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# The HERC proteins and the nervous system

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ABSTRACT

The HERC protein family is one of three subfamilies of Homologous to E6AP C-terminus (HECT) E3 ubiquitin ligases. Six *HERC* genes have been described in humans, two of which encode Large HERC proteins -*HERC1* and *HERC2*- with molecular weights above 520 kDa that are constitutively expressed in the brain. There is a large body of evidence that mutations in these Large *HERC* genes produce clinical syndromes in which key neurodevelopmental events are altered, resulting in intellectual disability and other neurological disorders like epileptic seizures, dementia and/or signs of autism. In line with these consequences in humans, two mice carrying mutations in the Large *HERC* genes have been studied quite intensely: the *tambaleante* mutant for *Herc1* and the *Herc2*$^{+/530}$ mutant for *Herc2*. In both these mutant mice there are clear signs that autophagy is dysregulated, eliciting cerebellar Purkinje cell death and impairing motor control. The *tambaleante* mouse was the first of these mice to appear and is the best studied, in which the *Herc1* mutation elicits: (i) delayed neural transmission in the peripheral nervous system; (ii) impaired learning, memory and motor control; and (iii) altered presynaptic membrane dynamics. In this review, we discuss the information currently available on HERC proteins in the nervous system and their biological activity, the dysregulation of which could explain certain neurodevelopmental syndromes and/or neurodegenerative diseases.

## 1. Introduction

The development of nervous system and its homeostasis throughout the lifetime of the individual is the result of the coordinated interplay of many genes that not only control the differentiation of the distinct regions of the nervous system [1,2] but also, the main processes driving their histogenesis, such as the cell cycle [3], migration [4], neurite outgrowth [5], synaptogenesis [6] and cell death [7,8]. A vast number of proteins are implicated in these processes, whose correct orchestration depends on maintaining the balance between newly formed proteins and those that age and/or are misfolded. In this context, protein degradation is an important part of proteostasis, and the autophagy/lysosome and proteasome pathways are the main mechanisms through which proteins are degraded. Ubiquitination participates in both these pathways, and it is an event that may also regulate protein activity independently of their degradation [9–11]. Indeed, altered ubiquitination can damage the structure and affect the activity of both the central and peripheral nervous systems (CNS/PNS) [12–14].

E3 ubiquitin ligases act in the third step of the ubiquitination pathway, recognizing the protein target (substrate) and catalyzing the

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addition of ubiquitin by E2 ubiquitin-conjugating enzymes. E3 ubiquitin ligases are classified into three major classes based on the catalytic mechanism they employ: RING (really interesting new gene), HECT (homologous to E6AP C-terminus), and RBR (RING-between-RING). In humans, the HECT family is composed of 28 members, each containing a characteristic catalytic domain of approximately 350 amino acid (aa) residues near their C-terminus. The HECT family has been sub-divided into three groups: NEDD4 (neuronal precursor cell-expressed developmentally downregulated 4), HERC, and "Others" [15–17].

HERC proteins are ubiquitin ligases that contain a HECT domain and a RCC1 (Regulator of Chromosome Condensation 1)-like domain (RLD). The HERC proteins have traditionally been classified into two sub-groups based on their molecular mass and domain structure: Large and Small HERCs [18] (Fig. 1A). Curiously, the differences between these sub-groups are thought to derive from the convergent evolution of ancestors belonging to distant families [19]. Large HERCs (HERC1 and HERC2) are proteins with molecular weights above 520 kDa and they contain characteristic domains that include multiple RLD domains. Small HERCs (HERC3–6) are almost 20% smaller, and they possess a single HECT and RLD domain. Human HERC5 and mouse HERC6 also act as E3 ligases for ISG15, a ubiquitin-like protein that is expressed upon stimulation of cells with interferon [20–22]. These E3 ligases are important regulators in cells, influencing neurodevelopment, DNA damage repair, cell proliferation and migration, and the immune

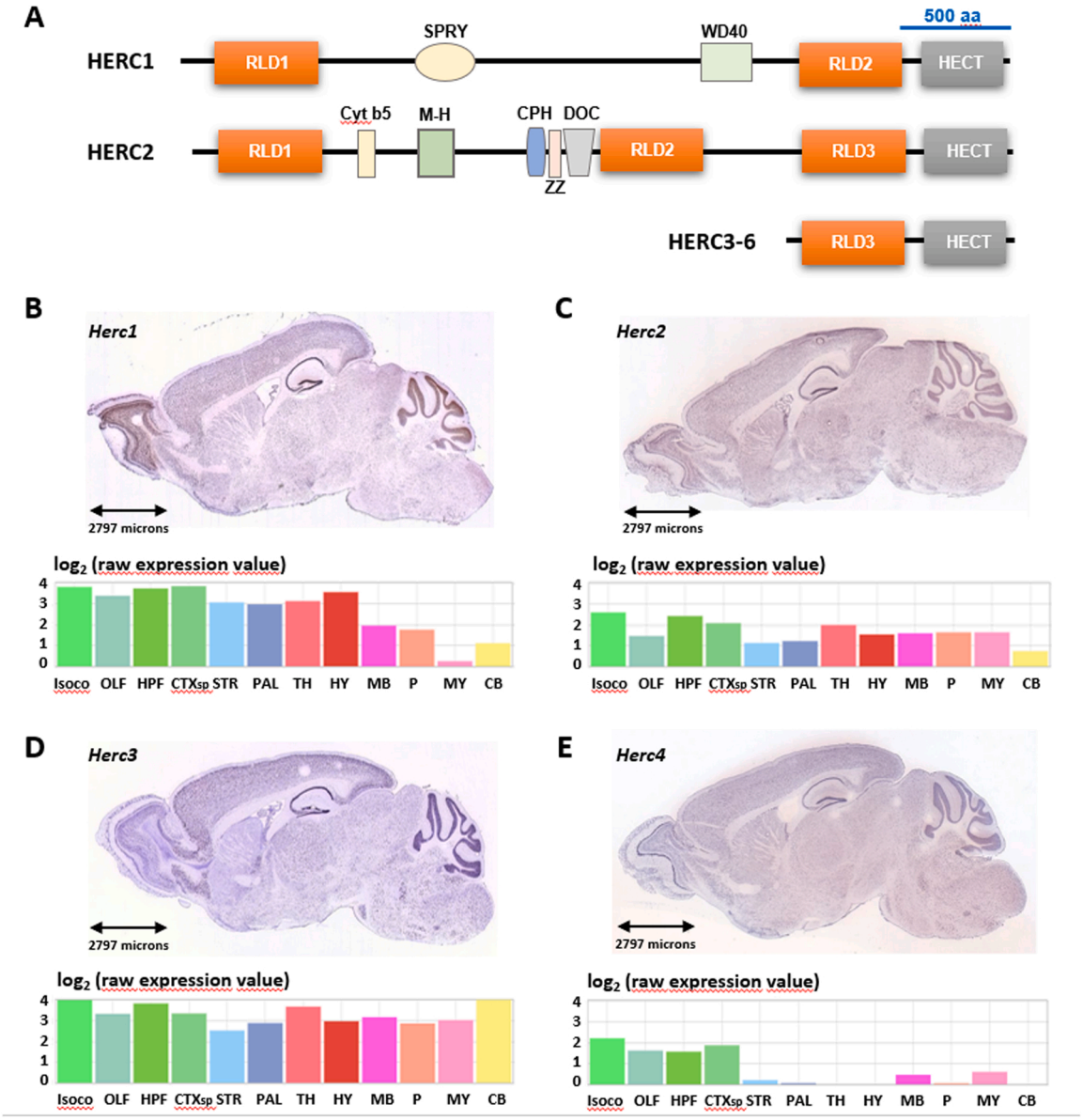


**Fig. 1.** HERC expression in the mouse nervous system. (A) Structural domains of the HERC protein family: HECT (homologous to the E6AP carboxyl terminus), RLD (RCC1 (regulator of chromosome condensation 1)-like domain), SPRY (spl A and RyR), BH3 (Bcl-2 homology domain 3), WD40 (G protein β-subunit like repeats), Cyt b5 (cytochrome b5-like region), M-H (mind-bomb/HERC2 domain), CPH (conserved domain within Cullin 7, Parkin-like cytoplasmic -PARC- and HERC2), ZZ (ZZ-type zinc finger), DOC (domain homologous to subunit 10 of the anaphase promoting complex -APC). (B-E) *In situ* hybridization of the *Herc1–4 genes* in the mouse brain. The *Herc5* gene is absent in the mouse genome and *Herc6* expression is not detected. Expression is shown by areas: isocortex (Isoco), olfactory areas (OLF), hippocampal formation (HPF), cortical subplate (CTXsp), striatum (STR), pallidum (PAL), thalamus (TH), hypothalamus (HY), midbrain (MB), Pons (P), medulla (MY) and cerebellum (CB). For B-E, images were obtained from the Allen Institute. © 2015 Allen Institute for Brain Science, Allen Brain Atlas API, available at: B, https://mouse.brain-map.org/experiment/show/632501. C, https://mouse.brain-map.org/experiment/show/69734983. D, https://mouse.brain-map.org/experiment/show/68845496, E https://mouse.brain-map.org/experiment/show/69028677. For *Herc6* expression see: https://mouse.brain-map.org/experiment/show/74635138.

response [23–26].

There is ever increasing evidence of the importance of HERC proteins in nervous system development and function, particularly regarding Large HERCs. Moreover, studies with mutant mouse models and human genetic studies have provided compelling evidence implicating Large HERCs in multiple neurodevelopmental disorders. In this review, the contribution of the HERC family to nervous system development, function and pathologies is discussed.

## 2. Expression of HERCs in the nervous system

Large HERC proteins are HECT ubiquitin ligases that in addition to the catalytic HECT domain and the RLD domains present in all HERC proteins, they contain other structural features (Fig. 1A). HERC1 contains two RLDs (RLD1 and RLD2), a spl A and RyR (SPRY) domain, a Bcl-2 homology domain 3 (BH3), seven WD40-repeats that are characteristic of the β-subunit of heterotrimeric G proteins, and other minor motifs like putative SH3-binding proline-rich sequences, a potential leucine zipper and several regions enriched in polar and acidic side chains. HERC2 contains three RLDs (RLD1–3), a cytochrome b5-like region, a mind-bomb/HERC2 (M-H) domain, a CPH domain, a ZZ-type zinc finger and a DOC domain [18]. A striking feature of both these proteins are their size, as both have almost 5000 aa residues in a single polypeptide chain. Although the function of some of these domains has been studied (RLD, CPH, HECT), that of the rest of the domains remains virtually unexplored. The HECT domain is the catalytic domain involved in the ubiquitination or the ISGylation of target proteins [15–17]. The HERC1 RLD domains interact with several proteins (clathrin, ARF and Rab) and with phosphoinositides involved in membrane trafficking (reviewed in [23]). The CPH domain is exclusive to HERC2 and it binds to p53 to regulate its transcriptional activity [27–29].

HERC proteins are expressed in several tissues (Genecards for *HERC1–6* genes: https://www.genecards.org/) and *in situ* hybridization studies in mice have provided a more detailed analysis of *Herc* gene expression in different areas of the nervous system (Fig. 1B-E). Large HERCs are expressed throughout the adult brain, with a higher relative expression of *Herc1* than *Herc2*, particularly in the isocortex, olfactory bulb, hippocampal formation, cortical subplate, striatum, pallidum, thalamus and hypothalamus (Fig. 1B-C). The expression of the Small HERCs is more heterogeneous (Fig. 1D-E), with *Herc3* expressed strongly in all areas of the adult brain analyzed, and with a relative expression even stronger than *Herc1* in the midbrain, Pons, medulla and cerebellum (Fig. 1D). *Herc4* is expressed very weakly in the brain, generally limited to the isocortex, olfactory bulb, hippocampal formation and cortical subplate (Fig. 1E). A homolog of the *Herc5* gene has not been found in the mouse genome and no *Herc6* expression is detected in nervous tissue. The broad expression of *Herc1* and *Herc2* in the mouse nervous system is consistent with it fulfilling widespread, important roles in this structure, as will be discussed below. Regarding Small HERCs, expression studies indicate that *Herc3* is the most relevant candidate in the nervous system, which should be further analyzed in future studies.

## 3. HERC1

HERC1 was the first HERC family member identified in a search for human oncogenes [30]. It is the largest member of the family (4861 aa in Homo sapiens -Hs- and 4859 aa in Mus musculus -Mm) and it is involved in membrane trafficking due to its activity as a guanine nucleotide release factor (GRF), as well as through its ability to bind clathrin and ARF proteins [18,30,31]. HERC1 binds to phosphatidylinositol 4, 5-bisphosphate, which is required for its GRF activity on ARF and Rab GTPases [18]. HERC1 interacts with the tuberous sclerosis complex (TSC) 2 protein [32], a protein that forms the TSC complex with TSC1 and TBC1D7. The TSC complex stimulates the GTPase activity of Rheb, which in its active GTP-bound state is a positive regulator of the mTOR (mammalian target of rapamycin) complex 1 (mTORC1) kinase activity. Thus, the TSC complex negatively regulates the activity of mTORC1, leading to cell growth inhibition and enhanced autophagy [33]. As the carboxyl-terminal domain of HERC1 destabilizes TSC2, HERC1 may be involved in processes regulated by mTORC1 [32]. HERC1 also acts as an E3 ubiquitin ligase and although several such substrates have been proposed, C-RAF was the first substrate shown to be ubiquitinylated by HERC1 [34]. Through C-RAF, HERC1 is involved in mitogen-activated protein kinase (MAPK) signaling pathways, regulating cell proliferation and migration, and suggesting it may act as a tumor suppressor [26,34–37]. Recently, HERC1 was identified as a quality-control factor that monitors failures in proteasome assembly [38]. Unassembled PSMC5, a subunit of the proteasome base, was identified and targeted for degradation by HERC1. Through this mechanism, HERC1 facilitates the removal of unassembled intermediates from the proteasome [38].

### 3.1. HERC1 mutations and genetic disorders

The *HERC1* gene contains 78 exons spanning 225 kb on chr15q22.31, these encoding a large and highly conserved ubiquitin ligase (>96% aa identity in mouse) [18]. *HERC1* mutations have been found in cancer and neuronal disorders, and while somatic mutations have been reported in leukemia, breast cancer and non-melanoma skin cancer [26], germline mutations have been associated to neuronal disorders (Table 1). The common features observed in patients with homozygous or compound heterozygous mutations in the *HERC1* gene led to the identification of the autosomal recessive neurodevelopmental disorder called MDFPMR syndrome (Macrocephaly, Dysmorphic Facies and PsychoMotor Retardation: Online Mendelian Inheritance in Man -OMIM- #617011) [39–43]. Several genetic studies have also associated *HERC1* mutations with autism spectrum disorders (ASDs), considering it a predictor of autism risk [44,45].

## 4. The *tambaleante* mouse

The recessive *tambaleante* (*tbl*) mutation appeared spontaneously in the 1980s in a mouse at the animal facility center of the Pasteur Institute. Early morphological studies of these *tbl* mice identified the almost complete loss of cerebellar Purkinje cells, which began in 2-month-old mice and continued throughout adulthood [46] (Fig. 2).

A more detailed study of this mutant described the degenerative changes in Purkinje cell axons and dendrites during the process of neuronal death [47], and later studies demonstrated that Purkinje cells die through autophagy [48] (Fig. 3A-C). A genetic analysis of these animals demonstrated that the *tbl* phenotype is due to a single nucleotide change (G1448A) in exon 5 of the *Herc1* gene. The G1448A mutation results in a Gly483Glu aa substitution within the RLD1 domain of HERC1. Overexpression of this mutant HERC1 protein was observed in the brain, suggesting enhanced stability, in conjunction with a decrease in mTORC1 activity and an increase in autophagy [49]. This altered mTORC1 activity is believed to be the cause of Purkinje cell autophagic death [49], although other mechanisms may also be involved, such as the dysregulation of proteasome assembly produced by the mutated HERC1 protein [38]. Therefore, from the very earliest studies [46–48] a role for HERC1 in normal nervous system development became clear. In this context and strengthening this idea, several members of the family of WD40-repeat proteins, including HERC1, have been proposed to play a relevant role in brain connectivity by regulating corpus callosum morphology [50].

Later studies extended the effects of the *tbl* mutation beyond the cerebellar sphere, demonstrating that other neurons in the CNS display signs of anomalous autophagy, including projection neurons like hippocampal pyramidal, neocortical pyramidal and spinal motor neurons. This effect was a consequence of derailed proteostasis, evident as an increase in autophagosomes and lysosomes, altered mitochondria, and anomalous expression of the markers of the autophagosome cycle like Beclin1, LC3 and p62 [51]. Therefore, these data indicate that the *Herc1*

**Table 1**
Main features of human *HERC1* mutations in neuronal disorders.

| **Reference** | [37] **Ortega-Recalde et al. (2015)** | | [38] **Nguyen et al. (2016)** | [39] **Aggarwal et al. (2016)** | | [40] **Utine et al. (2017)** | [41] **Schwarz et al. (2020)** | |
|---|---|---|---|---|---|---|---|---|
| **Gender** | Male (patient 1) | Female (patient 2) | Male | Male (patient 1) | Female (patient 2) | Male | Female (patient II:1) | Female (patient II:3) |
| ***HERC1* mutation (RefSeq: NM_003922)** | Compound heterozygous c.2625G>A (p.Trp875*) and c.13559 G>A (p. Gly4520Glu) | Compound heterozygous c.2625G>A (p.Trp875*) and c.13559 G>A (p. Gly4520Glu) | Homozygous c.9748C>T (p. Arg3250*) | Homozygous c.4906–2A>C (p. Leu1636Argfs*24) | Homozygous c.4906–2A>C (p. Leu1636Argfs*24) | Homozygous c.8678_8679insC (p. Ala2894Serfs*30) | Homozygous c.14072G>C (p. Arg4691Pro) | Homozygous c.14072G>C (p. Arg4691Pro) |
| **Mutation effect** | Truncated protein of 874 aa (variant 1) and altered HECT domain (variant 2) | Truncated protein of 874 aa (variant 1) and altered HECT domain (variant 2) | Truncated protein of 3249 aa. Not protein detection | Truncated protein of 1635 aa fused to 25 aa tail | Truncated protein of 1635 aa fused to 25 aa tail | Truncated protein of 2893 aa fused to 31 aa tail | Missense mutation in HECT domain | Missense mutation in HECT domain |
| **Age (last examination)** | 29 years | 24 years | 18 years | 7 years | 3 years | 8 years | 18 years | 17 years |
| **Overgrowth at birth** | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **Macrocephaly** | Yes | Yes | Yes | Yes | Yes | No | Yes | Yes |
| **Prominent forehead** | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **Long face** | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **Long fingers** | Yes | Yes | Yes | Yes | Yes | No | Yes | Yes |
| **Abnormality of vertebral column** | Yes (kyphoscoliosis, lumbar hyperlordosis) | Yes (kyphoscoliosis, lumbar hyperlordosis) | No | Yes (kyphoscoliosis) | Yes (kyphoscoliosis) | Yes (kyphoscoliosis) | Yes (lumbar scoliosis) | No |
| **Palpebral fissures** | Downslant | Downslant | NR | Upslant | Upslant | No | Downslant | Downslant |
| **Ocular hypertelorism** | Yes | Yes | NR | Yes | Yes | No | Yes | Yes |
| **Prognathism** | Yes | Yes | Yes | NR | NR | NR | NR | NR |
| **Hypotonia** | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **Bone age** | Normal | Normal | Normal | Normal | Normal | NR | NR | NR |
| **Motor delay** | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **Intellectual disability** | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| **Autistic features** | NR | NR | NR | NR | NR | Yes | NR | NR |
| **Hyperactivity** | NR | NR | NR | NR | NR | Yes | Yes | NR |
| **Neuroimaging (Brain MRI)** | Communicating hydrocephalus, megalencephaly, ventriculomegaly | Communicating hydrocephalus, megalencephaly, ventriculomegaly | Bilateral megalencephaly, thick corpus callosum, enlarged white matter, septum pellucidum cyst, small cerebellum | Normal | Normal | Normal | Normal | Normal |

(NR: not reported)

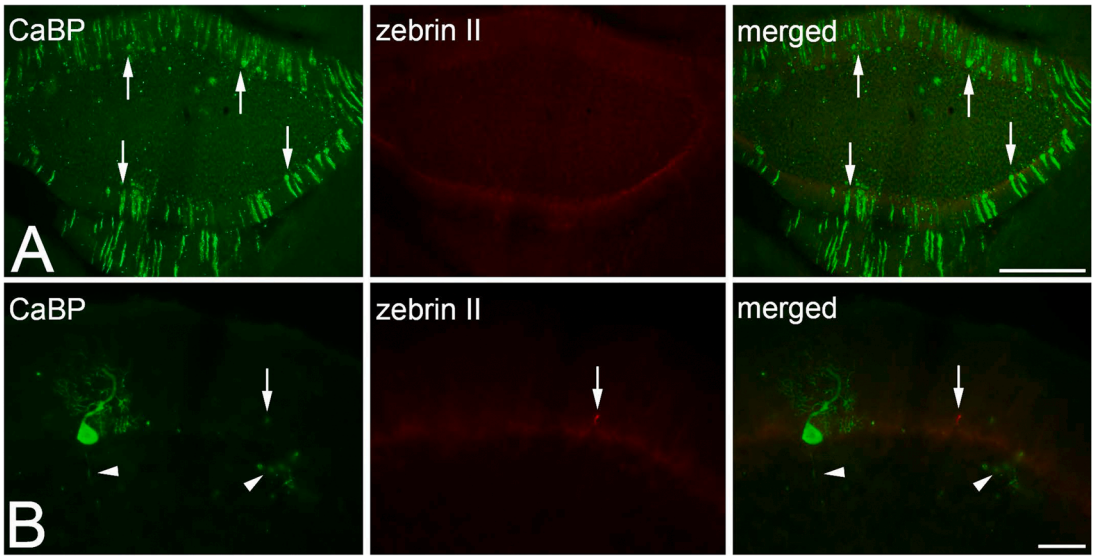


**Fig. 2.** The cerebellum of *tbl* mouse. Microphotographs of immunohistochemical staining with antibodies against calbindin (CaBP) and aldolase C/zebrin II [see 46, 112]. Coronal (A) and parasagittal (B) sections through a 4-month-old *tbl* cerebellum. Purkinje cells die in parasagittal bands (A, arrows). Dendritic debris (B, small arrows) and axonal torpedoes (B, arrowheads) were often observed. Bars = 1000 μm (A) and 50 μm (B).

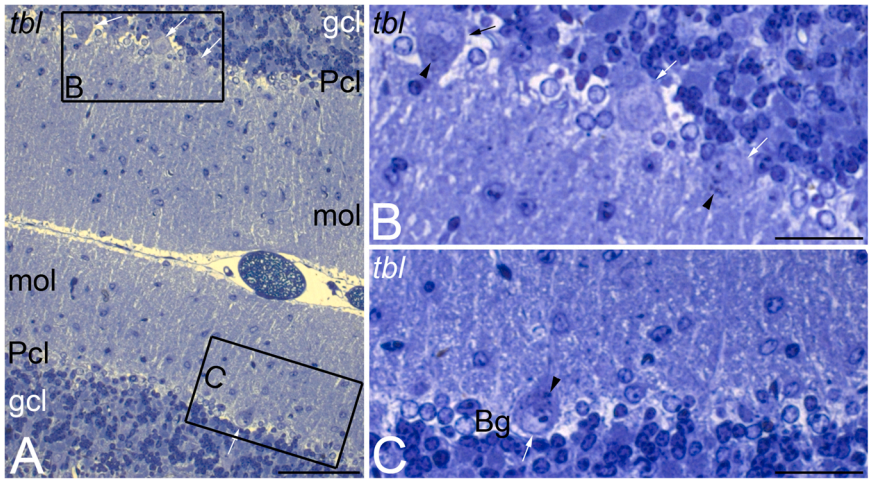


**Fig. 3.** Radial glia occupy the sites lacking Purkinje cells in the *tbl* cerebellum. Microphotographs of parasagittal semithin (1 μm thick) sections through the cerebellar cortex of adult *tbl* mice stained with toluidine blue [see 51]. A-C. Few surviving Purkinje cells are found in the *tbl* cerebellum (A, arrows) and the spaces left by the dead cells in the Purkinje cell layer (Pcl) are occupied by the clear cell nuclei of radial or Bergmann glia (Bg, C). Dark granules, represent the lysosomes that fill the cytoplasm of Purkinje cells (B-C, arrowheads). Bars = 300 μm (A) and 50 μm (B-C).

mutation alters neuronal proteostasis by dysregulating the mTOR/-autophagy pathway. In addition, while there are no data on the effect of the *tbl* mutation on GABAergic neurons at a distance from Purkinje cells, it seems conceivable that the alterations to proteostasis preferentially affect those CNS neurons with a higher rate of activity, such as Purkinje cells and glutamatergic projection neurons. Hence, it is likely that the *Herc1* mutation is also responsible for the decrease in glutamatergic innervation found in the *tbl* CNS [52,53] (see Section 4.2).

### 4.1. HERC1 and motor performance

Cerebellar Purkinje cell death in *tbl* mice was accompanied by clear motor symptoms, including tremor, unstable gait and abnormal posture of the hind limbs [48,54], as well as mild effects on motor learning capacities [54]. These early studies were carried out on adult *tbl* mice (older than 2 months of age) when Purkinje cells loss was prominent. However, young mice displayed impaired motor performance, even at 15 days of age, associated with morphological anomalies including smaller motor end-plates, and functional deficits in neurotransmitter release at the neuromuscular junction (NMJ) in fast (*Levator Auris Longus*, LAL), slow (*Transversus Abdominis*, TVA) and mixed muscles (*Gastrocnemius*, GN). Furthermore, at one month of age these alterations were accompanied by an incapacity to sustain the compound muscular action potential amplitude (CMAP) in the GN muscle after sciatic nerve stimulation as measured by electromyography (Fig. 4) [55]. Indeed, intracellular recordings of the LAL muscle indicated that neurotransmitter release was also altered in *tbl* mice. Altogether, these results show that the impairments to the motor system and in motor performance occur in young *tbl* mice, before Purkinje cell death would have taken place [55]. In addition, the velocity of axon conduction in adult *tbl* mice, when measured as the latency of the evoked response, was slower than in control mice [56].

In addition to the aforementioned alterations to neurotransmitters, adult *tbl* mouse had anomalous terminal Schwann cells that were interposed between the presynaptic and the postsynaptic sides of the NMJ, hindering normal neurotransmitter diffusion at the synaptic cleft [56]. They also suffered defects in the maintenance and formation of myelin sheaths, and in their wrapping, with degenerative changes similar to those found in other neuropathies [56]. Furthermore, signs of mitophagy are often found in the presynaptic regions of the *tbl* NMJ (Fig. 5). This dysregulated mitophagy could be a morphological correlate of the loss of $Ca^{2+}$ homeostasis [57], in part explaining the

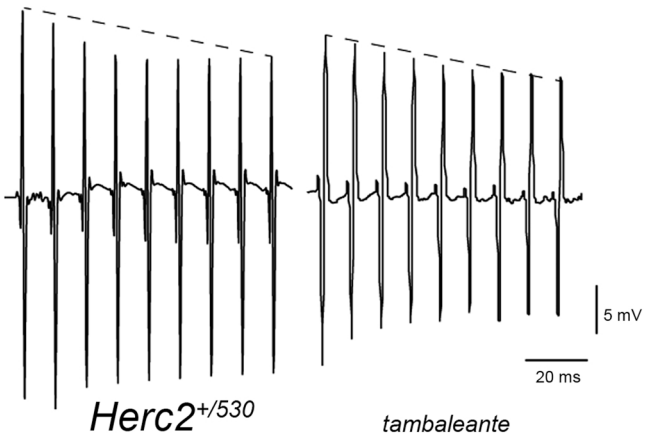


**Fig. 4.** NMJ transmission is affected in the *tbl* but not in *Herc2*$^{+/530}$ mice. Representative recordings of the amplitude of compound muscle action potentials (CMAPs) in the Gastrocnemius muscle during a train of stimuli delivered to the sciatic nerve at 100 Hz in a *Herc2*$^{+/530}$ and a *tambaleante* mouse [see 55 and 99]. While in *Herc2*$^{+/530}$ mice the response is similar to that of controls, *tambaleante* CMAP amplitudes decrease as the stimulus train progresses.

dampened neurotransmitter release (see Section 4.3).

Recently mTOR has been considered to be essential for the physiological activity of NMJs [58] and their misfunction could be at least partially explained by the alterations found in the *tbl* mouse NMJ. Furthermore, mTOR plays a key role in the development of PNS axons and myelin thorough the PI3K/Akt/mTOR signaling pathway [59,60], given that phosphorylated Akt is upregulated in the *tbl* mouse sciatic nerve [56]. Thus, in addition to the deleterious effect of dysregulated autophagy on NMJ synaptic transmission (see Section 4.3), our data suggest that a compensatory increase in phosphorylated Akt could directly alter Schwann cell homeostasis and be responsible for the myelin defects in *tbl* mice [56].

### 4.2. HERC1 and spinogenesis

The dendritic spines initially described by Ramón y Cajal [61] are considered a morphological correlate of learning processes [62]. The location and number of dendritic spines are altered in the *tbl* mouse, in which the appearance of ectopic dendritic spines is accompanied by a decrease in the total number of dendritic spines and in the number of mature spines [52,53]. These alterations are coincident with a loss of memory, and of associative and spatial learning, altered or absent long-term potentiation (LTP), fewer synaptic vesicles (SVs) and a clear decrease in glutamatergic inputs [52,53].

From studies over the past decades, alterations to dendritic spine populations have been associated with intellectual disability (ID) [63]. Moreover, in the last decade autophagy was seen to participate in dendritic spine homeostasis [64]. Thus, the dysregulation of autophagy driven by the HERC1 mutations [49] and described in distinct brain regions [for a review see 65] could alter normal spine proteostasis, justifying the morphological changes found in *tbl* amygdala and hippocampus [52,53]. In fact, deletion of the ubiquitin ligase 3a (Ube3a) provokes a decrease in the number of spines [66,67], while more dendritic spines are produced following its overexpression [68]. Furthermore, alterations to mTORC1 activity through changes to the TSC [69] or Akt [70] could also explain the defects in spinogenesis found in ASD [69] and bipolar disorders [70]. Thus, defective spinogenesis would appear to be clearly related to altered autophagy.

However, there is evidence that the changes to dendritic spines in the *tbl* mice might be related to their anomalous glutamatergic innervation. In fact, the glutamate AMPA receptor (AMPAR) is a postsynaptic modulator of plasticity [71] and the renewal of its subunits is regulated by autophagy in which ubiquitin E3 ligases proteins are involved (see Fig. 2 of ref. 63). The stability of AMPARs and NMDA receptors (NMDARs) [65,72–74] is essential for correct synaptic transmission, and the dysregulation of autophagy might alter the subunit composition of postsynaptic AMPARs and NMDARs. However, we have been unable to detect major changes in the expression of the main ionotropic subunits of AMPARs and NMDARs in the amygdala and hippocampus of *tbl* mice by immunohistochemistry [52,53]. Thus, more detailed studies will be necessary to establish whether HERC1 plays a role in postsynaptic AMPA and NMDA dynamics, perhaps *via* NEDD4 [65]. The fact that LTP is altered in the amygdala [52] and the hippocampal CA1 region [53] of *tbl* mice, and in both cases glutamatergic input to these *tbl* brain areas is reduced by half [52,53], suggests that the changes to dendritic spines are due to a loss of excitatory inputs in *tbl* mice, as occurs in other experimental conditions [75–77], rather than to the direct action of the mutated protein at the postsynaptic terminal.

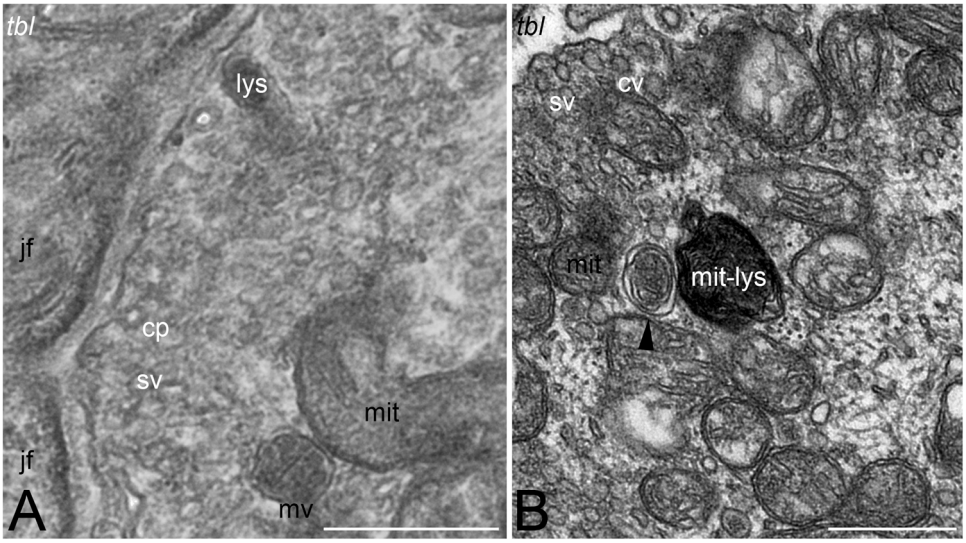


**Fig. 5.** Mitophagy is evident in *tbl* mouse NMJs. Transmission electron microscope microphotographs showing the cytoplasm of the presynaptic region of an adult *tbl* mouse NMJ. Signs of autophagy like lysosomes (A, lys) and of mitophagy, such as mitophagosomes (B, arrowhead) and mitolysosomes (mit-lys), are often seen: cv, coated vesicle; sv, synaptic vesicle; cp, endocytic coated pit; mit, mitochondria; mv, multivesicular body. Bars = 500 nm.

### 4.3. HERC1 and synaptic transmission

Molecular biology analysis of the HERC1 RLD1 domain indicated that it might participate in intracellular vesicle trafficking due to its interaction with ARF/Rab GTPases [23,25]. Moreover, the RLD2 domain of HERC1 interacts with clathrin [31], which plays a pivotal role in SV recycling [78,79]. Together with data from *in vivo* experiments in *tbl* mice, this mutation appears to elicit: (i) a reduction in the readily releasable pool (RRP) of SVs at the NMJ [55]; (ii) a loss of immunoreactivity for SV protein 2, concomitant with that of the marker of glutamate containing SVs VGLUT1 [52,53]; and (iii) altered LTP [52,53]. Hence, HERC1 would appear to influence synaptic transmission and more specifically, SV dynamics.

Neurotransmitter release is a complex process (reviewed in [80]) that begins by filling vesicles with the neurotransmitter cargo and the ensuing maturation of these SVs. Subsequently, these vesicles enter the RRP of SVs that approximates to the presynaptic active zones (AZs) where the vesicles dock. The entry of $Ca^{2+}$ elicited by presynaptic membrane depolarization provokes the fusion of the SVs to the presynaptic membrane and the release of their neurotransmitter cargo into the synaptic cleft. Finally, the SVs are recycled [81] through different pathways [80,82,83] and any damaged components are eliminated by lysosomal degradation [84,85]. *In vitro* experiments with cultured hippocampal neurons [86] demonstrate that the presynaptic terminals of *tbl* mouse neurons possess fewer docked SVs and a smaller RRP, smaller AZs, weaker clathrin immunoreactivity, and more endosomes and autophagosomes.

The data obtained *in vitro* explain the low number of glutamatergic SVs present in the amygdala and hippocampus of *tbl* mice *in vivo* [52,53], although how this decrease comes about remains unclear. Three possible explanations have been considered, the first of which is that mutated RLD1 alters ARF/Rab GTPase activity, thereby enhancing SV autophagy [86] or deregulating the endosomal recycling pathway [87]. A second possibility is that a dysregulation of autophagy alone explains the reduction in SV number [88]. Furthermore, the reduction of mTORC1 activity [49] could explain the decrease in vesicles, as well as the presence of endosomes and vacuoles, as demonstrated by altering the Fab1 complex [89]. The third possible explanation is that the clathrin mediated endocytotic pathway could be affected [86], essential for normal SV recycling [78,79,82], and in fact, GFP pull-down experiments demonstrated that the mutated HERC1 RLD1 domain was unable to interact with clathrin [86]. While none of these three possibilities have been confirmed, it remains possible that the altered SV recycling is due to a combination of these effects. Experimental data that mutated HERC1 interacts with clathrin independently of its RLD2 domain [31] strongly suggest that the dysregulation of clathrin mediated endocytosis is the best candidate to explain the effect of HERC1 mutation on SV dynamics [86].

The alterations to SV dynamics affect both the number of glutamatergic SVs and vesicular glutamate filling, two events seen to be diminished *in vitro* [86]. These changes could potentially explain the decrease in glutamatergic innervation observed in *tbl* mice [52,53], which could in turn be responsible, at least partially, for the morphological alterations to dendritic spines, the absence or depletion of LTP, and the memory impairments seen in these mutants [52,53]. Another interesting finding from the *in vitro* studies was the smaller AZ of presynaptic *tbl* synapses [86]. This alteration might be a direct consequence of the dysregulated SV turnover [86], as proposed in other experimental models [90]. In addition, the *tbl* mutation might affect the regulation of constitutive autophagy proteins in the AZ, as observed in other mutations and brain conditions [90–96].

## 5. HERC2

The *HERC2* gene was identified as the gene responsible for the mouse syndrome called *rjs* (runty, jerky, sterile) or *jdf2* (juvenile development and fertility-2), characterized by reduced viability, small size, neuromuscular defects and sterility [97,98]. This gene encodes an unusually large protein (4834 aa in Hs and 4836 aa in Mm) that participates in the ubiquitination of proteins like XPA, BRCA1, USP20, RPA, NEURL4, USP33, FBX15 and NCOA4. Through post-translational modification of these and other proteins, HERC2 may regulate cellular processes such as DNA repair, DNA replication, checkpoint control, ciliogenesis, centrosome architecture, iron metabolism and ferritinophagy [23,26]. HERC2 can also regulate cellular processes independently of its ubiquitin ligase activity. For example, HERC2 interacts with p53, stimulating its tetramerization and transcriptional activity [26–28].

### 5.1. HERC2 mutations and genetic disorders

The *HERC2* gene spans 211 kb on chr15q13.1 and contains 93 exons, encoding a ubiquitin ligase (>95% aa identity in mouse) [18] that is essential during embryonic development [99]. HERC2 regulates motor coordination and genetic variations in the *HERC2* gene are associated with variability in skin/hair/eye pigmentation [23]. Like *HERC1*, *HERC2* mutations have been described in cancers, including leukemia, breast cancer, gastric and colorectal carcinomas, cutaneous melanoma and uveal melanoma [26]. The *HERC2* gene is located close to an imprinting region of chromosome 15 that is associated with neurodevelopmental disorders like Prader–Willi and Angelman syndrome [100,101]. The complex genomic structure of the proximal 15q results in unequal homologous recombination during meiosis, which in turn leads to recurrent duplications and deletions. Although the *HERC2* gene is frequently deleted in patients with Prader-Willi or Angelman syndromes, it is not subject to imprinting [101–104]. Missense mutations in *HERC2* have been associated with an autosomal recessive neurodevelopmental disorder with some phenotypic similarities to Angelman syndrome and ASDs, features ranging from cognitive delay, speech disorders, ataxia, microcephalia, facial dysmorphism, seizures and hypopigmentation, as well as other secondary signs like infections and behavioral alterations [105–108]. A homozygous deletion spanning *HERC2* and *OCA2* causes a more severe neurodevelopmental phenotype [109], and novel loss-of-function mutations in HERC2 have been associated with severe developmental delay and pediatric lethality [110]. For reference, an updated summary of the existing clinical and molecular data from patients with biallelic loss of function and missense variations in *HERC2* has been published recently [111].

### 5.2. The $Herc2^{+/530}$ mouse model

The mutant allele of *Herc2*, called $Herc2^{530}$, was originally generated by insertion of a gene trap β-galactosidase/neomycin (β-geo-Neo) cassette between exons 2 and 3 of the mouse *Herc2* gene on a C57BL/6J genetic background. Homozygous mice were not viable, dying before day 7.5 of embryonic development. Behavioral analysis of heterozygous mice ($Herc2^{+/530}$) demonstrated impaired motor synchronization with normal neuromuscular function. Morphological analysis showed HERC2 to be present in Purkinje cells and a specific loss of these neurons in the cerebellum of $Herc2^{+/530}$ mice. Moreover, in these heterozygous animals there was a clear increase in autophagosomes and lysosomes. These findings together reveal a crucial role for HERC2 in embryonic development and motor coordination [99].

### 5.3. $Herc2^{+/530}$ and motor performance

Behavioral studies showed that like *tbl* mice, $Herc2^{+/530}$ mice fail to successfully perform the rotarod test. However, the $Herc2^{+/530}$ mutant does not present evident alterations in the NMJ-muscle interactions (Fig. 4) [99]. Impaired motor performance is related to cerebellar Purkinje cell damage, which show signs of autophagic cell death [99] in the cerebellum of the $Herc2^{+/530}$ mutant (Figs. 6 and 7).

All Purkinje cells express HERC2 as demonstrated by its co-

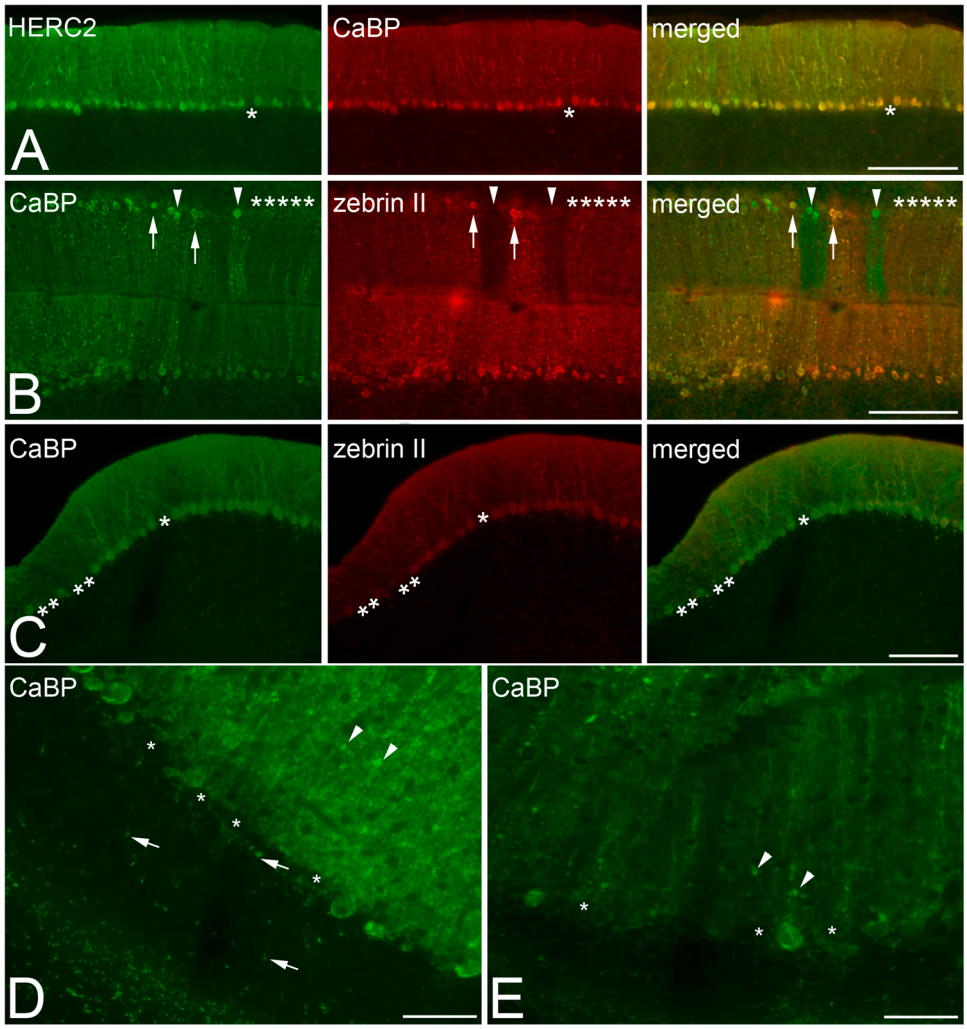


**Fig. 6.** Early cell death of zebrin II Purkinje cells in *Herc2*$^{+/530}$ mutants. Microphotographs of immunohistochemical staining with antibodies against HERC2, calbindin (CaBP) and aldolase C/zebrin II in coronal sections through the vermis (A-B), and paravermis (C-E) [see 99, 112] of the 9-month-old *HERC2*$^{+/530}$ cerebellum. Purkinje cells express HERC2 protein (A) and cell death follows a parasagittal gradient in which the number of dead Purkinje cells increases from the vermis to the paravermal zones (compare asterisks A-C). Signs of Purkinje cell damage like axonal torpedoes (D, arrows) and thick dendritic debris (D-E, arrowheads) were consistently found. The arrows in B indicate calbindin (CaBP) and zebrin II double labeled Purkinje cells, while arrowheads show single CaBP expressing Purkinje cells. Bars = 1000 μm (A-C) and 50 μm (D-E).

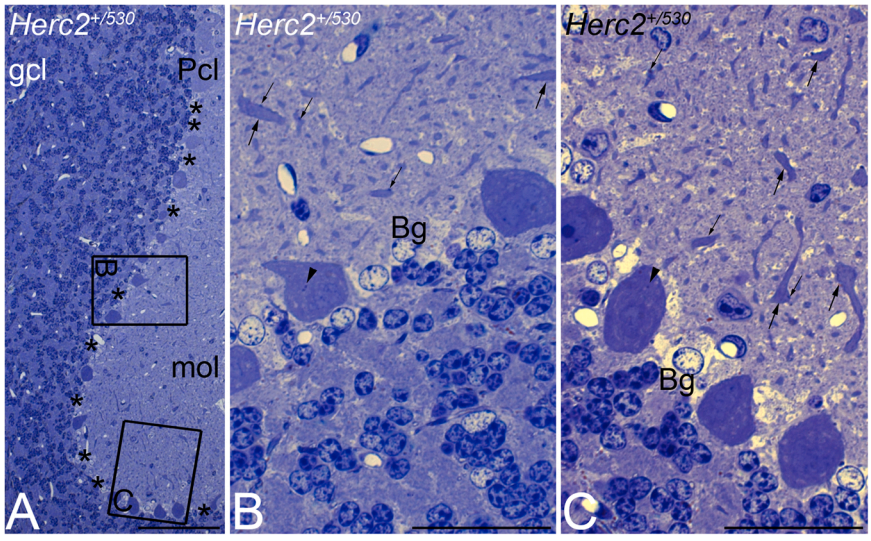


**Fig. 7.** Radial glia also occupied the sites lacking Purkinje cells in the *Herc2*$^{+/530}$ cerebellum. Microphotographs of parasagittal semithin Section (1 μm thick) through the cerebellar cortex of 9-month-old *Herc2*$^{+/530}$ mice [see 99]. A-C. Dead Purkinje cells (A, asterisks) intercalated with healthy ones, with the spaces left by the dead cells occupied by the clear cell nuclei of radial or Bergmann glia (Bg, B-C). Dark granules representative of lysosomes, fill the cytoplasm of Purkinje cells (B-C, arrowheads), while thick dendrites with lysosomes are found in the molecular layer (B-C, arrows): gcl, granule cell layer; mol, molecular layer; Pcl, Purkinje cell layer. Bars = 300 μm (A) and 50 μm (B-C).

expression with the Purkinje cell marker calbindin (Fig. 6A). Like the *tbl* mutant, cell death appeared in parasagittal patches of affected Purkinje cells, subsets of cells that in the *Herc2*$^{+/530}$ mutant seem to express the early heterogeneous Purkinje cell marker zebrin II (reviewed in [112]) (Fig. 6B-C). However, while Purkinje cell death was almost complete in *tbl* mice (Fig. 2), the *Herc2*$^{+/530}$ cerebellum contained a considerable number of healthy Purkinje cells. Indeed, those that die follow a medio-lateral gradient in which the number of Purkinje cells dying

increases from the cortex of the vermis (Fig. 6B) to the paravermis (Fig. 6C) and lobules (see figs. 8 and 9 of [99]).

## 6. Conclusions

Clinical observations and experimental evidence are accruing regarding the key roles that Large HERC ligases play in neurodevelopment [23,26,99]. The *tambaleante Herc1* mutation has profound deleterious effects in several parts of the CNS, impairing learning, memory and motor control [51–54]. Moreover, in the PNS this mutation impairs NMJ transmission and disrupts normal myelination [55,56]. Although the pathways through which this mutation acts are not fully understood, normal neuronal synaptic transmission is weakened in *tbl* mice due to dysregulated autophagy or other effects of the mutated RLD1 domain. Moreover, the neurodevelopmental defects of these mice resemble those reported in human recessive *HERC1* mutations, such as the MDFPMR syndrome [39–43]. Furthermore, *HERC1* mutations are considered to predict ASD [44,45]. *HERC2* mutations have been implicated in several pathologies with neurodevelopmental alterations, and behavioral and mental deficiencies [105–111]. The lethality of the *Herc2* homozygous mouse shows the important role of HERC2 in development, and heterozygous mice display altered motor coordination [99]. These findings help to understand some effects reported in individuals with recessive mutations, such as neurodevelopmental delay and unstable gait [111]. Interestingly, the $Herc2^{+/530}$ mutation has a similar but milder effect on the motor system as the *tbl* mutation [49,99], whose Purkinje cell death seems to be induced by the dysregulation of autophagy.

In conclusion, although detailed experimental studies are now ongoing to define the pathways that each Large HERC protein mutation alters, it is clear that mice carrying mutations in either HERC1 or HERC2 are faithful models to analyze the neurodevelopmental disorders provoked by these.

## Ethics approval

Not applicable.

## Authors' contributions

JAA and JLR wrote the manuscript and provided the final approval of the published version. Figures and tables were elaborated by JAA and JLR. EMP-V, RR, SB and FV revised critically the manuscript.

## Conflicts of interest

Authors declare no competing interests.

## Availability of data and material

Not applicable.

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