Published in final edited form as:

Cerebellum. 2010 September ; 9(3): 272–283. doi:10.1007/s12311-010-0169-6.

Novel Approaches to Studying the Genetic Basis of Cerebellar Development

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Abstract

The list of genes that when mutated cause disruptions in cerebellar development is rapidly increasing. The study of both spontaneous and engineered mouse mutants has been essential to this progress, as it has revealed much of our current understanding of the developmental processes required to construct the mature cerebellum. Improvements in brain imaging, such as magnetic resonance imaging (MRI) and the emergence of better classification schemes for human cerebellar malformations, have recently led to the identification of a number of genes which cause human cerebellar disorders. In this review we argue that synergistic approaches combining classical molecular techniques, genomics, and mouse models of human malformations will be essential to fuel additional discoveries of cerebellar developmental genes and mechanisms.

Keywords

Cerebellum; Neurological and targeted mouse mutants; Congenital human cerebellar malformations; Genomics; Genetics; Ataxia

Introduction

In the last several decades, various approaches have contributed to our understanding of the molecular basis of cerebellar development. The study of spontaneous neurological mouse mutants aided many initial discoveries that are further reviewed below [1–5]. Significant advances in mouse genetics have allowed for more targeted studies using engineered gene knockouts and transgenic mice. These mice have facilitated the examination of more subtle phenotypes such as mild behavioral abnormalities and small disruptions in cerebellar circuitry [6–8]. Advances in brain imaging techniques and improvements in the classification of human cerebellar malformations have further aided the discovery of genes regulating cerebellar development. Genetics has recently enabled the identification of genes causing human pontocerebellar hypoplasia, Joubert syndrome, and Dandy–Walker malformation (DWM). When combined with studies in mouse, a variety of molecular mechanisms, including transcriptional regulation, mitochondrial function, and ciliary signaling have been implicated in homeostasis, patterning, and cell proliferation during cerebellar development. Concurrently, the application of new genomic techniques, which amass vast amounts of biological information, is just beginning to unravel the systems biology of the developing cerebellum. Here we discuss these issues and advocate the integrated use of human and mouse systems to

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further advance our knowledge of the molecular and developmental processes that form the cerebellum.

The Cerebellum as a Genetic System

The mature cerebellum has exquisite, stereotypical morphology, foliation, and lamination, which are consistent between individuals and highly conserved across vertebrates. At the cellular level, unlike other regions of the CNS, the cerebellum is composed of very few neuronal types, each with distinct morphology, arranged in discrete lamina, and connected in stereotypical circuits [9]. The cerebellum has essential roles in motor coordination, but is not essential for viability. Thus, compared with other regions of the central nervous system (CNS) the cerebellum has been more amenable to genetic studies since disruptions in development, which lead to abnormal morphology or function, are readily observed in obvious neurological and behavioral phenotypes. Because of this, it has been possible to obtain a precise understanding of cerebellar development [10–14]. The mechanisms deciphered from the study of cerebellar development have broad applicability to other CNS regions such as the cerebral cortex. For example, while initial insights regarding the function of the *Reelin* gene were gleaned from studying the cerebella of *reeler* mice ([15]; discussed below), recent studies have revealed that this gene is required for the emigration of dentate gyrus progenitors from a transient subpial zone and into the subgranular zone [16]. Also, while *Foxc1* controls normal cerebellar and posterior fossa development by regulating secreted growth factor signals from the mesenchyme ([17]; discussed below), it is also required for the development of meningeal structures that in turn influence skull and cortical development [18].

Developmental Insights from Mouse Cerebellar Mutants

Spontaneous Neurological Mouse Mutants

A search of Mouse Genome Informatics (MGI) at The Jackson Laboratory (www.informatics.jax.org) reveals just over 170 spontaneous, ENU- or X-ray-induced mutant alleles with altered cerebellar function or development. Most spontaneous cerebellar mutants were identified due to their behavioral or morphological phenotypes and, as such, are severely affected. Phenotype-to-gene approaches, commonly referred to as forward genetics, enabled an unbiased search for genes involved in cerebellar development, since the phenotype indicated that the mutation by definition affected a gene important for the disrupted process. There are no prior assumptions regarding gene function. Here we highlight several classical mutants to demonstrate that they have been pivotal to our current knowledge of cerebellar development and continue to be a rich resource for the cerebellar research community.

The *staggerer* mutation spontaneously arose at The Jackson Laboratory in 1955 and was first described in 1962 [19]. The cerebellum of these mice is small and there is pronounced post-natal loss of Purkinje and granule cells. The discovery that parallel fiber activity is important for the pruning and refinement of climbing fiber–Purkinje cell synaptic arrangement [1] was derived from these mutants prior to the identification of the causative deletion of *retinoid-like orphan receptor alpha (Rora)* in 1996 [20]. *Staggerer* chimeric analysis provided key evidence for the interdependence of Purkinje and granule cells during early post-natal development [2, 21]. Subsequent genome-wide expression analysis in *staggerer* mutants confirmed that *Rora* acts as a transcriptional regulator of Purkinje cells and regulates the secretion of sonic hedgehog (SHH), a mitogen for adjacent granule cell progenitors in the external granule layer [22].

The *lurcher* mouse, harboring a gain-of-function mutation in the *delta 2 ionotropic glutamate receptor, Grid2*, normally expressed in Purkinje cells, has also been an important model [3, 23]. The cerebellum of this mutant is hypoplastic due to severe post-natal degeneration of Purkinje, granule, and olivary neurons. The absence of Purkinje cells causes a significant

reduction in the proliferation of granule cell precursors primarily due to a lack of the mitogenic effects of SHH. Other factors, such as IGF-1, FGF2, and EGF are known to act as mitogens in the cerebellum, but SHH has been shown to be over two orders of magnitude more potent [24]. Any granule cells that are produced due to the proliferative effects of these other factors end up dying since there is a paucity of Purkinje cells with which to form synaptic contacts. Indeed, there is a linear relationship between the number of Purkinje and granule cells in the cerebellum, and in the absence of enough Purkinje cells there occurs a concomitant death of the “extra” granule cells [25]. Thus, this mutant showed that target neurons likely provide trophic support to pre-synaptic contacts.

Leaner and *weaver* mice, harboring mutations in the *alpha-1A calcium channel subunit* gene, *Cacna1a*, and the potassium inwardly rectifying channel gene, *Girk2*, respectively, also exhibit Purkinje and granule cell death [5,26,27]. The *leaner* mouse has been useful in demonstrating the role of intracellular calcium ion concentrations and neuronal apoptosis in cerebellar development [4]. And even though *Girk2* is expressed in both granule and Purkinje cells, the *weaver* mutation preferentially affects the former, demonstrating that some neurons are more susceptible to this particular mutation.

Not all mutations cause cerebellar cell death. The *dreher* (*Lmx1a*) mutant mouse fails to form the fourth ventricle roof plate, an essential embryonic signaling center adjacent to the developing cerebellar anlage. Loss of this transcription factor in the roof plate causes secondary mis-specification of adjacent cerebellar neurons [28,29]. Disruption of neuronal migration also occurs in *reeler* and *scrambler* mutants, resulting in small cerebella with no foliation and ectopic clusters of Purkinje cells beneath the granule cell layer [30,31]. Disruption of the large *Reelin* gene, which codes for a secreted extracellular matrix serine protease, is responsible for the *reeler* phenotype [15] whereas mutations in the *Dab1* gene, coding for a cytoplasmic adapter protein, cause the *scrambler* phenotype [32]. It appears that *Reelin* acts as an inhibitory signal for migration because ectopic Purkinje cells form in both *reeler* and *scrambler* [33, 34]. The *rostral cerebellar malformation* mutant contains a mutation in the Netrin receptor *Unc5h3* gene that results in over-migration of granule and Purkinje cells into the midbrain and fewer cerebellar folia [35]. Experiments with chimeras demonstrated the co-dependence of different cell types during cerebellar development as wild-type Purkinje cells exhibit inappropriate migration under the influence of *Unc5h3* mutant granule cells [36].

Notably, there are many spontaneous and ENU-induced cerebellar mutants which remain to be characterized and are certain to add new, interesting, and likely unpredictable pieces to the puzzle of cerebellar development.

Targeted Mouse Mutants

The advent of transgenesis and gene-targeting technology has greatly aided the discovery of genes regulating cerebellar development. Gene expression patterns initially identified many genes likely to be involved in cerebellar development. Essential roles for these genes were verified in knockout mice. For example, insights on the function of the isthmus organizer and its role in defining the cerebellar territory along the early neural tube were found from analysis of targeted alleles of *Gbx2*, *Otx2*, *En1/2*, *Wnt1*, *Fgf8*, and other genes [37–40].

Modern molecular genetics methods have also provided an understanding of genes that cause subtle phenotypes not likely to be found in forward genetic screens. These phenotypes affect higher order roles of the cerebellum, such as learning, memory, and synaptic plasticity. For example, targeted mutants with a deletion of *Grid2* result in Purkinje cell dendrites that form reduced synaptic contacts with granule cell parallel fibers. Whole-cell patch-clamp demonstrated that mutant Purkinje cells lack long-term depression (LTD) [6]. Mice with a targeted mutation of the *metabotropic glutamate receptor 1* (*mGluR1*) also shed light on the

molecular basis of cerebellar synaptic plasticity as they showed a spatial learning deficiency and a lack of cerebellar LTD [7]. Neuronal calcium ion concentration plays a role in synaptic plasticity through a variety of mechanisms, which include regulation of neurotransmitter release, gene transcription, and altered ion channel permeability. Mice lacking the gene for Calbindin1, an intracellular calcium-binding protein that acts as a calcium ion buffer in Purkinje cells, exhibit impaired movements only when challenged, suggesting an impairment of the cerebellum to adapt to a changing environment [8]. Patch-clamp recordings further revealed an alteration of synaptic calcium transients in mutant Purkinje cells. Moreover, an antisense transgenic approach to diminish *Calbindin1* mRNA levels demonstrated that this gene is required for the maintenance of hippocampal long-term potentiation (LTP) and spatial learning [41]. Mice lacking the *Calbindin2* gene (alias: *Calretinin*), which is expressed in granule cells, exhibit motor defects and increased granule cell excitability in the absence of abnormal morphology [42].

Current state-of-the-art mouse molecular genetics technologies now give us the ability to precisely and selectively modulate gene function. Thus, genes with essential roles throughout the developing embryo can be disrupted specifically in the cerebellum, leaving their non-cerebellar roles unaffected. Conditional gene ablation can be accomplished by the use of Cre-LoxP or other related recombinase systems, where a cell- or tissue-specific promoter drives the expression of Cre recombinase, which then excises any DNA flanked by recombinase recognition LoxP sequences. The tamoxifen-inducible Cre-LoxP system, which utilizes a fusion of Cre with an estrogen receptor ligand-binding domain, makes it possible to study gene function during a specific time and place in development [43]. A wide variety of regulatory sequences have been identified which can drive recombinase expression to individual cell types of the cerebellum and the developing CNS (reviewed in [44,45]). For example, *Pcp2* regulatory sequences have been widely used to target Cre expression to Purkinje cells [46], whereas *Atoh1* regulatory elements are often used to drive Cre expression in granule cells [47]. These regulatory sequences can also be used to overexpress genes/alleles of interest in specific cell types. For example, the *Gbx2* gene, which is critical for the placement of the mid-hindbrain junction, was ectopically expressed in the midbrain and rhombomere 1 of the hindbrain by using Cre expression driven by the regulatory elements of *Engrailed1* [48]. This study showed that *Gbx2* gain-of-function in this region of the brain eliminates the expression of isthmus organizer gene *Fgf8*, and results in the absence of the midbrain and cerebellum at later stages.

Additional studies in mouse and other model organisms such as zebrafish, can be further utilized to understand the molecular mechanisms of cerebellar development [49–53]. It is now feasible to generate specific models of human cerebellar malformations which allow for the study of underlying developmental biology of clinically important disorders.

Advances in Cerebellar Development from the Study of Human Cerebellar Malformations

In addition to spontaneous and targeted mouse mutants, the study of human cerebellar malformations is beginning to provide new insights regarding the basic developmental principles of the cerebellum. Currently, human patient populations with congenital developmental disorders are largely underutilized in basic research but they have proven to be valuable for identifying novel, significant developmental genes. As in the mouse, disruption of human cerebellar development is often severely handicapping but not lethal, making it amenable to genetic analysis. Also similar to mice, the structure of the human cerebellum facilitates the easy identification of malformations as its morphology, foliation, and lamination are stereotypical across individuals and its morphogenesis is well understood. In conjunction with advances in imaging techniques, this allows patients to be diagnosed with malformations at early post-natal or even fetal stages. While patient populations provide a great resource for

researchers, they are not often employed due to several difficulties, including a lack of routine brain imaging on patients with developmental abnormalities, genetic heterogeneity among cerebellar patients resulting in the requirement of large sample sizes, and difficulties recruiting patients. Despite these obstacles, human cerebellar malformations have been used to identify cerebellar developmental genes [11,54]. Gratifyingly, mutations in human *RELN* cause cerebellar hypoplasia, similar to the phenotype seen in the *reeler* mouse [55], demonstrating the validity of cross species comparisons. Once genes have been identified in human cerebellar malformation syndromes, mouse models have proven essential for deciphering the underlying developmental disruptions [11].

Types of Human Cerebellar Malformations

Advances in imaging, genetics, and classification are enabling previously consolidated malformations to be delineated into distinct categories [56,57]. Here we will discuss cerebellar vermis hypoplasia (CVH), DWM, Joubert syndrome and related disorders (JSRD), and pontocerebellar hypoplasia (PCH) (Fig. 1). The defining features of these diagnoses are based on imaging criteria rather than clinical outcome, with most of these diagnoses associated with intellectual and motor disabilities. CVH is characterized by a small hypoplastic cerebellum with the vermis more affected than the hemispheres. DWM includes CVH; however, there is also an upward rotation of the cerebellar vermis that results in an enlarged fourth ventricle, and an increased size of the posterior fossa. DWM is the most common cerebellar malformation, with an estimated incidence of approximately 1 in 5,000 [57,58]. CVH is also relatively common and often confused with DWM, making estimations of incidence problematic. CVH and DWM often present as sporadic cases, although there are several CVH loci with known recessive or X-linked inheritance [59–61]. Mendelian inheritance for DWM is rare, and the genetics are likely oligogenic [58,62]. In contrast, JSRD are most often autosomal recessive disorders and are rare, with a population incidence estimated to be 1/100,000 [57]. As a group, JSRD are characterized by cerebellar vermis hypoplasia plus the presence of elongated cerebellar peduncles and a deepened interpeduncular fissure that appear as a “molar tooth” on axial brain scans. In addition, these patients exhibit axon guidance defects that include a decussation failure of the pyramidal tract and superior cerebellar peduncles. Patients with PCH exhibit a heterogeneous set of malformations characterized by hypoplasia and atrophy of the cerebellum, inferior olive, and ventral pons. This degenerative disorder often begins with embryonic atrophy of these regions.

Causative Genes in Human Cerebellar Malformations

In the last decade, there has been considerable effort in defining the genetic basis of human cerebellar malformations. Causative genes include those involved in cerebellar patterning, cell fate specification, and other developmental processes (Table 1).

Pancreas specific transcription factor 1a (Ptf1a) was initially implicated as a basic helix–loop–helix transcription factor in pancreatic development since mice with a targeted deletion lacked pancreatic tissue [63]. However, its role in brain development was not investigated until truncations of this gene were found to result in cerebellar agenesis in multiple families [54]. Further investigations determined that loss of Ptf1a causes a failure to generate GABAergic cerebellar neurons in the embryonic cerebellar anlage in both human and mouse [64]. Since Purkinje cells, which are GABAergic, are also required for the proliferation of cerebellar granule neurons, humans and mice lacking Ptf1a exhibit profound cerebellar agenesis [64].

Transcription factors have also been implicated in other types of cerebellar malformations. Heterozygous loss of the *ZIC1* and *ZIC4* genes encoding zinc finger transcription factors can cause DWM, a phenotype which is mimicked in *Zic1* and *Zic4* double heterozygous mutant mice [65]. Mutations in *FOXC1*, a transcription factor gene located in the 6p25.3 locus, have

recently been shown to contribute to human DWM [17]. Mouse models have demonstrated that *Foxc1* is developmentally expressed in the mesenchyme adjacent to the cerebellum, where it is critical for normal posterior fossa development [66]. In addition to regulating skull development, *Foxc1* controls mesenchymally expressed signaling molecules including *Bmp2* and *Bmp4* [17]. Loss of these signaling molecules causes the adjacent cerebellar rhombic lip to lose *Atoh1* (*Math1*) expression, a gene critical for normal granule cell differentiation. These findings, based on studies in both human and mice, have surprisingly implicated mesenchymal signaling as a critical regulator of early cerebellar anlage development.

Studies of JSRD patients have also provided surprising insights into new developmental mechanisms. Of the nine loci linked to JSRD, eight have been cloned and the following causative genes identified: *AH11* [67,68], *ARL13B* [69], *CC2D2A* [70,71], *CEP290* [72,73], *INPP5E* [74,75], *NPHP1* [76,77], *RPGRIP1L* [78,79], and *TMEM67* [80]. Many of these genes are implicated in normal ciliary function and their protein products localize to the cilia or basal bodies. One such cilia-related protein is Nephrocystin, the product of *NPHP1*, which interacts with beta-tubulin and localizes to primary cilia [81]. In cell culture, CEP290, centrosomal protein 290, is involved in ciliogenesis, localizes to centrioles in a microtubule-dependent manner, and regulates the microtubule network, as shown through RNAi. Furthermore, CEP290 interacts with the protein product of *CC2D2A* both genetically and physically [71]. Most recently, mutations in the *INPP5E* gene, which codes for inositol polyphosphate-5-phosphatase E, were found in patients with Joubert syndrome. While it was known that this enzyme hydrolyzes phosphatidylinositols, INPP5E was found to be localized to cilia and mutations resulted in premature destabilization of cilia after stimulation [74,75]. Thus, examination of human patients led to a novel role for INPP5E in both cilia signaling and Joubert syndrome. Mutations in many components of this single biological pathway result in similar cerebellar defects. The actual purpose of cilia in the cerebellum is likely to be linked to SHH signaling. Significantly, loss-of-function mutations in murine *Kif3a* and *Ift88*—genes encoding intraflagellar transport proteins for the formation and maintenance of cilia—cause SHH-dependent proliferation defects of granule cell progenitors. This loss of SHH signaling results in cerebellar phenotypes resembling those seen in JSRD [82,83]. JSRD now provide a model for how studies of human cerebellar malformations can lead to the discovery of causative genes and expand our knowledge of the pathways involved in cerebellar development.

Additional molecules have been implicated in human cerebellar malformations, which are certain to illuminate new cerebellar developmental mechanisms. Deletions of the Rho-GAP protein encoding gene *Oliogophrenin-1* (*OPHN1*) have been found in multiple families with X-linked CVH [59,60]. While *Ophn1* is required for the stabilization of glutamatergic spines [84], it has not been implicated in regulating earlier developmental events such as cell division. Interestingly, mice with a targeted deletion of *Ophn1* exhibit learning deficits and have dilated lateral and third ventricles, but their cerebellar size and morphology are normal [84]. This suggests that the mental retardation (MR) seen in human patients may not be due to cerebellar defects. However, until the connectivity and plasticity of the mutant mouse cerebellum are examined in detail this only remains a speculation. Recently *Ophn1* has been shown to facilitate clathrin-mediated endocytosis of post-synaptic vesicles, including the AMPA receptor, by repressing the RhoA/ROCK pathway [85]. Because of this, mutant mice lack LTD in the hippocampus. Cerebellar LTD still remains to be examined.

Mutations of another molecule with a known role in synapse development have also been seen in PCH [86]. CASK is a calcium/calmodulin-dependant serine/threonine kinase localized to synapses via membrane-associated molecules, including Neurexin. CASK also regulates gene transcription during cell proliferation [87]. Although mouse *Cask* mutants have cerebellar hypoplasia, the developmental basis for this pathology has not yet been studied [86]. Genes from the tRNA splicing pathway have also been observed to cause PCH when mutated in

humans. One family has been found with three members containing mutations in the *RARS2* gene, which encodes mitochondrial arginine-transfer RNA synthase [88]. Individuals with PCH have also been found to have mutations in *TSEN54*, *TSEN34*, and *TSEN2*, which all encode tRNA splicing proteins [89]. The study of mouse models will be essential to determine why developing cerebellar and pontine cells are particularly sensitive to the loss of these genes even though they are ubiquitously expressed.

Human studies have demonstrated that patient clinical phenotypes associated with severe congenital cerebellar malformations described here can be highly variable. Less severe cerebellar malformations have been reported in patients with non-syndromic MR [57], Autism Spectrum Disorders, and schizophrenia [90–93]. Evidence of Purkinje cell dysfunction in cerebella from autistic patients has been demonstrated by reduced levels of *glutamate decarboxylase (GAD67)*, which codes for a GABA-synthesizing enzyme [94]. In addition, levels of various gene transcripts involved in GABAergic transmission are altered in lateral cerebellar hemispheres of schizophrenic patients. Specifically, *GAD67*, *GAD65*, *GAT-1*, *MGLUR2*, and *NOS1* were downregulated whereas *GABA_A-alpha6*, *GABA_A-delta*, *GLUR6*, and *GRIK5* were upregulated [95]. Thus, it is likely that the genes underlying these more common and genetically complex neurodevelopmental disorders also influence cerebellar development. Notably, most patients with MR, autism, and other neurodevelopmental disorders rarely undergo brain imaging. Therefore, the coincidence of these disorders with cerebellar malformation is often missed. In order to fully and accurately delineate clinical phenotypes, we strongly advocate routine brain imaging of all human neurodevelopmental disorders. Further, given the extremely fine resolution with which cerebellar phenotypes can now be characterized in mice at the molecular, cellular, and systems level, mouse models for these common neurodevelopmental disorders are certain to be highly informative regarding their underlying pathology.

High-Throughput/Genomic Approaches for Identifying Novel Genes with Cerebellar Functions

While the study of single gene traits has been critical to our understanding of the molecular mechanisms regulating cerebellar development, it is clear that genes do not function in isolation. Consequently, if cerebellar and indeed all biological research is to advance, the generation of new hypotheses will require the integration of large genome-wide datasets with both systems level and molecular networks. What follows is a description of some recent genomic studies that have contributed towards our understanding of nervous system development, in general, and cerebellar development, in particular.

SNP Arrays for Gene Discovery via Copy Number Analysis, Linkage Mapping and Genome-Wide Association Studies

Clinical karyotype analysis has shown that chromosomal aberrations over 4 Mb in size are associated with ~10% of MR cases [96] and with 17% of DWM cases [97]. However, high resolution array genomic hybridization, as well as high density single-nucleotide polymorphism (SNP) microarrays, now reveal that smaller structural variations are also frequently associated with neurodevelopmental disabilities. For instance, a 250-kb deletion overlapping the *NRXN1* gene and a 1.4-Mb duplication including *APBA2* were found in patients with schizophrenia [98]. Recently several copy number variants (CNVs) smaller than 50-kb were also discovered in patients with Autism Spectrum Disorders encompassing genes of the ubiquitin family (such as *PARK2*, *RFWD2*, *PAPPA2*, and *FBXO40*) [99]. Similar studies of patients with cerebellar defects are likely to yield causative genes. An additional advantage of SNP arrays is the ability to detect large regions of homozygosity that can be of consequence in inbred populations and imprinted loci. SNP arrays can be especially useful in performing

linkage mapping on families with segregating cerebellar malformations as seen in JSRD [100], as well as carrying out population based genome-wide association studies (GWAS) such as for schizophrenia and autism [101,102]. However, there are currently no GWAS of cerebellar malformations most likely due to extensive genetic heterogeneity and the need to utilize a large number of subjects in such studies. Efforts to recruit additional patients are likely to make GWAS possible.

Identifying causative genes in large CNV regions is particularly challenging. Recently, the phenotype dataset from a collection of 5,000 mouse gene knockouts was exploited in a systematic genome-wide analysis to forge links between genotype and disease [103]. Given that gene content within MR-associated CNVs and benign CNVs is essentially the same, gene function, rather than density, may better prioritize candidate genes. Indeed, the major finding of this study was that genes functioning in the nervous system were significantly enriched in MR-associated CNVs [103]. This clearly indicates that integration of human and mouse data will be essential for the identification of causative genes for developmental disorders. As detailed in the next sections, there are many mouse resources that can be exploited for this analysis.

Genome-Wide Gene Expression Datasets

Expression microarrays have become increasingly widespread in the last few years and, when employed sensibly [104], yield useful information in a short time. They have specifically been employed to address questions regarding cerebellar development. For instance, *Ctnd2* (*catenin delta 2*) was found to be a downstream target of Pax6 in the developing mouse brain, including the cerebellum, since it was downregulated in *Pax6* mutants in the external granule layer, and its promoter was found to contain a binding site for Pax6 [105–107]. Interestingly, hemizygous deletions of human *CTNND2* are associated with severe MR in cri-du-chat syndrome [108]. Whereas mice that lack *Ctnd2* are viable and display normal behavior, they exhibit deficits in learning and motor coordination [109]. It will be worthwhile to examine the cerebella of these mouse mutants in parallel with brain imaging studies of human patients. Another study compared microarray data from the pontine nuclei of wild-type mice with those from *weaver* and *lurcher* mutants, and demonstrated that the target environment of the cerebellum induces specific transcriptional changes in presynaptic pontine neurons [110].

There are a growing number of genome-wide datasets describing patterns of gene expression in the wild-type developing cerebellum. A microarray screen of early post-natal mouse cerebellum using only transcription factor probes revealed 227 genes expressed in neuronal and glial cells, 24 of which showed cell-type and stage-specific expression patterns [111]. These markers, therefore, serve as candidates for the examination of mutations in human cerebellar malformations. Gene expression profiling of the developing human brain from four gestational stages (18, 19, 21, and 23 weeks) has revealed that more than three-quarters of all known genes are expressed in the cerebellum [112]. There were 139 genes overexpressed in the cerebellum by two-fold or more when compared with other areas of the brain, some of which included *HEY2*, *MSX2*, *LMX1A*, *FGFR1*, *FGF5*, and *ZNF536*. Unsupervised clustering showed that, relative to other regions of the brain, the cerebellum was the most distinct structure, reflecting differences in cellular composition and developmental processes [112]. In situ hybridization patterns of a limited number of genes in the developing human brain can be found at <http://hbatlas.org/>. However, there are more extensive catalogs of gene expression in the mouse cerebellum. The Cerebellar Gene Regulation in Time and Space database (www.cbgrits.org) provides datasets of gene expression from wild-type and select cerebellar mutant mice during embryonic and early post-natal development. Additionally, the Cerebellar Development Transcriptome Database (CDT-DB; <http://www.cdtdb.brain.riken.jp/CDT/>) provides spatiotemporal gene expression profiles of the post-natal mouse cerebellum.

determined by methods such as RT-PCR, microarrays and in situ hybridizations. GENSAT (www.gensat.org) is a database containing verified transgenic mice that fluorescently label specific brain cells and regions. The BGEM database (www.stjudebgem.org) provides gene expression images from radioactive in situ hybridization in the mouse brain across embryonic, post-natal, and adult stages. The Allen Brain Atlas (<http://www.brain-map.org/>) contains in situ images of many genes in the developing mouse brain as well as ~20,000 genes in the adult, whereas Eurexpress (www.eurexpress.org) contains in situ images primarily from E14.5 mouse embryos.

Given the availability of these extensive gene expression datasets, it is now possible for investigators to assign functions to unannotated genes by correlating their expression with groups of known genes/markers. Computationally generated networks of interacting genes can provide additional clues about novel tissue-specific functions of a gene based on the number and combination of other genes present in the network [113].

Proteomics

While a cell's transcriptome can be identified with relative ease, identification of the proteome is more difficult. Lack of protein amplification methods, the need for large quantities of biological samples, and equipment costs are hindrances to such attempts. In spite of these drawbacks, the method has been used to study the cerebellum. One study sought to identify the protein composition of the parallel fiber to Purkinje cell synapse through a proteomic approach [114]. This synapse was affinity-purified from 50 cerebella of transgenic mice and over 60 proteins were identified, including many of the known ones. Novel proteins included those involved in phospholipid metabolism and signaling such as Synaptojanin 1 and 2, Phospholipase B, ATP-binding cassette sub-family A (ABC1) member 12, and CDC42 binding protein kinase gamma (MRCK-gamma). It was also shown that MRCK-gamma is involved in maturation and shortening of dendritic spines. Proteomics, in combination with bioinformatics, has also been applied to identify ciliary proteins with the successful identification of Bardet–Biedl Syndrome (BBS)-causing genes [115–117]. Specifically, this was accomplished by comparing in silico the proteomes of non-flagellated organisms with those of ciliated/flagellated ones and obtaining a candidate list of proteins with likely ciliary or flagellar roles. Given that ciliary proteins have been implicated in cerebellar defects such as JSRD, extensive mining of these datasets as well as generation of additional ones will undoubtedly propel future cerebellar disease gene discoveries.

Newly Emerging Avenues of Cerebellar Developmental Research

Analysis of mutant mouse cerebella has historically driven our understanding of the molecules and processes that form the functional cerebellum. While the classification of human cerebellar malformations and subsequent identification of their causative genes has advanced the field, integration of genome-wide transcription and proteome data will allow the construction of cerebellar developmental molecular networks. Looking to the future, we see three emerging avenues of research, which will enhance our knowledge of cerebellar development.

Firstly, recent descriptions of zebrafish cerebellar development and the identification of markers for cerebellar neuronal populations will now facilitate more detailed examination of mutant fish phenotypes [50,51,118,119]. While zebrafish cerebellar development differs in some ways from mouse and human, many developmental mechanisms are conserved. For example, morpholino-based loss-of-function experiments have demonstrated gene function of candidate JSRD genes, providing evidence for their involvement in human disease. Of particular note are the zebrafish mutants *scorpion* and *sentinel*, which correspond to human JSRD-causing mutations in the genes *ARL13B* and *CC2D2A*, respectively [69,71].

Secondly, whole-genome sequencing and improved understanding of gene expression regulation will facilitate gene discovery in humans, mice, and zebrafish. Notably, there has been a historical bias towards the identification of mutations in protein-coding genes since detrimental changes are relatively easy to identify and interpret. There has been a paucity, however, of identified mutations in non-coding regions such as microRNA genes and other regulatory sequences. The advent of whole genome sequencing will enable us to identify yet more pathogenic mutations which will point to additional cerebellar genes.

Finally, brain imaging technology using functional MRI, diffusion tensor imaging, and other emerging modalities will be important to augment our understanding of cerebellar dysfunction even in the absence of obvious cerebellar malformations in humans and model organisms.

Conclusion

Our current understanding of the molecular and genetic basis of cerebellar development is derived primarily from the study of spontaneous and targeted mouse mutants. Only recently have human patients with cerebellar malformations begun to contribute to the discovery of genes that regulate the development of the cerebellum. Continued advances in the genomic technologies described here will facilitate the identification of other causative genes in human cerebellar malformations. In conjunction with continued use of model vertebrates, these novel approaches will yield additional genes—and hence networks—required in normal cerebellar development.

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Fig. 1.

Magnetic resonance images (*MRI*) showing sagittal views of the cerebellar vermis from a subset of human cerebellar malformations. The image of a patient with cerebellar vermis hypoplasia (*CVH*) shows decreased vermis size that does not reach the obex, the narrowing of the fourth ventricle in the caudal medulla (*white line*), as occurs in normal subjects. In addition to vermis hypoplasia, subjects with Dandy–Walker malformation (*DWM*) also exhibit an increased posterior fossa size and an upward rotation of the vermis. The parasagittal image of a patient with Joubert syndrome shows vermis hypoplasia and an elongated superior cerebellar peduncle (*white arrowhead*). The plane of this off-midline image is designated with a *dotted white line* in the corresponding axial image. The “molar tooth” malformation of Joubert syndrome and related disorders can be seen in the axial *MRI* as elongated cerebellar peduncles (*white arrowhead*) and deepened interpeduncular fossa (*black arrow*) compared with a normal subject (*N*; inset). Subjects with pontocerebellar hypoplasia (*PCH*) exhibit both decreased vermis size and pontine hypoplasia (*arrows*). *Cb* cerebellum, *PF* posterior fossa

Table 1

List of genes and suspected cellular processes that have been implicated in human cerebellar malformations (see text for discussion)

Cerebellar malformations	Implicated human genes	Likely disrupted process
Cerebellar vermis hypoplasia (CVH)	<i>OPHN1</i> [59,60]	Spine morphogenesis
Dandy–Walker malformation (DWM)	<i>ZIC1</i> , <i>ZIC4</i> [65], <i>FOXC1</i> [17]	Granule cell differentiation
Joubert syndrome and related disorders (JSRD)	<i>AHI1</i> [67,68], <i>ARL13B</i> [69], <i>CCD2A</i> [70,71], <i>CEP290</i> [72,73], <i>INPP5E</i> [74,75], <i>NPHP1</i> [76,77], <i>RPGRIP1L</i> [78,79], and <i>TMEM67</i> [80]	Granule cell proliferation
Pontocerebellar hypoplasia (PCH)	<i>CASK</i> [86], <i>RARS2</i> [88], <i>TSEN54</i> , <i>TSEN34</i> , and <i>TSEN2</i> [89]	Spine development, cell proliferation, tRNA splicing, cellular maintenance.