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Current Opinion in Behavioral Sciences

Impact of cerebellar-specific genetic and circuit manipulations on the behavioral phenotype and cerebellar physiology in murine autism models

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Clinical evidence suggests that developmental cerebellar injury and cerebello-cortical connectivity abnormalities are often present in autism. In mouse models, cerebellar-specific deletions of autism risk genes, or temporally constrained, developmental manipulations of cerebellar circuits, elicit autistic-like behaviors. Nonetheless, behavioral and electrophysiological findings are inconsistent within and across models. Additionally, while cerebellar manipulations during development can induce autistic phenotypes, studies of early cerebellar function and connectivity are scarce.

In this review, we discuss the impact of cerebellar-specific genetic mutations and circuit manipulations on adult behavior and cerebellar neuronal activity in murine autism models. We also explore how cerebellar development can impact the establishment of mature circuits, and we consider the existing gaps regarding the use of murine models to elucidate the cerebellar role in autism.

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Current Opinion in Behavioral Sciences 2024, **55**:101330

This review comes from a themed issue on **Role of the Cerebellum in Cognition and Behavior**

Edited by **Sheeba.Arnold-Anteraper** and **Wietske van der Zwaag**

Available online xxxx

Received: 28 June 2023; Revised: 4 October 2023;
Accepted: 28 November 2023

https://doi.org/10.1016/j.cobeha.2023.101330

Introduction

Autism spectrum disorder is one of the most frequently diagnosed neurodevelopmental conditions, involving a range of characteristics, including deficits in social interaction and communication, along with inflexible and repetitive behaviors. It has long been known to have a strong genetic causation [1] and, according to the Simons Foundation Autism Research Initiative database, at the time of writing this review, over 1100 genes have been identified that significantly increase the possibility of receiving an autism diagnosis. However, despite the breadth of genetic knowledge on autism susceptibility, studies have failed to identify a shared molecular substrate. Therefore, research on autism has been increasingly focusing on identifying common molecular and anatomical pathways that may underlie core autistic phenotypes.

While there is no single brain abnormality evident in all autistic people, clinical evidence has placed developmental cerebellar injury and cerebello-cortical connectivity abnormalities at the forefront of the pathological findings observed in children and adults with an autism diagnosis [2–4]. Furthermore, several high-confidence autism risk genes are prominently expressed in the cerebellum [5,6], with some showing a significant enrichment in this region [7]. Researchers have hypothesized that because the cerebellum begins cellular differentiation very early on in development and is one of the last structures to fully mature, it might be particularly vulnerable to genetic and environmental factors that derail its developmental trajectory [3].

The aim of this review is to discuss how autism mouse models representing (1) cerebellar-specific deletions of high-confidence autism risk genes, and (2) cerebellar circuit manipulations, impact adult mouse behavior and electrophysiological properties of the cerebellar neurons. We also explore the developmental mechanisms of how cerebellar-specific manipulations could lead to brain-wide abnormalities and autism-like phenotypes. Finally, we consider future research directions using mouse models that could further elucidate the role of cerebellar development in autism.

Of note, in this review, we alternate between person-first (person with autism) and identity- first (autistic person) language, as both of those terms are currently preferred by the autism community [8].

Cerebellar-specific mutations of autism risk genes show commonalities between phenotypic impairments in autism-like behaviors

In recent years, several studies employing the targeted deletion of known autism risk genes in the cerebellum

Table 1

Behavioral overview of autism-like phenotypes in mouse models with cerebellar-specific mutations.

	Spatial learning	Fear conditioning	Motor learning	Sensorimotor learning	Motor coordination	Locomotion	Reversal learning	USV emission rate	Stereotypes	Social interactions	Anxiety
Shank2 (PC specific)			↓ 9	↓ 9	↓ 9,10	- 9,10		- 10	↑ 9,10	↓ 9,10	- 9,10
Pten (PC specific)	- 12		↓ 12		↓ 12	- 12	- 12		~ 12	↓ 12	
Tsc1 (PC specific)	- 16,17	↓ 14	↓ 16	↑ 14	↓ 13,16	↓ 16	↓ 16	↑ 16	~ 13,16	↓ 16	
mTORC1 GOF* (PC specific)			↓ 19		↓ 19	↓ 19				- 19	- 19
Tsc2 (PC specific)	- 18				↓ 18	- 18	- 18		↑ 18	↓ 18	~ 18
p75NTR (PC specific)									↑ 20	↓ 20	
Bmal1 (PC specific)			↓ 22		↓ 22				~ 22	↓ 22	
Scn8a (PC specific)	↓ 24,25		↓ 23,24	↓ 25	↓ 23,24	- 24	↓ 24		↑ 24	↓ 24	↑ 24
Grip1/2 (PC specific)						- 11			↑ 11	- 11	- 11
Auts2 (En1-cre)**			↓ 21		↓ 21			↓ 21			

*GOF— gain-of-function mutation leading to mTORC1 hyperactivation; **En1-Cre — localized deletion to the rhombomere-1-derived brain area including the cerebellum; ↑, significantly higher in mutants than in WT animals; ↓, significantly lower in mutants than in WT animals; -, mutants did not differ significantly from WT; ~, differences between mutant and WT mice were mixed. Numbers denote references.

have been employed to unravel the intricate genetic mechanisms underlying cerebellar dysfunction. The targeted deletion of these genes within the cerebellum has provided valuable insights into their functional significance in cerebellar development, maintenance, and overall neurological function. Many studies have successfully reproduced phenotypes resembling human autism-like characteristics, including motor coordination deficits, affected social interactions, and cognitive impairments (Table 1).

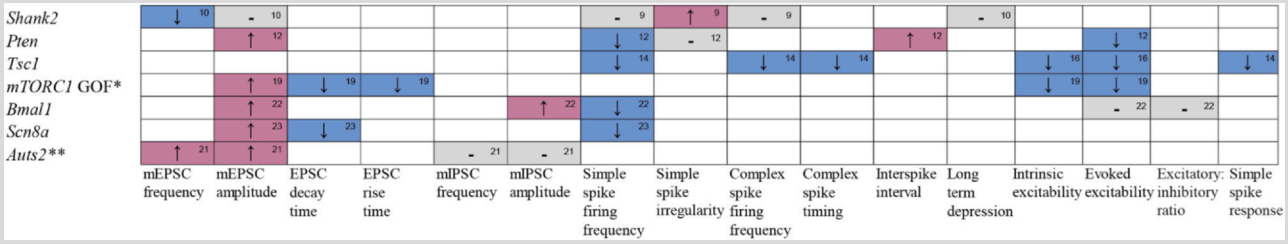
Targeted, conditional deletions of autism risk genes in the cerebellum have so far focused on genes encoding scaffolding proteins, which are members of the signaling cascade downstream of cell surface receptors (*Shank2* [9,10] and *Grip1/2* [11]), genes in the mechanistic target of rapamycin (mTOR) signaling pathway, responsible for cell growth and migration (*Pten* [12], *Tsc1* [13–17], *Tsc2* [18], and *mTOR* [19]), the *p75NTR* gene encoding the p75 neurotrophin receptor [20], which mediates cell survival and regulates axonal growth and proliferation, the *Auts2* gene, a key regulator of the transcriptional network during brain development [21], as well as the clock gene *Bmal1* [22] and *Scn8a* that encode a voltage-gated sodium channel [23–26].

In most cases, cerebellar-specific deletions of above-listed genes result in impairments in cerebellar-dependent behaviors, as seen by affected motor coordination [10,12,16–19,21–24] and motor learning [9,12,16,17,21–24]. Additionally, when examined for autism-like behaviors, cerebellar-specific targeting of autism risk genes showed clear effects on the behavioral phenotype with impairments in social interactions [9,12,16–18,20,22,24] and increased presence of stereotypes [9–11,16–18,20,24], providing important evidence for the role of cerebellar functioning in autism-like phenotypes.

Notably, although cerebellar autism models showed strong similarities in behavioral patterns, stereotypical behavior was not consistent within nor across models. Mice with a Purkinje cell (PC)-specific *Pten* mutation showed decreased grooming duration, yet significantly increased upright scrabbling and jumping [12]. Additionally, PC-specific *Tsc1* mutant mice were found to show increased levels of grooming behavior in a study by Tsai et al. in 2012 [16], but presented with a far lower grooming frequency than wild-type (WT) mice in a study by Klibaite et al. in 2022 [13]. Mice with PC-specific mutations in the *Bmal1* gene were found to have an increased number of both spontaneous grooming bouts and water puff-induced grooming compared with WT mice, while displaying strong impairments in both the marble-burying test and the nestlet shredding test [22]. The available data thus suggest that stereotypic behavior exhibits distinct regulatory patterns associated with various genetic mutations, in contrast to the majority of other autism-like behaviors. Grooming, marble burying, and nestlet shredding are all commonly described as 'stereotypic behaviors'. In a recent study by Silverman et al. [27], the authors urge caution in attributing results from a single task to a general behavioral domain (such as 'stereotypic behaviors' or 'sociability'). Instead, the authors suggest that the results from a task should be considered solely as a measure of a group's performance in that task, as different tasks may measure different aspects of a behavioral domain. The conflicting findings in stereotypic behaviors underscore the importance of evaluating multiple assays across multiple domains of behavior, which will facilitate comparisons between available models. Interestingly, the manifestation of anxiety-like behavior showed sexually divergent expression in mice with PC-specific *Tsc2* deletion. Female *Tsc2* mutant mice spent slightly less time in the center of an open-field arena, whereas male *Tsc2* mutant

Table 2

Overview of changes in PC activity in genetic autism mouse models with cerebellar-specific mutations.



*GOF— gain-of-function mutation leading to mTORC1 hyperactivation; ↑, significantly higher in mutants than in WT animals; **En1-Cre — localized deletion to the rhombomere-1-derived brain area including the cerebellum; ISI, interspike interval; mIPSC, miniature-inhibitory postsynaptic currents; mEPSC, miniature-excitatory postsynaptic currents; EPSC, excitatory postsynaptic currents; SS, simple spike; CS, complex spike. ↓, significantly lower in mutants than in WT animals; -, mutants did not differ significantly from WT; ~, differences between mutant and WT mice were mixed. Numbers denote references.

animals were found to spend slightly more time in the center of the apparatus [18].

Cerebellar signaling is impaired in autism mouse models with targeted genetic mutations

To gain deeper insights into the impact of targeted deletions of autism risk genes in the cerebellum, several of the available studies also used these mouse models to conduct electrophysiological recordings (Table 2). Although there is sparse overlap between the exact measurements analyzed, some commonalities can be found. For example, cerebellar-specific mutant mice for *Pten*, *Bmal1*, *Scn8a*, *mTORC1*, and *Auts2* showed significant increases in (m)EPSC amplitude [12,19,21–23]. Additionally, lower SS firing frequency was observed in mice with PC-specific deletion of *Tsc1*, *Bmal1*, and *Scn8a* [14,16,22,23]. Reduced evoked excitability was observed in mice with PC-specific mutations in the *Pten*, *Tsc1*, and *mTORC1* genes [12,16,17,19], which could potentially be attributed to their shared involvement within the mTOR pathway [28]. Short-term plasticity was observed to be reduced in mice with PC-specific knockout of the *Scn8a* gene, while those with PC-specific mutations in the *Shank2* and *Tsc1* genes displayed no alterations in synaptic plasticity [10,16,23]. These studies show that cerebellar signaling abnormalities in autism mouse models with targeted genetic mutations can provide insights into the underlying mechanisms of cerebellar dysfunction in autism. These findings contribute to our understanding of how behavioral and cognitive impairments in individuals with autism might originate from cerebellar defects.

Targeted cerebellar circuit manipulations and interventions provide a substrate for behavioral defects in autism

Acute, targeted cerebellar manipulations such as lesions, optogenetic silencing, and stimulation, as well as rescue experiments, have been used in addition to genetic models to unravel the functional principles of the influence of cerebellar dysfunction on the cognitive and behavioral features of autism (Table 3). In PC-*Tsc1* mutant mice, continuous treatment with the mTOR inhibitor (rapamycin) initiated at P7 showed amelioration of all behavioral deficits and normalized PC excitability [16]. A follow-up study identified a critical period from P7 to P35 in PC-*Tsc1* mice for the onset of social and motor learning deficits, where rapamycin treatment rescued behavioral deficits as well as PC survival, tonic firing frequency, and intrinsic excitability [17]. However, no critical period was identified for stereotyped behavior as rapamycin treatment did not affect the repetitive grooming phenotype. The authors propose that the critical period for the contribution of mTOR signaling on repetitive behavior and behavioral inflexibility extends past the tested period and/or remains plastic into adulthood, while also being controlled by different circuitry from other autism-like impairments such as social behavior. This could aid in explaining the previous finding that the expression of stereotypical behavior exhibits significant variability exclusively within mutant mouse models with PC-specific mutations (Table 1). Similarly, rapamycin treatment was able to rescue social behavior and social novelty preference in PC-specific *Tsc2* mutant mice [18].

Using selective optogenetic stimulation of cerebellar axons in the ventral tegmental area of WT C57BL/6 mice showed cerebellar inputs to this area are required for intact social preference but cannot, on their own, promote social interactions [29]. In C57BL/6 mice, both social associative memory and social recognition were shown to be impaired after manipulations using neurotoxic lesion or chemogenetic excitation of PCs in lobule IV/V in a study by Chao et al. [30]. The targeted lesion of lobule-IV/-V PCs also induced excessive repetitive grooming behavior in treated animals. In an earlier paper, Chao et al. were able to rescue excessive grooming behavior in BTBR *T*$^{+}$*tf*/J (BTBR) mice, by using the Kv1.2 agonist docosahexaenoic acid (DHA) to

Table 3

Overview of changes in behavior and cerebellar firing patterns as a result from targeted cerebellar manipulations and rescue experiments.

Reference #	Mouse model	Manipulation	Result
[16]	PC-specific *Tsc1*	Rapamycin P7 to adult	Rescue of all behavioral deficits, tonic firing frequency, and intrinsic excitability
[17]	PC-specific *Tsc1*	Rapamycin P7–P35 P7–P63	Rescue of social and motor deficits, tonic firing frequency, and intrinsic excitability No rescue of repetitive behaviors and behavioral inflexibility
[18]	PC-specific *Tsc2*	Rapamycin P10 to adult	Rescue of social behavior and social novelty preference
[29]	C57BL/6	Optogenetic stimulation of cerebellar axons in the ventral tegmental area	Cerebellar inputs to this area are required for intact social preference
[30]	C57BL/6	Neurotoxic lesion or DREADD excitation of lobule-IV/-V PCs	Impaired socio-associative memory and social recognition and induced excessive repetitive grooming behavior
[31]	BTBR	Kv1.2 agonism with DHA	Partially rescued reduced intrinsic excitability and firing regularity
[32]	C57BL/6	Acute (adults) DREADD inhibition of PCs [crus I bilateral, crus I left, crus I right, and lobule VI]	Strongly impaired reversal learning in lobule VI and bilateral crus I PC inhibition
[33]	C57BL/6	Developmental (juveniles) or acute (adults) DREADD inhibition of MLIs [crus I right, crus II right, lobule VI, and lobule VII]	Inactivation of crus I, but not crus II or lobule VI, impaired learning during eyeblink conditioning Developmental inactivation of crus I or II in impaired social preference Acute inactivation of lobule VI, VII, or crus I or II did not affect social preference Developmental, but not acute, inactivation of lobule VII caused impairments in repetitive grooming

Numbers denote references.

partially alleviate the reduced intrinsic excitability and firing regularity [31]. These results demonstrate the importance of anterior cerebellar functioning on social and repetitive behavior in autism. Likewise, healthy cerebellar functioning in lobule IV and bilateral crus I was shown to be essential for behavioral flexibility as designer receptors exclusively activated by designer drug (DREADD)-inhibited PCs in C57BL/6 mice led to strong impairments in reversal learning [32]. Similarly, a study by Badura et al. found that both developmental and acute inactivation of molecular layer interneurons (MLIs) in crus I, but not crus II or lobule VI, was able to disrupt learning during eyeblink conditioning performance. However, while developmental DREADD MLI inactivation of crus I or II in juvenile C57BL/6J mice abolished the preference for a social stimulus over an object in the three-chambered test, acute inactivation of MLIs in lobule VI, VII, and crus I or II in adult C57BL/6J mice did not affect social preference [33]. Juvenile disruption of lobule VII caused impairments in repetitive self-grooming, while adult manipulations did not show the same effect [33]. Therefore, cerebellar manipulations consistently show both region-specific and developmental critical periods for the development of autism-like behavior, providing anatomical and developmental substrates for the involvement of cerebellar development in the behavioral repertoire in autism.

Cerebellar development — a susceptibility window

In humans, cerebellar development is initiated early in gestation, with distinguishable macrostructures identified during early fetal development, at 6 postconception weeks, that grow linearly with the cerebrum [34,35]. Between 15 and 22 weeks of gestation, the absolute volume of the cerebellum increases 5.3-fold, and at a higher rate than the supratentorial brain from week 17, with the anterior lobe appearing to have a faster growth rate than the posterior one [36]. Later, the cerebellum continues to grow *in utero* and its volume increases 5-fold between gestation week 24 and birth [34]. In mice, cerebellar development follows similar steps to that of humans, albeit at a disparate rate. The cerebellar primordium can be seen from embryonic days 7–8, followed by a smooth cerebellar-like structure at E15, with foliation beginning at around E16.5 [37,38].

In both species, the cerebellum undergoes extensive growth after birth. In the first 90 days of human development, the cerebellum shows the largest increase in overall volume compared with other areas of the brain [39]. This is not a constant linear growth, rather a U-shaped one, with a peak in volume changes found during infancy, at around 10–12 years of age, which appears to be lobule- and sex-dependent, and that continues until adolescence [40]. This growth is also lobe-dependent, as

myelin content tends to increase over time in cerebellar lobules involved in high-association processes, while it remains constant or decreases in lobules involved in sensory processing [40]. In addition to region- and sex-dependent growth variation, cerebellar white matter development is also modulated by placental hormone production, with structural abnormalities being found both in genetic models of placenta allopregnanolone reduction and in brain tissue from preterm infants [41]. In the mouse, the majority of cerebellar cell types are still undergoing generation and migration during early postnatal development. This sequential development and the maturation of distinct cell types leads to progressive modifications of firing properties and gene expression, which ultimately leads to the appearance of a mature cerebellum at around one month postnatally [42,43].

This long window of time during which the cerebellum develops, extending from the protected *in utero* environment to later postnatal periods, makes it a particularly sensitive structure, susceptible to developmental insults that can affect not only its own development but also the connectivity with other cerebral areas. Although data on connectivity during cerebellar development are scarce, in the mouse brain, cerebello-thalamic tract axons can already be seen invading the thalamic anlage from E17.5 and, one day later, these are also seen in layer VI of the developing cortex [44]. Conversely, afferent fibers into the cerebellum are found from E12 and these all seem to be established at the time of birth [45]. In infants, functional connectivity between the cerebellum and the frontoparietal and default mode networks can be detected during very early postnatal stages, including in preterm children [46,47]. Of note, although connectivity with sensorimotor cortical components is more salient during this period, cerebello-cortical networks encompassing default mode, executive control, and planning systems become stronger during childhood, exhibiting representations similar to those found at later stages [46,48]. Ultimately, the adult brain presents with networks that connect distinct cortical primary motor, sensory, and association areas with the cerebellum and cerebello-cortical connections that primarily innervate forebrain-associative areas [49,50].

The early anatomical involvement of the cerebellum with nonmotor areas is also reflected on the effects of cerebellar manipulations in preadult stages. Although murine studies investigating the effects of cerebellar manipulation during development are very scarce, as previously mentioned, these have been shown sufficient to elicit autism-like behaviors [33]. Of note, some of these altered behaviors, including impaired social communication, have been detected at very early ages (P7) [51,52], suggesting an important role of the cerebellum in driving these behaviors before the establishment of fully mature networks with the cerebrum. Furthermore, given the large number of autism mouse models where cerebello-cortical connectivity is altered [53], these early cerebellar manipulations are expected to cause considerable behavioral impact. Accordingly, we observe significant behavioral changes in adult mice with the cerebellar-specific mutations described above. However, whether atypical cerebellar development contributes to autism-like behavioral deficits in more genetically translatable models (i.e. in the presence of haploinsufficient genetic mutations) remains to be investigated.

Conflicting evidence and outstanding questions

Autism is highly complex and multifactorial, with both environmental and genetic factors contributing to its establishment. As a result of these heterogeneous phenotypes, evidence from the investigation of shared features as possible biomarkers for autism has been highly conflicting. A number of reports have indicated PC loss in the cerebellum, and hypoplasia of the vermis in people with autism [54]. However, many of these studies were considerably underpowered [55], particularly for cell counting analysis that relied on access to postmortem cerebellar tissue. A recent imaging study with a large cohort of 219 controls and 274 autistic people reported no evidence for gross structural changes in cerebellar morphology between the two groups [56]. Notably, this study does not inform on putative connectivity changes, which have indeed been previously reported [4], nor does it have the cellular resolution to quantify potential cellular abnormalities (i.e. cell count or migration deficits). Large longitudinal studies, reporting both structural and functional data from the same participants, will be key to resolve these conflicting reports and understand the contribution of cerebello-cortical networks to the development of autistic phenotypes over time. In accordance with human studies, total cerebellar volume, albeit scarcely studied in depth in autism mouse models, also appears to be intact, although lobule-specific volumetric changes have been reported [57]. Nonetheless, a recent study using monogenic mouse models of autism was able to cluster 16 genetic groups into 4 distinct cohorts based on functional connectivity patterns between distinct brain areas [53]. Although it remains unclear whether these multiparametric brain connectivity signatures can also be found in autistic individuals, the separation of apparent heterogeneous phenotypes into distinct autism subgroups could aid the identification of autism anatomical and functional biomarkers.

In light of the phenotypic variability described in this review, the emergence of novel behavioral analysis tools could further aid the classification of heterogeneous and variable presentations. Methods, such as Motion Sequencing (MoSeq) that uses unsupervised machine learning to identify and group discrete mouse behaviors, have proven highly effective in the identification of behavioral patterns that are usually outside the scope of

traditional behavioral assays [58]. This detection and clustering of even subtle differences in behavior signatures are crucial to advance the identification of autism risk genes and dissect the effects of interventions on behavior [27].

Finally, our knowledge of cerebellar circuitry development in health and disease remains limited, particularly due to the lack of techniques to manipulate early cerebellar function during perinatal development. Recently, a new method for wireless light emitting diode (LED) implantation in newborn mice was described, potentially enabling optogenetic control in mice as young as 10-days-old [59]. The combination of novel methods for early cerebellar manipulation, combined with direct comparisons of murine and human imaging data, will provide a great opportunity to expand our knowledge of cerebellar circuitry development in health and disease [4,41]. The ultimate goal of future research would be to causally test the hypothesis stating that developmental injury limited to the cerebellum is sufficient to alter circuit formation within cortical, autism-relevant areas at an anatomical and/or functional level [54].

Funding

This work was supported by Netherlands Organization for Scientific Research (NWO) Grants VIDI/917.18.380,2018/ZonMw and Convergence Health & Technology Integrative Neuromedicine Flagship Program 2022.

Declaration of Competing Interest

Nothing to declare.

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- •• of outstanding interest

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