Review

Programmed cell death in cerebellar Purkinje neurons

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Apoptosis, autophagy and necrosis are the three main types of programmed cell death. One or more of these types of programmed cell death may take place in neurons leading to their death in various neurodegenerative disorders in humans. Purkinje neurons (PNs) are among the most highly vulnerable population of neurons to cell death in response to intrinsic hereditary diseases or extrinsic toxic, hypoxic, ischemic, and traumatic injury. In this review, we will describe the three main types of programmed cell death, including the molecular mechanisms and the sequence of events in each of them, and thus illustrating the intracellular proteins that mediate and regulate each of these types. Then, we will discuss the role of Ca^{2+} in PN function and increased vulnerability to cell death. Additionally, PN death will be described in animal models, namely lurcher mutant mouse and shaker mutant rat, in order to illustrate the potential therapeutic implications of programmed cell death in PNs by reviewing the previous studies that were carried out to interfere with the programmed cell death in an attempt to rescue PNs from death.

Keywords

Neurodegeneration; Purkinje neurons; Programmed cell death; Apoptosis; Autophagy; Necrosis; Mutations; Necroptosis

1. Introduction

Neurodegenerative disorders result from the progressive loss of particularly susceptible populations of neurons [1]. Neurodegenerative disorders include Parkinson disease (PD), Alzheimer disease (AD), Huntington disease (HD), and ataxias [2, 3]. Neurons die during development, aging and consequent to a number of pathological factors, such as genetic mutations and traumatic injury.

Neuronal death can occur by one or more of three chief programmed death pathways, which are apoptosis, autophagy, and necrosis [4–6]. Apoptosis and autophagy are referred to as programmed cell death type I and type II, respectively [7]. Necrosis had been considered as a non-programmed cell death for a long time. However, necrosis has more recently been included as a type of programmed cell death [7, 8]. Thus, this review will provide an overview on the chief types of programmed cell death and on the organelles involved in these cell death types. Then, the vulnerability of cerebellar PNs to cell death will be discussed. Then, PN death in rodent mutants, namely shaker mutant rat and lurcher mutant mouse and the therapeutic implications of the programmed cell death in PNs will be discussed.

2. Programmed cell death

2.1 Apoptosis

Apoptosis or programmed cell death type I can be triggered by various death stimuli, such as stress [9, 10], activation of death receptors [11], genetic disorders [12], and abnormally increased intracellular calcium concentration ($[Ca^{2+}]_i$) [13–15].

Neuronal apoptosis occurs commonly during development and maturation and appears necessary for shaping and eventual appropriate circuitry formation of the nervous system [16].

Apoptosis is characterized by distinct morphological and biochemical alterations. Morphologically, the cell shrinks, chromatin condenses, nuclear DNA fragments, cell membrane maintains its integrity, and apoptotic bodies containing nuclear material are formed. Apoptotic bodies are ultimately engulfed by phagocytes without eliciting any inflammation [17]. Biochemically, protein degradation and caspase activation are augmented characterizing apoptosis [18], which is regulated by the B-cell lymphoma 2 (Bcl-2) family proteins that comprise both anti- and pro-apoptotic proteins that should be maintained in balance, because otherwise apoptosis can be either promoted or inhibited [19, 20].

Caspases are a family of cysteine proteases, which are present in normal cells as inactive zymogens that become enzymatically active only in response to apoptotic stimuli. Active caspases are large and small fragments that result from the cleavage of the single peptide precursor, and they mediate apoptosis leading to the distinctive morphological changes [21]. According to their function, caspases can be classified as inflammatory caspases and apoptotic caspases. *Inflammatory caspases are caspases-1, -4, -5, -11, -13 and -14,* which mediate the proteolytic activation of inflammatory cytokines [22]. Apoptotic caspases can be either initiator or executioner caspases [21]. *Apoptotic initiator caspases are caspases-2, -8, -9 and -10,* that have a caspase activation and recruitment domain such as caspase -2 and -9 or a death effector domain such as caspase -8 and -10. Initiator caspases can initiate apoptosis by cleaving themselves leading to their activation and subsequent cleavage of the common downstream executioner caspases resulting in their activation [21]. *Executioner caspases-3, -6 and -7,* have short prodomains and lack the ability of auto-cleavage, and consequently need to be cleaved and activated by initia-

tor caspases. Executioner caspases perform the downstream execution steps of apoptosis by cleaving multiple cellular substrates [21].

Caspases have 5 cumulative effects during apoptotic events. First, caspases can disable processes involved in homeostasis and repair, such as DNA repair processes, to prevent counterproductive events from occurring simultaneously [23]. Second, caspases can stop cell cycle progression [24]. Third, caspases can cause signal amplification and inhibitor inactivation by cleaving pro- and anti-apoptotic proteins [25]. Fourth, caspases can mediate nuclear and cytoskeletal disassembly and morphological changes [26]. Finally, caspases are involved in the recognition of dying cells for their phagocytosis and subsequent clearance [26]. Caspases cleave Ca^{2+}-AMPA glutamate receptors, and subsequently inhibit Ca^{2+} build up and overload in a neuron that would lead to excitotoxicity and subsequent necrosis [27]. Caspases have also been reported to cleave and inactivate the plasma membrane Ca^{2+} pump (PMCA) in neurons and non-neuronal cells undergoing apoptosis [28], leading to Ca^{2+} build up and subsequent Ca^{2+} overload, which may trigger Ca^{2+}-dependent death pathways such as necrosis, even when Ca^{2+} signals are not the initial death trigger. These studies have also shown that necrosis can be reduced or prevented by caspase inhibitors in brain ischemia due to caspase-mediated cleavage and inactivation of PMCAs [28]. On the other hand, inhibition of caspase activation, in the presence of cell death stimuli, can expose or even augment underlying caspase-independent death programs [29].

Caspases may also have non-apoptotic functions, such as synaptic plasticity [30, 31], and proliferation and differentiation of non-neuronal cells [32]. For instance, apoptotic-like morphological changes have been demonstrated in differentiating cells, and T-cell proliferation has been shown to be prevented by caspase inhibitors [33].

Caspase activation can be initiated by two well described apoptotic pathways (Fig. 1): the mitochondria-mediated pathway and the cell surface death receptor pathway [17]. The mitochondria-mediated apoptotic death pathway is also called the intrinsic apoptotic pathway. Permeability transition pore (PT-pore) and Bcl-2 family are important regulators of the mitochondria-mediated death pathway [34, 35]. Bcl-2 is a family of proteins that can be either pro-apoptotic, such as Bcl-2-associated X protein (Bax), or anti-apoptotic, such as Bcl-2 [36]. Members of Bcl-2 family exist on the cytoplasmic surface of different organelles, such as mitochondria, endoplasmic reticulum, and the nucleus [37]. Members of the Bcl-2 family act as regulators of the permeability transition pore (PT-pore) [38]. When the PT-pore at the mitochondrial intermembraneous contact spots is opened, small molecular weight substances, such as charged ions, are lost from the matrix causing mitochondrial depolarization and rupture of the outer mitochondrial membrane due to osmotic mitochondrial swelling [38]. The pro-apoptotic Bcl-2 family proteins regulate outer mitochondrial membrane permeabilization, causing irreversible release of apoptotic molecules, such as cytochrome c, which might be the primary regulatory step for mitochondria-mediated caspase activation [39]. Cytochrome c normally exists entirely in the intermembraneous space of mitochondria [40]. A cytosolic protein called aoptotic protease activating factor 1 (Apaf-1) binds to cytochrome c in the cytosol, in the presence of ATP [41]. Consequently, a multimeric Apaf-1/cytochrome c complex is formed, and it subsequently recruits procaspase-9, which becomes activated by proteolysis [42]. Subsequently, activated caspase-9 is released from this complex to cleave and activate executioner caspases, -3, -6 and/or -7. Cell commitment to apoptosis cannot be caused by occasional leakage of cytochrome c, due to a relatively high threshold of caspase activation set by the formation of a multimeric Apaf-1/cytochrome c complex [42].

The cell surface death receptor-mediated apoptotic pathway is also called the extrinsic apoptotic pathway. Cell surface death receptors are transmembrane proteins that form a family belonging to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily. These death receptors are activated by structurally linked ligands that belong to the TNF gene superfamily [43, 44]. Cell surface death receptor-mediated caspase activation starts by trimerization of ligand-induced receptor [43, 44]. As a consequence of ligand-death receptor binding, particular intracellular proteins that are associated with the death receptor, such as procaspase-8, are recruited. After its recruitment, procaspase-8 is immediately activated through proteolysis. Then the active form of caspase-8 cleaves and activates downstream executioner caspases [43–45].

Apoptosis is morphologically characterized by chromatin condensation, DNA fragmentation, nuclear breakup [4, 46], membrane blebbing [47], cell shrinkage [46, 48], and formation of apoptotic bodies [49]. Apoptotic bodies are membrane-bound fragments of the dying cells that are eventually cleared by phagocytosis without any associated inflammation [50].

The principal molecular components of apoptosis in neurons are the same as those in other non-neuronal cell types [51]. And the occurrence of apoptosis has been reported in many neurodegenerative diseases [51].

Autophagy and necrosis are other cell death types that have been suggested to contribute to the pathogenesis of neurodegenerative diseases in addition to apoptosis.

2.2 Autophagy

Autophagy, also called type II programmed cell death, is self-destruction of the cell [52]. Autophagy occurs normally at low basal levels as the degradative mechanism for removal of long-lived proteins and organelles [53]. Autophagy occurs in neurons in physiological processes, such as regulation of metabolism through removal of particular enzymes, morphogenesis, and tissue remodeling due to differentiation [54, 55]. Unlike apoptosis, autophagy can promote either survival or death of the cell [55]. Cells that undergo excessive

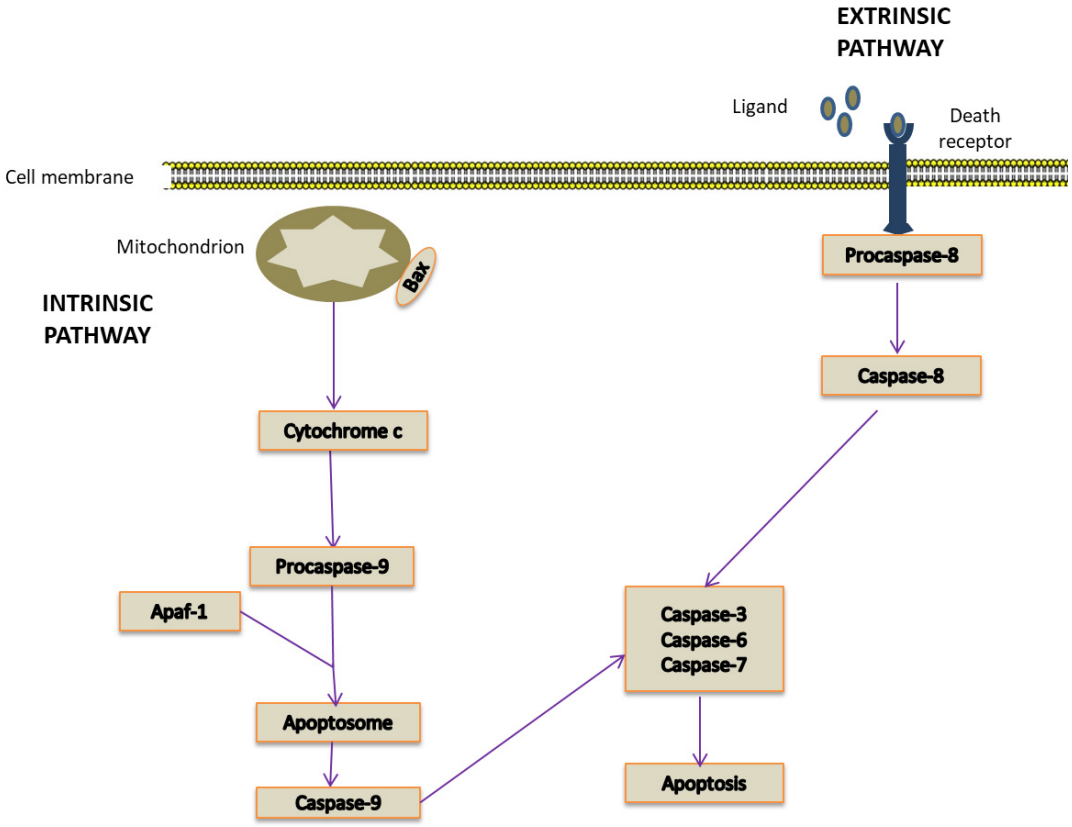

Fig. 1. Pathways of apoptosis. The intrinsic apoptotic pathway is triggered when apoptotic stimuli stimulate proapoptotic proteins, such as Bax, to induce the permeabilization of the outer mitochondrial membrane, and the subsequent discharge of cytochrome c from the mitochondrial intermembranous space. Cytochrome c then participates in the formation of a multimeric Apaf-1/cytochrome c complex, which then forms the apoptosome by recruiting procaspase-9 in order to activate it. Once activated, caspase-9 is dissociated from this complex, and it activates executioner caspases-3, -6, and/or -7. On the other hand, extrinsic apoptotic pathway starts by trimerization of ligand-induced receptor subsequrnt to the binding of particular death ligands to death receptors. Subsequently, particular intracellular proteins that are associated with the death receptor, such as procaspase-8, are recruited. After its recruitment, procaspase-8 is immediately activated through proteolysis. Then the active caspase-9 and active caspase-8 cleave and activate downstream executioner caspases-3, 6, and/or 7 that carry out the proteolytic events of cellular proteins and structures ultimately leading to apoptosis.

autophagy, where short-lived organelles and proteins are degraded, are caused to die in a non-apoptotic manner, which is independent of caspases [55].

Based on the mechanism of autophagic vacuole formation and material transport to lysosomes, autophagy can be classified into 3 types: macroautophagy, chaperon-mediated autophagy (CMA), and microautophagy [56].

Macroautophagy is the classic autophagy, which is stimulated by stress conditions, such as nutrient deprivation, infections and toxins [57]. Intracellular stress situations, including aggregation of misfolded proteins and accumulation of damaged organelles can also induce macroautophagy [58]. Macroautophagy is characterized morphologically by the formation of double membrane-bounded vacuoles, called autophagosomes [59]. The origin of the autophagosome membrane is unknown, but it is suggested to derive from the endoplasmic reticulum, trans Golgi, and specialized membrane cisternae, called phagophores [60].

Chaperon-mediated autophagy does not require vesicle formation [61]. It is selective for a specific group of cytosolic proteins containing a pentapeptide motif [62]. Chaperon heat shock cognate 70 (Hsc70) transfers protein substrates, after recognizing that motif, to the lysosomal membrane. After binding to the lysosome-associated membrane protein 2a (lamp2a), Hsc70 translocates those proteins into the lysosomal lumen, where they are degraded by lysosomal hydrolases [62–64].

Microautophagy is the direct uptake of soluble proteins and organelles by lysosomes without autophagosome formation. The lysosomal membrane itself invaginates to engulf and subsequently degrade the cytoplasmic component [65]. These lysosomal invaginations can be observed in the electron microscope [66]. The exact physiological function of microautophagy is still to be elucidated. However, microautophagy has been suggested to play a prominent role in maintaining membrane homeostasis, by removing the outer autophagosome membrane following autophagosome-lysosome fusion in macroautophagy [65, 66].

Macroautophagy proceeds through 4 stages, which are: induction and cargo selection, vesicle formation, vesicle

docking and fusion with lysosomes, and finally vesicle breakdown and recycling [56]. For the induction stage, a serine/threonine protein kinase (mTOR) acts as an important regulator of autophagy [67]. Under nutrition-rich conditions, phosphorylated mTOR negatively controls autophagy, primarily by acting on the signaling cascade that controls general translation and transicription. Under starvation conditions, mTOR is dephosphorylated and consequently inactivated leading to disinhibition of autophagy [68, 69]. Another major upstream signaling pathway that regulates starvation-induced autophagy is phosphoinositide 3-kinase (PI3K) [70]. Beclin 1 exists with class III PI3K in a complex producing one product, phosphatidylinositol 3-phosphate (PtdIns(3)P) [71]. Autophagy is stimulated by the administration of PtdIns(3)P, while it is inhibited by inhibition of class III PI3K [72, 73]. Once induced, the autophagic process begins with cargo selection. Cargo selection is the selection of the cytoplasmic part of the cell to be degraded by the autophagic process [56].

In the vesicle formation stage, a membrane sac isolation membrane or phagophore wraps around the degradation substrates and ultimately fuses to become an autophagosome or autophagic vacuole [74]. Most of the proteins involved in vesicle expansion and maturation dissociate from the mature autophagosome. Microtubule-associated protein light chain 3 (LC3) recruited on the surface of autophagosomes remains on the autophagosomal membrane. It has been shown that 2 ubiquitin-like conjugation systems are required for autophagosome formation [75, 76]. Once the autophagosome is formed, its outer membrane completely fuses with the outer lysosomal membrane making a path for the autophagic body, which is the inner membrane-bound autophagic vacuole [77]. Thus, the autophagic body is released into the lysosomal cavity [78]. Then, contents of the autophagosome are degraded by the hydrolytic enzymes in the acidic lysosomal environment. Eventually, essential cytoplasmic contents are recycled [77, 79].

Autophagy can be most reliably detected using the transmission electron microscope that illustrates the autophagic vacuoles and multilamellar whorls as the principal ultrastructural features of autophagy [80]. Multilamellar whorls form as a consequence of the compaction of engulfed organelle membranes, which typically occurs at the autolysosomal stage [81].

Disaggregated protein accumulation due to autophagic dysfunction contributes to the pathogenesis of neurodegenerative diseases such as Parkinson disease, Alzheimer disease, Huntington disease and Niemann-Pick type C disease [54, 82–84]. Niemann-Pick type C disease is a lysosomal storage disease, produced by nPN1 or nPN2 gene mutations, where neurons display characteristics of autophagic cell death [84]. Initiation of autophagy, including the induction of the transcriptional level of the autophagic genes, may use apoptotic mechanisms for the execution of cell death. For example, autophagy related 5 (Atg5) is a protein, which is the main regulator of the sequestration stage in autophagy, seems to be an important mediator of apoptosis [85, 86]. Atg5 triggers cytochrome c discharge from the mitochondrial intermembraneous space promoting mitochondria-mediated apoptosis [87]. The anti-apoptotic protein Bcl-2 inhibits Beclin-1-mediated autophagic cell death by binding to Beclin1/Atg6 [88]. On the other hand, caspase inhibition prevented cell death but did not affect vacuole formation [89].

2.3 Necrosis

Necrosis was initially called “non-lysosomal vesiculate”, since it is characterized by the destruction of the cytoplasm by non-lysosomal degradation [90]. Recently, necrosis has been recognized as a third type of programmed cell death [90]. Necroptosis is the most deeply characterized form of necrotic programmed cell death, which was first recognized as a cell death backup to apoptosis when apoptosis was inhibited by endogenous or exogenous factors. It is induced by interleukin-1β (IL-1β), TNF, particular viral infections and other factors [91]. Additionally, necroptosis is mediated by receptor-interacting serine/threonine kinase RIPK 1 to RIPK3, which assemble subsequent to the engagement of the death receptor pathway in the absence of caspase 8 forming an RIPK1/RIPK3 complex, which is called the Necrosome that is essential for activating the necroptotic pathway [92] (Fig. 2, Ref. [93, 94]). Subsequently, RIPK1 and RIPK3 undergo auto- and trans-phosphorylation, and thus permitting recruitment and activation of the pseudokinase, and eventually leading to the disruption of plasma membrane integrity, and its subsequent rupture [93]. Accordingly, endogenous molecules, which are referred to as damage-associated molecular patterns (DAMPs), are relaesed [94]. DAMPs, which include IL-1 family members, activate inflammasome, trigger inflammation and elicit immune responses [94].

Necroptosis has been shown to mediate a variety of human diseases, such as ischemic brain injury, systemic inflammation, ischemic reperfusion injury and neurodegenerative diseases [95].

Morphological features of necrosis include disruption of the plasma membrane early during the death process, resulting in leakage of cellular contents and subsequent inflammation, which involves microglia activation acquiring increased motility and phagocytic activity [96]. Other morphological features include enlargement of cytoplasmic organelles and detachment of ribosomes from the endoplasmic reticulum and fragmentation of the nuclear membrane [96].

2.4 Organelles involved in programmed cell death

In order to understand the interaction between the various types of cell death, this section reviews the organelles participating in different types of programmed cell death. Mitochondria, lysosomes, and endoplasmic reticulum play a significant role in the discharge and activation of death factors, as summarized in Table 1 (Ref. [77–79, 97–108]). Death factors are factors signaling and mediating death pathways such as cathepsins, calpains, and other proteases.

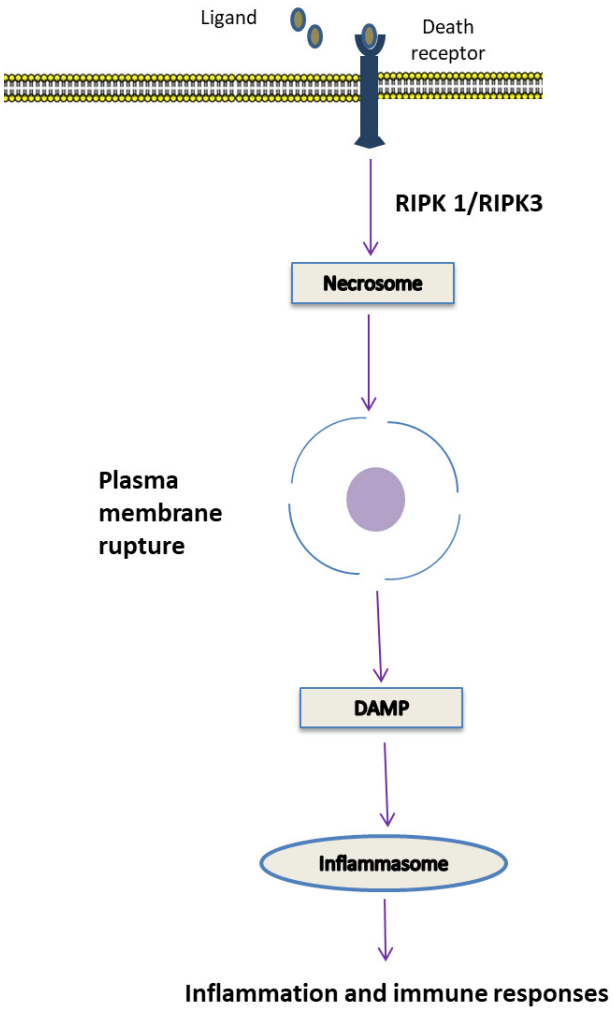

Fig. 2. Sequence of events in necroptosis. Necroptosis is mediated by receptor-interacting serine/threonine kinase RIPK 1 to RIPK3, which assemble subsequent to the engagement of the death receptor pathway in the absence of caspase 8 forming an RIPK1/RIPK3 complex, which is called the Necrosome that is essential for activating the necroptotic pathway that eventually leads to the disruption of plasma membrane integrity, and its subsequent rupture. Accordingly, endogenous molecules, which are referred to as damage-associated molecular patterns (DAMPs), are released [93, 94]. DAMPs, which include IL-1 family members, activate inflammasome, trigger inflammation and elicit immune responses.

2.4.1 Mitochondria

Increased permeability (permeabilization) of the outer mitochondrial membrane triggers the release of toxic proteins from the mitochondrial intermembrane space, such as cytochrome c [97]. Permeabilization of the outer mitochondrial membrane can occur by 2 mechanisms. Firstly, the outer mitochondrial membrane can rupture due to opening of a permeability transition pore at the communication areas between the inner and outer mitochondrial membranes [98]. Secondly, permeabilization of the outer mitochondrial membrane can be caused by activation of proapoptotic Bcl-2 proteins, including Bax and Bak. Proapoptotic Bcl-2 proteins are normally controlled by anti-apoptotic Bcl-2 proteins, which can prevent the permeabilization of the outer mitochondrial membrane by heterodimerization with Bax-like proteins [109].

Cytochrome c is a vital constituent of the respiratory chain that normally exists in the mitochondrial intermembranous space [110]. The inhibitor of apoptosis protein family (IAP), which inhibits apoptotic pathways, controls the catalytic function of cytochrome c [111]. When released into the cytosol, cytochrome c participates in the formation of apoptosome which triggers the apoptotic cascade as reviewed earlier [112].

Apoptosis-inducing factor (AIF) normally performs oxidoreductase function in the mitochondrial intermembranous space [113]. When released in the cytosol, AIF performs apoptotic functions, which can be dependent or independent of caspases [114]. Apoptosis-inducing factor release from the intermembranous space can be triggered by lysosomal protease cathepsin D [115]. Once outside the intermembranous space, cytosolic AIF translocates to the nuclei and stimulates chromatin condensation independently of caspase cascade [116]. On the other hand, AIF release can be triggered downstream of cytochrome c due to particular proapoptotic stimuli, probably dependent on caspase cascade [117]. Additionally, Mitochondria-derived reactive oxygen species (ROS) reinforces the stability of the necrosome [99]. Additionally, mitochondria serve as membrane source of autophagosomes in starvation-induced autophagy [100]. Furthermore, depolarization of mitochondria can induce autophagy [101].

2.4.2 Endoplasmic reticulum

The endoplasmic reticulum (ER), which is the stress sensor of the cell, performs an important function in alleviating cellular stress, due to its ability to synthesize proteins [102]. Misfolded and aggregated proteins, cytosolic Ca^{2+}overload or Ca^{2+} depletion from the ER lumen can stress ER function [102]. Endoplasmic reticulum stress can initiate programmed cell death by selective activation of caspase-12, which is normally present at the cytosolic face of the ER in its inactive state [103]. Members of Bcl-2 family are present on mitochondria and ER and thereby can mediate an interaction between the two organelles [118]. As a result of this interaction, ER stress can induce mitochondrial membrane permeabilization, which is the rate-limiting step in the mitochondria-mediated apoptosis [104]. ER stress can also induce necroptosis [105]. Additionally, endoplasmic reticulum–Golgi intermediate compartment (ERGIC) can contribute membrane to the forming phagophore [106].

2.4.3 Lysosomes

The last organelles involved in programmed cell death are lysosomes containing different proteases, such as cathepsin B, L, and D [119]. Cathepsin B and D are most steady at physiological cytoplasmic pH, and appear to have a noticeable role in apoptotic and necrotic programmed cell death

[119]. Oxidative stress, TNF-α and chemotherapeutic drugs can activate lysosomal proteases leading to cell death [120]. Lysosomal proteases trigger programmed cell death via multiple pathways [121]. Partial and selective permeabilization of the lysosomal membrane triggers apoptotic-like programmed cell death, whereas massive breakdown of lysosomes induces unregulated necrosis [122]. Lysosomal proteases can cause cell death directly by cleaving and activating caspases [107]. Lysosomal proteases can also endorse cell death indirectly by eliciting mitochondrial dysfunction and successive discharge of mitochondrial proteins [104]. The discharge of lysosomal cathepsins and consequent cell death can be induced by activated calpains [123]. Calpains, which are a family of Ca^{2+}-activated neutral cytosolic proteases that normally reside in the cytosol as inactive zymogens, have been reported to perform downstream of caspase activation [124]. Furthermore, lysosomal release of cathepsin D into cytoplasm can stimulate necroptosis, probably by its contribution to the cleavage of caspase-8 [108].

And as mentioned formerly, lysosomes fuse with autophagosomes, so that the the autophagic body is released into the lysosomal cavity [77, 78]. Then, contents of the autophagosome are degraded by the hydrolytic enzymes in the acidic lysosomal environment [79].

In summary, the literature reviewed above strongly suggests that organelles involved in programmed cell death interact with each other. As a consequence, one can hypothesize that more than one type of programmed cell death may occur in the same dying cell.

3. Cerebellar PNs are among the most vulnerable neurons to cell death

Purkinje neurons are the sole output neurons of the cerebellar cortex, and they are involved in coordination of voluntary movements [125]. Death of PNs results in ataxia [126]. Purkinje neurons are the most highly susceptible population of neurons to intrinsic hereditary diseases [127] or extrinsic toxic [128], hypoxic [129], ischemic [130], and traumatic injury [131]. In addition, numerous genetic mutations directly affect PN survival and cause PN degeneration [126].

The role that Ca^{2+} plays in PN function and the afferents that project to PNs might be the reason why PNs are among the most vulnerable neurons to cell death [132]. Purkinje neurons receive excitatory input from two afferent fiber types, namely climbing fibers and parallel fibers [133]. Each climbing fiber makes hundreds of synaptic connections on the proximal dendrites of a single PN, resulting in a very powerful excitatory input and subsequent dramatic increase in $[Ca^{2+}]_i$ [134]. On the other hand, parallel fibers arise from granule cells within the cerebellum, which receive the excitatory mossy fibers from different parts of the central nervous system [135]. Each PN receives input from about 175,000 parallel fibers synapsing on the distal dendritic branches of that PN [136].

Intracellular Ca^{2+} concentrations can be divided between the cytosolic compartment and Ca^{2+} storage sites such as the endoplasmic reticulum (ER) [137]. Resting $[Ca^{2+}]_i$ is low [138]. However, there is very rapid increase in PN $[Ca^{2+}]_i$ due to its strong stimulation by the excitatory inputs of parallel and climbing fibers afferents resulting in complex patterns of Ca^{2+} dynamics in PN dendrites, in which metabotropic glutamate receptor signaling pathway mediates Ca^{2+} influx through voltage-gated Ca^{2+} channels and Ca^{2+} release from intracellular stores [139, 140]. On the other hand, Ca^{2+} ions are pumped out of the cytosol by plasma membrane calcium ATPase2 (PMCA2), which is abundantly expressed in PN compared to other neurons [141]. PMCA2 uses energy in order to maintain a relatively low intracellular net Ca^{2+} load [141]. Reduced calcium clearance perturbs calcium dynamics in PN dendrites and disrupts the relay of information from PN to other neurons leading to neural dysfunction [141]. Indeed, partial reductions in PMCA2 levels have been shown to perturb the function and integrity of PNs and the overall outcome on PN survival [142].

Furthermore, Ca^{2+}-binding proteins, which buffer the Ca^{2+} in the cell by binding to it, also participate in maintaining $[Ca^{2+}]_i$ that are essential for normal Ca^{2+} signaling, and thus preventing the accumulation of pathologic quantities of Ca^{2+} [138, 143–145].

Although PNs are highly susceptible to cell death, not all PNs respond similarly to death stimuli. For example, only specific PNs that are zebrin 2- and excitatory amino-acid transporter 4 (EAAT4)-negative are particularly sensitive to hypoxic stress caused by cerebral ischemia [146]. Additionally, while some PNs die in response to a death stimulus; other PNs survive indicating the presence of heterogeneous PN populations.

4. PN death in neurodegenerative diseases in animal mutants

Mechanisms underlying PN neurodegeneration is better investigated in animal model studies, because autopsy delay of post-mortem cerebella leads to artifacts and shows end-stage disease only. Neurodegenerative diseases affect the cerebellum in many species, in which many single mutations totally affect different genes. These animal mutants have demonstrated distinctive age of onset of PN degeneration, in addition to distinctive temporal and spatial patterns of PN degeneration. Such animal models have been used to illustrate the type(s) of programmed cell death causing PN loss and the pathogenesis of the particular neurodegenerative disease (Table 2, Ref. [147–152]). Additionally, those animal models allow targeted studies of different pathological mechanisms underlying PN death. Thus, PN death in lurcher mutant mice and shaker mutant rats, as examples of animal mutations that lead to PN death, will be reviewed in this section.

4.1 Lurcher mutant mouse

Lurcher is an autosomal semidominant mutation. Homozygous lurcher mice die shortly after birth due to a massive

loss of mid- and hindbrain neurons during late embryogenesis. In heterozygous lurcher mutant mice, starting during the second postnatal week, PNs degenerate in a rapid and extensive manner, so that 90% of PNs have already degenerated by 26 postnatal days of age [153]. No PNs survive at 90 postnatal days of age [154]. PNs have been suggested to die by an excitotoxic mechanism, since the lurcher mutation is a gain of function mutation that transforms the glutamate receptor delta2 (GluRδ2) subunit into a constitutively open channel that maintains PNs at a depolarized resting membrane potential [153]. In addition to the cell autonomous PN death, target-related cell death occurs in 90% of granule cells and 75% of inferior olivary neurons in the lurcher mutant mouse [155]. Both apoptosis and autophagy have been reported in lurcher mutant PNs. The GluR$\delta 2^{Lc}$ mutation, naturally occurring postnatal cell death, and the physical trauma of making organotypic slice cultures have been shown to induce the multiple PN death pathways [156].

Morphological abnormalities indicative of apoptosis including the appearance of perinuclear clumps of chromatin, axonal swellings (torpedoes) and a delayed maturation process, have been observed in lurcher PNs at 8 postnatal days of age [147]. Delayed maturation process of the lurcher mutant PNs was revealed by the incomplete development of the basal polysomal mass of PN bodies and the late development of proximal and distal compartments of the dendritic trees, which were hyperspinous [157]. Swollen mitochondria with dilated cristae, reported in some models of apoptosis [158], were also observed in dying lurcher PNs [157]. Norman *et al.* [159] demonstrated markedly condensed nuclear chromatin, and nuclear and cell membrane blebbing in dying lurcher PNs at 12 postnatal days of age. The presence of deoxyribonucleic acid (DNA) fragmentation, detected by TUNEL, active caspase-3 expression, and increased Bax expression have been reported in lurcher mice, suggesting that PNs die by apoptosis [148, 149].

Autophagy was also reported in lurcher PNs when autophagic macromolecular complexes were observed to be formed by interactions between GluRδ2 and Beclin1 via the neuronal isoform of protein-interacting specifically with TC10 (nPIST) [150]. Beclin1 is the human ortholog of the yeast autophagic gene Atg6 [160]. Autophagy was introduced in cells transfected with the lurcher mutant glutamate receptor (GluR$\delta 2^{Lc}$) but not wild type GluRδ2, and that the death of those transfected cells could be partially rescued pharmacologically by inhibiting autophagy with 3-methyladenine. The occurrence of moderate autophagy has, indeed, been suggested by demonstrating autophagosomes in the electron micrographs of degenerating PNs in the lurcher mutant mouse [150].

Furthermore, an attempt to rescue lurcher PNs from death was by the genetic elimination of tissue plasminogen activator (tPA) [161], which is a serine protease member of the fibrinolytic system that mediates excitotoxic neuronal cell death. Lu and Tsirka (2002) crossed lurcher mutant mice with tPA-null mutant mice to eliminate tPA in lurcher mutant mice (Lc/+; tPA–/–) [161]. Lurcher homozygotes lacking tPA (Lc/Lc; tPA–/–) died shortly after birth. At 12 and 30 postnatal days of age, significantly lower numbers of surviving PNs in both lurcher mutant mice (Lc/+; tPA+/+) and tPA-null lurcher double mutants (Lc/+; tPA–/–) compared with the wild-type mice were reported [161]. However, PN death was clearly attenuated in tPA-null lurcher double mutants (Lc/+; tPA–/–) compared with the lurcher mutants (Lc/+; tPA+/+) [161]. Additionally, the tissue plasminogen activator elimination resulted in a decrease in active caspase-8 expression but had no effect on active caspase-9 expression at 12 and 30 postnatal days of age [161].

Moreover, previous studies critically tested the role of various cell death pathways in lurcher mutant PN degeneration with respect to evidence for the molecular heterogeneity of Purkinje cells [162]. The expression of putative survival factors, such as heat shock proteins, in a subset of cerebellar PNs was proposed to influence cell death pathways and attribute to the pattern and diverse mechanisms of lurcher mutant PN degeneration [162].

4.2 Shaker mutant rat

Shaker mutant rats have an x-linked recessive mutation [163], in which missense change is identified in ATPase plasma membrane Ca^{2+} transporting 3 (Atp2b3) gene, which encodes PMCA3 that is highly expressed in the cerebellum [164]. However, the resultant PMCA3 variant has been shown in the *in vitro* analysis not to have any functional effects, suggesting that this genetic change probably remains not causative, but very closely associated with the causative shaker mutation [165]. The shaker mutation naturally results in the degeneration of spatially limited populations of cerebellar PNs at an interval of seven to fourteen weeks of postnatal age [163]. Nevertheless, PN degeneration was reported to occur at earlier [166] or later ages [167] in response to experimental conditions. Purkinje neuron degeneration results in gait ataxia and whole body tremor, which develop concomitantly with PN degeneration [163]. At risk PNs always degenerate and are located in two cortical areas that are known as anterior (ADC) and posterior (PDC) degeneration compartments [163]. The ADC comprises lobules I-VIb and d, whereas the PDC comprises lobules VIIb-VIII and IXa-c. At risk PNs in the PDC normally degenerate 1–2 weeks later and slower compared to the ADC at risk PNs [163]. On the other hand, secure PNs usually survive, and they exist outside the ADC and PDC forming an intermediate (ISC) and a flocculonodular (FNSC) survival compartments [163]. The ISC comprises lobules VIc-VIIa, while the FNSC comprises lobules X and IXd [163]. Shaker mutant PNs have been shown to die by multiple death pathways including apoptosis and autophagy [151, 152].

Table 1. Roles of mitochondria, endoplasmic reticulum and lysosomes in apoptosis and autophagy.

	Mitochodria	References	Endoplasmic reticulum	References	Lysosomes	References
Apoptosis	Increased permeabilization of the outer mitochondrial membrane triggers the release of toxic proteins from the mitochondrial intermembrane space, such as cytochrome c	[97, 98]	cytosolic Ca^{2+} overload or Ca^{2+} depletion from the ER lumen can stress ER function, leading to caspase-12 activation and mitochondrial membrane permeabilization	[102–104]	Partial and selective permeabilization of the lysosomal membrane leading to release of lysosomal proteases that can cause cell death directly by cleaving and activating caspases or indirectly by eliciting mitochondrial dysfunction and successive discharge of mitochondrial proteins	[104, 107]
Autophagy	Serve as membrane source of autophagosomes in starvation-induced autophagy	[100]	Endoplasmic reticulum–Golgi intermediate compartment (ERGIC) can contribute membrane to the forming phagophore	[106]	Lysosomes fuse with autophagosomes, so that the the autophagic body is released into the lysosomal cavity	[77–79]
	Depolarization of mitochondria can induce autophagy	[101]			Then, contents of the autophagosome are degraded by the hydrolytic enzymes in the acidic lysosomal environment	
Necroptosis	Mitochondria-derived reactive oxygen species (ROS) reinforces the stability of the necrosome	[99]	ER stress can induce necroptosis	[105]	Lysosomal release of cathepsin D into cytoplasm induces necroptosis, probably by cleaving caspase-8	[108]

Table 2. Experimental evidence for the occurrence of apoptosis and autophagy in lurcher mutant rat and shaker mutant rat.

	Lurcher mutant mouse	Reference	Shaker mutant rat	Reference
Apoptosis	Morphological features of apoptosis in the electron micrographs of degenerating PNs	[147]	Morphological features of apoptosis in the electron micrographs of degenerating PNs	[151, 152]
	TUNEL labeling in PN	[148]	TUNEL labeling in PN	[152]
	Active caspase-3 expression in PN	[148]	Active caspase-3 expression in PN	[152]
	Increased Bax expression in PN	[149]		
Autophagy	Autophagic macromolecular complexes were formed by interactions between GluRδ2 and Beclin1 via nPIST	[150]	Morphological features of autophagy in the electron micrographs of degenerating PNs	[151]
	Morphological features of autophagy in the electron micrographs of degenerating PNs	[150]		

Previous studies sought to identify epigenetic factors that may modify the events leading to hereditary PN degeneration in the shaker mutant rat. These studies included chronic infusion of neurotrophic factors [167] and inferior olive chemoablation [166].

Trophic factors play critical roles for neuronal survival developmentally, post-developmentally, and therapeutically [168]. Trophic factors were used in an attempt to protect at risk PNs against cell death in the shaker mutant rat [167].

Exogenous glial derived neurotrophic factor (GDNF) and/or insulin-like growth factor 1 (IGF-1) were chronically infused intraventricularly by osmotic pumps to the 5 week 3 day old shaker mutant rat cerebellum [167]. Four weeks of continued infusion of GDNF or IGF-1 delayed the degeneration of many at risk PNs in the ADC [167]. Unexpectedly, the number and spatial distribution of surviving PNs were reported [167] to be comparable to that found in age-matched non-saline and saline-infused controls 2 weeks and 4 weeks after stopping GDNF or IGF-I infusion. Eight weeks of continued trophic factor infusion did not support the continued survival of most PNs in the ADC (165). In addition, four weeks of continued infusion of both GDNF and IGF-1 delayed the degeneration of many more PNs than with either neurotrophic factor alone, suggesting that GDNF and IGF-1 acted on disparate mutant PN populations, whose differential survival influences various aspects of locomotion [167]. Thus, trophic factors were proposed to be only partially and transiently neuroprotective for at risk PNs.

Climbing fibers, which originate from the inferior olivary neurons of medulla oblongata, dynamically control PN structure and function [169]. Climbing fiber deafferentation resulting from IO chemoablation causes temporal acceleration of the hereditary degeneration of at risk PNs in shaker mutant rats [166]. Temporally accelerated PN death caused by IO chemoablation in shaker mutant rats could not be blocked by chronic infusion of GDNF and IGF-1 prior to IO chemoablation suggesting that olivocerebellar deafferentation might have led to an overexcitation of parallel fibers due to the removal of inhibition by the climbing fibers [170].

Morphological evidence was provided for the occurrence of apoptosis and autophagy in at risk PNs during the natural phenotypic expression of the shaker mutation [151]. Additionally, active caspase-3 immunoreactivity and DNA fragmentation, which was detected by TUNEL labeling, were shown in at risk PNs, suggesting an evidence for active caspase-3-mediated apoptosis in at risk PNs during the ordinary phenotypic expression of the shaker mutation [152]. Furthermore, active caspase-3 expression was augmented in at risk PNs following IO chemoablation [171].

5. Therapeutic implications

Since the end-point of apoptosis, autophagy, and/or necrosis in PN is their death, therapeutic approaches that target the molecular and biochemical events of the ongoing one or more type of programmed cell death in PNs may protect against their loss.

Experiments have attempted to genetically rescue lurcher mutant PNs from death using transgenic mice overexpressing anti-apoptotic Bcl-2 protein [172], lacking pro-apoptotic Bax protein [173], or lacking tissue plasminogen activator (tPA) (Table 3, Ref. [161, 166, 167, 170, 172–175]) [161]. Zanjani *et al.* [172] bred lurcher mutant mice with human Bcl-2 transgenic mice, to produce lurcher mutant mice overexpressing Bcl-2 (+/Lc-Hu-Bcl-2), which is a key regulator of apoptosis. Zanjani *et al.* [172] used lurcher (+/Lc) mutant mice as controls. At 2 postnatal months of age, substantial numbers of PNs were reported to survive in the lurcher mutant overexpressing human Bcl-2 (+/Lc-Hu-Bcl-2), whereas virtually all PNs had degenerated in the control (+/Lc). Surviving PNs overexpressing Bcl-2 were distributed throughout all lobules and the different parasagittal planes of the cerebellum. However, by 5–6 postnatal months of age, only very few surviving PNs were reported and the number of surviving PNs in +/Lc-Hu-Bcl-2 was comparable to that observed in controls (+/Lc). These findings [172] suggested that Bcl-2 overexpression could temporarily delay PN degeneration, and Bcl-2 overexpression was not adequate to permanently protect lurcher mutant PNs from the effects of the leaky GluRδ2 receptor.

A different attempt to rescue lurcher PNs from death/degeneration used heterozygous lurcher mutant mice (+/Lc) bred with Bax knockout mice (Bax–/–) to generate Bax-knockout lurcher double mutant mice (+/Lc, Bax–/–) [173]. Bax deletion was reported to be insufficient to inhibit the death of brainstem neurons in lurcher homozygotes, which were never found in the litters. At 15 postnatal days of age, there were significantly more surviving PNs in +/Lc, Bax–/– than in control lurcher mutants. The increased number of surviving PNs was throughout all regions of the cerebellum in double mutants (+/Lc, Bax–/–), indicating that Bax deletion did not rescue particular subpopulation of PNs. By 30 postnatal days of age, the number of surviving PNs in the double mutant mice decreased to a low level, which was not significantly different from that found in control lurcher mutant mice [173]. These findings suggested that Bax deletion did not permanently inhibit apoptosis but only transiently delayed the degeneration of lurcher PNs for several weeks [173].

Moreover, lurcher mutation was suggested to cause depolarized resting membrane potentials due to the constitutively open GluRδ2 channels in PNs [161]. This depolarization triggered the intrinsic apoptotic pathway as well as Ca^{2+}-dependent secretion of tPA [161]. Secreted tPA initiated the extrinsic apoptotic pathway and caspase-8 activation, which facilitated the initial PN degeneration observed in lurcher mutant mice [161]. Therefore, tPA elimination was suggested to delay death receptor-mediated apoptotic pathway and to ultimately trigger other apoptotic pathways that were sufficient to elicit PN death [161].

Table 3. Potential implications in the lurcher mutant rat and shaker mutant rat.

	Therapeutic implications	Reference
Lurcher mutant mouse	Bcl-2 overexpression could temporarily delay lurcher PN degeneration	[172]
	Bax deletion only transiently delayed the degeneration of lurcher PNs	[173]
	tPA elimination could delay death receptor-mediated apoptotic pathway and to ultimately trigger other apoptotic pathways that were sufficient to elicit PN death	[161]
	Autophagic cell death in granule cells in vitro served as an alternate death pathway, when apoptosis was blocked by caspase inhibition	[174]
	3-methyladenine inhibition of autophagy in HeLa cell culture triggered apoptotic cell death	[175]
Shaker mutant rat	Trophic factors (GDNF and IGF-1) could be only partially and transiently neuroprotective for at risk PNs	[167]
	Climbing fiber deafferentation caused temporal acceleration of the hereditary degeneration of at risk PNs	[166]
	Temporally accelerated PN death caused by IO chemoablation in shaker mutant rats could not be blocked by chronic infusion of GDNF and IGF-1 prior to IO chemoablation	[170]

Autophagic cell death in granule cells *in vitro* served as an alternate death pathway, when apoptosis was blocked by caspase inhibition [174]. Alternatively, 3-methyladenine inhibition of autophagy in HeLa cell culture triggered apoptotic cell death [175]. Moreover, a study on Huntington disease suggested that prothymosin-α might cause cells to shift between apoptosis and autophagy by adverse regulation of the apoptosome activity [176].

Thus, treatments that interfere with the pathways of the various types of programmed cell death may denote promising therapeutic approaches in the protection against PN loss in various neurodegenerative diseases in patients. However, it must be recognized that there are concerns about targeting programmed cell death in neurodegenerative diseases; since it is still to be comprehended whether or not inhibition of the different types of programmed cell death in PNs can be effective and harmless. Thus, a careful assessment in this regard is essential.

6. Conclusions

Purkinje neurons are among the most vulnerable neurons to cell death due to intrinsic hereditary diseases or extrinsic factors. Purkinje neuronal death causes cerebellar abnormalities, which are manifested by abnormalities in gait and posture and thus can be clearly recognized. Programmed cell death is proposed to be involved in the pathogenesis of neurodegenerative diseases associated with PN loss, based on *in vitro*, *in vivo*, and human postmortem studies [151, 152, 162, 177]. A better understanding of the molecular events regulating the various types of programmed cell death would be extremely beneficial for disease therapy, as these mechanisms are implicated in a variety of human neurodegenerative diseases, by making it possible to identify the possible factors that can be targeted therapeutically in order to halt or slow the progression of the disease. Further research in this area can be anticipated to provide promising opportunities regarding therapeutic manipulation of cell death programs.

Abbreviations

PNs, Purkinje neurons; PD, Parkinson disease; AD, Alzheimer disease; HD, Huntington disease; Bcl-2, B-cell lymphoma 2; PMCA, plasma membrane Ca^{2+} pump; PT-pore, Permeability transition pore; Apaf-1, apoptotic protease activating factor 1; TNF, tumor necrosis factor; NGF, nerve growth factor; Hsc70, heat shock cognate 70; lamp2a, lysosome-associated membrane protein 2a; PI3K, phosphoinositide 3-kinase; PtdIns(3)P, phosphatidylinositol 3-phosphate; Atg5, autophagy related 5; RIPK, receptor-interacting serine/threonine kinase; DAMPs, damage-associated molecular patterns; ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; $[Ca^{2+}]_i$, intracellular calcium concentration; EAAT4, excitatory amino-acid transporter 4; GluRδ2, glutamate receptor delta2; DNA, deoxyribonucleic acid; nPIST, neuronal isoform of protein-interacting specifically with TC10; GluR$\delta 2^{Lc}$, lurcher mutant glutamate receptor; tPA, tissue plasminogen activator; Lc/Lc; tPA–/–, Lurcher homozygotes lacking tPA; Lc/+; tPA+/+, lurcher mutant mice; Lc/+; tPA–/–, tPA-null lurcher double mutants; Atp2b, ATPase plasma membrane Ca^{2+} transporting 3; ADC, anterior degeneration compartment; PDC, posterior degeneration compartment; ISC, intermediate survival compartment; FNSC, flocculonodular survival compartment; GDNF, glial derived neurotrophic factor; IGF-1, insulin-like growth factor 1; +/Lc-Hu-Bcl-2, lurcher mutant overexpressing human Bcl-2; +/Lc, heterozygous lurcher mutant mice; Bax–/–, Bax knockout mice; +/Lc, Bax–/–, Bax-knockout lurcher double mutant mice.

Ethics approval and consent to participate

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Conflict of interest

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