Alexander H. Hoon Jr., MD, Susan L. Connors, MD, and Pam K. Gillin, MSN, for their thoughtful reviews and helpful comments. Lisa A. Croen, PhD, provided the innovative graph on the prevalence of autism in Fig. 1. Sharon N. Blackburn, BA contributed creative graphic designs.

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Part I
Molecular and Clinical Genetics

Chapter 1

ENGRAILED2 and Cerebellar Development in the Pathogenesis of Autism Spectrum Disorders

Ian T. Rossman and Emanuel DiCicco-Bloom

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 myelencephalon (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;

Patterning genes compartmentalize the neural tube into functionally distinct domains.

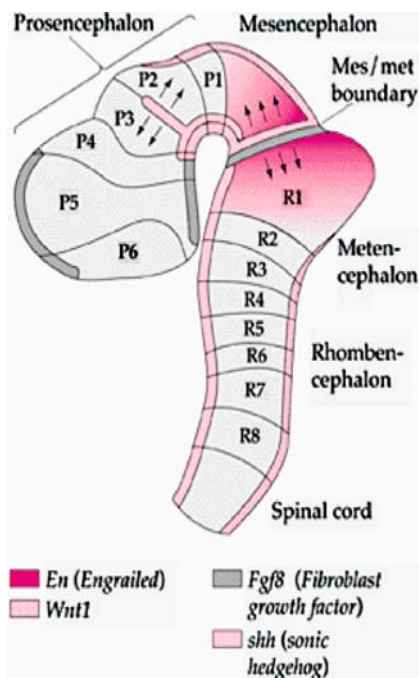


Fig. 1.1 Shortly after neural tube closure, patterning genes become expressed along the anterior-posterior, dorsal-ventral, and medial-lateral axes. These genes help compartmentalize the neural tube, giving rise to the forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). 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

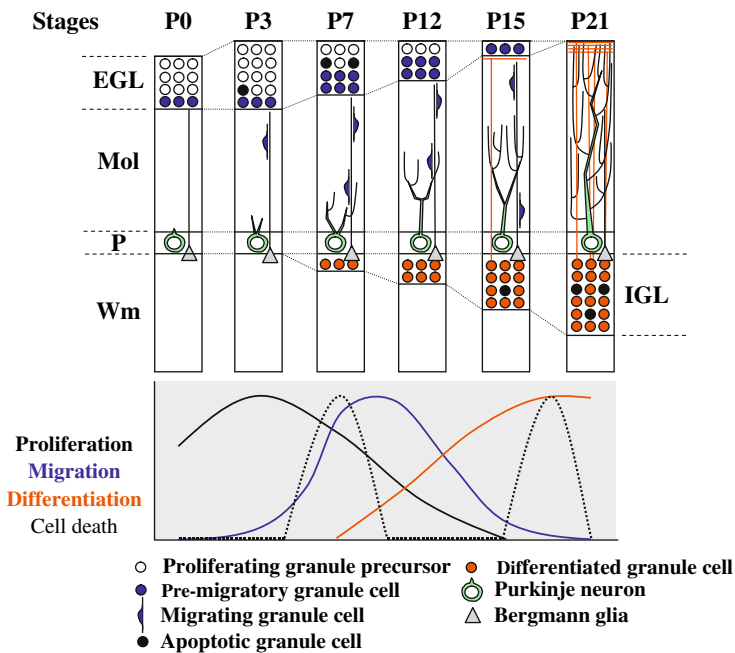


Fig. 1.2 Granule neuron precursors proliferate superficially in the external germinal layer (EGL), then migrate inwardly along Bergmann glia. During migration, the granule neuron precursors (GNPs) differentiate, producing an axon with parallel fibers that communicate with Purkinje cell dendrites, thereby 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

Patterning gene expression in the prenatal WT and *En2* KO cerebellum.

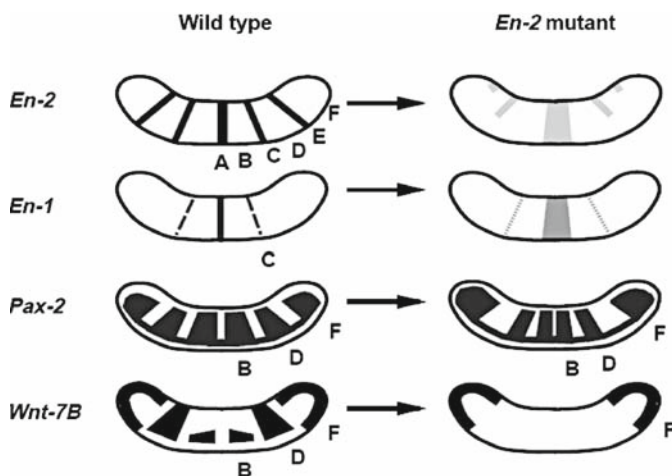


Fig. 1.3 Several patterning genes are expressed complementary to *En2* expression. While expression of cerebellar patterning genes is preserved in the *En2* knock-out (KO) mouse mutant, discussed below, their spatial expression patterns are disrupted. This suggests that positional information necessary 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.