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Neuronal death mechanisms in cerebellar Purkinje cells

by

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Abbreviations

3-MA	3-Methyladenine
ABC	Avidine Biotin Complex
Apaf1	Apoptosis protease activating factor-1
ATG	Autophagy gene
BH	Bcl homology
BSE	Bovine spongiform encephalopathy
CARD	Caspase recruitment domain
CJD	Creutzfeld Jacob disease
DED	Death effector domain
DFF45	DNA fragmentation factor 45kDa
DISC	Death-inducing signal complex
DIV	Days *in vitro*
DNA	Desoxyribonucleic acid
Dpl	Doppel
E13.5	Embryonic day 13.5
EGL	External germinal layer
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
FADD	Fas-associated death domain
Fas	Fibroblast-associated
FLIP	FADD-like-ICE-inhibitory protein
GABA	γ amino butyric acid
GluR	Glutamate receptor
ho	*hotfoot*
IP_3R	Inositol triphosphate receptor
IR	Immunoreactivity
JNK	c-jun N-terminal protein kinase
LTD	Long term depression
LTP	Long term potentiation
Lc	*Lurcher*
LYATT	Lysosomal amino acid transporter
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
MOMP	Mitochondrial outer membrane permeabilisation
NASP	1-Naphtyl-acetyl-spermine
NDUFS	Subunit of the mitochondrial respiratory complex I
Neo	Neomycine
NGS	Normal goat serum
NHS	Normal horse serum
ORF	Open reading frame

P2	Postnatal day 2
PB	Phosphate buffer
PBS	Phosphate buffer saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI3-K	Phosphoinositide 3-kinase
PKC	Proteine kinase C
PrP^{C}	Cellular prion protein
PrP^{res}	Prion protein resistant to proteinase K
PSD	Post-synaptic density
PTP	Protein tyrosine phosphatase
RML	Rocky mountain laboratory
ROS	Reactive oxygen species
rpm	Rotations per minute
Scrg1	Scrapie responsive gene 1
SOD	Superoxide dismutase
SQSTM	Sequestosome
SSCP	Single-strand conformation polymorphism
TNF-R1	Tumor necrosis factor receptor 1
TOR	Target of rapamycin
TRADD	TNF-R-associated death domain
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
TSE	Transmissible spongiform encephalopathy
UPR	Unfolded protein response

Introduction

During the course of my thesis, I investigated the participation of apoptotic and autophagic cell death programs in neuropathologies. Neuronal cell death mechanisms are known to play a major role in neurodegenerative diseases and the physiopathological significance of the interplay between apoptotic and autophagic cascades is still not understood. Insights into the complex patterns of neuronal cell death observed in nervous system diseases are critically needed to take up the challenge of designing novel neurodegenerative disease therapies, specifically targeting cell death pathways.

A million people worldwide are affected by neurodegenerative diseases, a heterogeneous group of degenerative conditions affecting specific areas of the central nervous system. The majority of neurodegenerative pathologies are age-related disorders, and these diseases are becoming an increasing health and socio-economical problem in industrialized countries (Mayeux, 2003). Neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer, Parkinson, Huntington and prion diseases induce progressive cognitive or movement impairment depending on the type of neuronal cells undergoing selective degeneration (Troncoso et al., 1996; Cleveland, 1999; Nunomura et al., 2007). Although these diseases are phenotypically well described, the molecular mechanisms leading ultimately to neuronal death remain unclear, and despite vigorous research efforts, therapy options have not been found.

In Alzheimer (Nakagawa et al., 2000; Nixon et al., 2005), Parkinson (Webb et al., 2003; Hayley et al., 2004), Huntington (Hickey and Chesselet, 2003) and prion (Lucassen et al., 1995; Liberski et al., 2008) diseases, autophagy and apoptosis have been shown to be activated in parallel. If apoptosis is an absolute programmed cell death mechanism, in most cases, autophagy constitutes cell defense mechanisms towards cellular dysfunction or stress. Thus, both apoptosis and autophagy may be triggered by common upstream

signals resulting in either combined autophagy and apoptosis or a switch between the two mechanisms in a mutually exclusive manner (Chu, 2006; Maiuri et al., 2007).

I focused my investigations on the cerebellar Purkinje cell in mouse models with mutations which specifically affect these neurons using a combination of biochemical, histological and cytological methods *in situ* as well as in organotypic cerebellar cultures. This approach has provided new insights into the complex molecular and cellular events underlying neuronal stress and degeneration.

The cerebellum presents several advantages for the anatomical study of neurodegeneration: *i*) it consists of the repetition of a single neuronal circuit made up of a few types of neurons and afferences centered on the Purkinje cell which emits the only output of the cerebellar cortex, *ii*) cerebellar abnormalities are usually straightforward to recognize because cerebellar damage manifests itself as abnormalities in gait and posture (ataxia), *iii*) many spontaneous mutations that affect cerebellar development and function have been recognized and lead to Purkinje cell degeneration (Appendix N°1). Purkinje cell death was investigated in *Lurcher* and *hotfoot* mice with mutations of the *Grid2* gene coding for the glutamatergic receptor GluRδ2 on one hand and, in the Nagasaki $Prnp^{0/0}$ mutant mouse deficient for the prion protein and overexpressing its neurotoxic paralogue Doppel on the other hand.

The aims of this project were

1) **To determine the mechanisms of *Lurcher* Purkinje cell death.** The *Lurcher* mutation transforms the GluRδ2 receptor into a constitutively opened channel. In *Lurcher* heterozygous mice, cerebellar Purkinje cells are permanently depolarized, a characteristic that has been thought to be the primary cause of their postnatal apoptotic death, although autophagy has been suggested to contribute to GluRδ2-induced death (Yue et al., 2002). The more dramatic phenotype of *Lurcher* homozygotes is probably due to a simple gene dosage effect of the mutant allele. We have analyzed the phenotype of *Lurcher*/*hotfoot* heteroallelic mutants bearing only one copy of the *Lurcher* allele and no wild-type *Grid2* to determine the effects of the absence of wild-type GluRδ2 receptors on Purkinje cell survival in these mutants. Using a pharmacological approach in organotypic cerebellar cultures, I further analyzed the respective contributions of autophagy

and excitotoxicity-induced apoptosis in the GluRδ2-*Lurcher* Purkinje cell survival and growth.

2) **To analyze the involvement of autophagy in the dendritic development and synaptogenesis of Purkinje cells.** The Purkinje cells of the *hotfoot* mutant mice are lacking GluRδ2 and display impaired climbing fiber (Kashiwabuchi et al., 1995) and parallel fiber (Kurihara et al., 1997) innervations indicating that GluRδ2 is involved in Purkinje cell excitatory synaptogenesis. During normal development, the postsynaptic spines deafferented by supernumerary climbing fiber elimination may involve GluRδ2-dependent autophagy. In the *hotfoot* adult cerebellum, Purkinje cells display many postsynaptic spines devoid of presynaptic innervation (Kashiwabuchi et al., 1995). The persistence of these spines may be due to the lack of GluRδ2-dependent autophagic mechanism. I analyzed Purkinje cell development in the *hotfoot* cerebellum to estimate the contribution of GluRδ2-dependent mechanisms (autophagy?) to the excitatory synaptogenesis of Purkinje cells. Firstly, the survival and dendritic development of Purkinje cells were compared between *hotfoot* and wild-type organotypic cerebellar cultures, and then climbing fiber development was examined during the postnatal period in the *hotfoot* cerebellum.
3) **To assess which neuronal death mechanisms are activated by Doppel during Purkinje cell degeneration in prion protein-deficient Nagasaki mutant mice.** In the Nagasaki mutant mouse, Purkinje cells prematurely die from toxicity induced by Doppel, a prion protein-like protein overexpressed in the absence of the cellular prion protein (PrP^C) (Moore et al., 2001; Wong et al., 2001; Cui et al., 2003; Sakudo et al., 2005b). To provide insight into the neuroprotective properties of PrP^C, as well as into the cell death programs triggered by Doppel, transgenic models were examined by biochemical and anatomical analysis.

The literature review of my thesis is divided into four parts which include an overview of apoptosis and autophagy – **the molecular basis of programmed cell death** - an overview of anatomy, development and physiological functions of the brain region under the focus of my research - **the mouse cerebellum** - and an overview of the mutant mice models of Purkinje cell degeneration analyzed in my thesis – **the *hotfoot* and the *Lurcher* Grid2 mutant mice** – and – **the Nagasaki prion protein-deficient mice**. The

results obtained in the *Grid2* mutant mice studies are presented in the format of unpublished data and a publication, and the data obtained in the Nagasaki mutant mice studies are presented in the format of publications. Afterwards, I shall integrate the results obtained from these models, and discuss the results in the light of other neuropathologies and highlight the importance of interplay between apoptosis and autophagy in neuronal response to pathological insults.

1. Molecular basis of programmed neuronal cell death

Cell death is a fundamental process involved in the regulation of tissue homeostasis and necessary for the elimination of supernumerary and diseased cells. This is achieved by two major active self-destruction mechanisms: the regulated and the unregulated pathways (Fig. 1). The unregulated cell death mechanism is a non-programmed cell death pathway often called necrosis and is caused by overwhelming stress. Characteristic features of necrosis include organelle swelling, mitochondrial dysfunction, massive oxidative stress and plasma membrane permeabilization. Necrosis irreversibly leads to the release of intracellular organelles and inflammation (Zong et al., 2004; Ditsworth et al., 2007). The regulated cell death mechanisms essentially comprise two programmed cell death (PCD) pathways: apoptosis (or type I PCD), the first characterized form of PCD, and autophagy (or type II PCD) which has been proposed to be an alternative cell death pathway, but is still controversial (Tsujimoto and Shimizu, 2005; Chu, 2006). Both are essential for discrete removal of supernumerary cells such as neurons during normal development. Under pathological conditions, apoptosis and autophagy may be activated in addition to necrosis.

1.1 ***Apoptosis: the type I programmed cell death***

Apoptosis is a highly conserved and complex cellular mechanism (Kerr et al., 1972). In mammals, external signals trigger two major pathways leading to the activation of caspases: the mitochondrial pathway (intrinsic pathway) and the death receptor pathway (extrinsic pathway).

1.1.1 Caspases, the main effectors of apoptosis

Caspases are a family of 14 cysteine-dependent aspartate-specific acid proteases that mediate and execute the apoptotic cell death program (Yuan et al., 1993; Salvesen and Dixit, 1997). All caspases exist as a latent pro-form of a single polypeptide chain, and are activated by specific cleavage at aspartic acid residues which leads to the formation of active tetramers and initiate apoptosis (Shi, 2002).

Caspases can be divided into 3 groups with respect to their structure and function (reviewed in Degterev et al., 2003):

- Group I or inflammatory caspases (Caspases 1, 4, 5, and 11) are not involved in apoptosis but play a role in the maturation of cytokines during inflammatory processes.
- Group II or initiator caspases (Caspases 2, 8, 9, 10, and 12) are long prodomain-containing caspases including DEDs-containing caspases (8 and 10) and CARD-containing caspases (2 and 9).
- Group III or effector caspases (Caspases 3, 6 and 7) are executioner caspases. These short prodomain-containing caspases are activated by upstream initiator caspases and cleave multiple cellular substrates.

Caspases 13 and 14 are still not characterized and are structurally close to inflammatory caspases.

1.1.2 The intrinsic pathway

The intrinsic apoptotic pathway is initiated in the mitochondria and the endoplasmic reticulum. The major event is the mitochondrial outer membrane permeabilization (MOMP). MOMP is mainly regulated by a specific class of proteins belonging to the B-cell/Lymphoma-2 family (Bcl-2 family), which play a pivotal role in the activation of the caspase cascade.

1.1.2.1 The Bcl-2 family

The Bcl-2 family of proteins can be divided into 2 groups:

- The anti-apoptotic proteins BCL-2, BCL-$_{XL}$, BCL-w, MCL-1.
- The pro-apoptotic proteins BAX, BAK, BOK, BID, BIM, BAD.

All Bcl-2 family members have at least one of the 4 known Bcl-homology domains (BH1 to 4) which correspond to α-helical segments (Adams and Cory, 1998). Some pro-apoptotic proteins called “BH3-only” proteins (BID, BIM, BAD) contain only a BH3 domain (Puthalakath and Strasser, 2002), and others such as “BH123”-containing proteins (BAX, BAK, BOK) share BH1 to 3 with BCL-2 (Adams and Cory, 1998). In this context, the BH3 domain is presumed to be an essential death domain and both BH3-only and BH123 domain proteins are required for the induction of apoptosis (Cheng et al., 2001).

1.1.2.2 The apoptotic mitochondrial cascade

In normal conditions, the pro-apoptotic proteins are cytosolic and anti-apoptotic proteins (Krajewski et al., 1993) are membrane-bound proteins of the ER and the mitochondria (Zhu et al., 1996). In the mitochondria, anti-apoptotic proteins sequester the pro-apoptotic ones in stable mitochondrial complexes, thereby preventing the activation of BH123 proteins such as BAX and BAK (Cheng et al., 2001). Apoptotic signals are able to activate BID by inducing homodimerization and translocation of BAX and BAK to the mitochondria (Fig. 2) (Wolter et al., 1997; Gross et al., 1998; 1999). BAX can be activated by another pathway which also involves activation of the transcription factor p53 when DNA is damaged (Lane, 1992; Miyashita and Reed, 1995). Homodimerization of BAX allows an efflux of cytochrome c due to the constitution of a pore in the mitochondrial outer membrane (Fig. 2) (Liu et al., 1996; Newmeyer and Ferguson-Miller, 2003). The binding of cytochrome c to the apoptosis protease activating factor-1, Apaf1 (Zou et al., 1997) causes the formation of a complex called the apoptosome in the presence of ATP. The subsequent recruitment of procaspase-9 will lead to the activation of the effector caspases 3, 6 and 7 (Fig. 2) (Li et al., 1997; Cecconi et al., 1998).

1.1.2.3 The ER stress apoptotic cascade

Oxidative stress can induce the unfolded protein response (UPR) in the endoplasmic reticulum (ER) which permits the elimination of misfolded protein aggregates. The UPR can be unable to counteract the stress which leads to the triggering of apoptosis (Fig. 3) (Breckenridge et al., 2003; Rao et al., 2004a). This mechanism requires the release of calcium from the ER through the binding of a small amount of cytochrome c released from the mitochondria. This positive feedback will result in the activation of the caspase cascade (Rao et al., 2004b). The Bcl-2 family members BCL-2, BAX and BAK themselves can also act at the ER level (Zong et al., 2003). BCL-2 is able to interrupt the described above crosstalk between ER and mitochondria, while BAX and BAK regulate the Ca^{2+} release from the ER (Scorrano et al., 2003) and promote caspase-12 activation. Activated caspase-12 will then translocate from the ER to the cytosol, directly cleave procaspase-9 to activate the effector caspase-3 (Szegezdi et al., 2003) and finally cause apoptotic cell death.

1.1.3 The extrinsic pathway

The extrinsic pathway is activated by ligand binding to death receptors such as tumor necrosis factor receptor 1 TNF-R1 (Tartaglia et al., 1993), fibroblast-associated Fas (Suda et al., 1993) and TNF-related apoptosis-inducing ligand receptor TRAIL-R (Griffith et al., 1998) which are transmembrane proteins (Fig. 4) with an extracellular cysteine-rich domain and an intracellular death domain. The ligand-bound TNF-R1, Fas and TRAIL-R transmit apoptotic signals through the binding of their death domain with the death domain of the TNF-R-associated death domain protein TRADD (Hussein et al., 2003) or the Fas-associated death domain protein FADD (Chinnaiyan et al., 1995; 1996) (Fig. 4). The N-terminal death effector domain DED (Lee et al., 2000) and caspase recruitment domain CARD (Hofmann et al., 1997) of FADD will be activated leading to the activation of caspases 8 and 10 (Fig. 4) (Weber and Vincenz, 2001) and the formation of the death-inducing signal complex DISC (Lee et al., 2000; Wang, 2001; Lavrik et al., 2003). This complex will finally activate effector caspase-3 (Fig. 4) (Stennicke et al., 1998). The caspase signaling initiated by the death receptors is regulated by the FADD-like-ICE-inhibitory protein FLIP which competitively inhibits the recruitment of procaspase-8 by FADD (Irmler et al., 1997; Scaffidi et al., 1999).

The c-jun N-terminal protein kinase JNK, a member of the mitogen-activated protein kinase (MAPK) is involved in TNF-induced apoptosis and may play a pro-apoptotic role in neurons. JNK can phosphorylate and thereby inactivate BCL-2 and BCL-$_{XL}$ (Fig. 4) (Basu and Haldar, 2003) and can activate the proapoptotic BH3-only protein BIM (Becker et al., 2004).

An intrinsic, mitochondrial-dependent apoptotic pathway has been shown to be activated by TNF-R1 and Fas (McKenzie et al., 2008) through the cleavage of the Bcl-2 family member BID by caspase-8 (Li et al., 1998; Scaffidi et al., 1998). The resulting truncated form tBID has been shown to induce the release of cytochrome c (Luo et al., 1998) and apoptosis. On the basis of the recruitment of the mitochondrial pathway by death receptors, two types of responses have described. Type I cells respond by activation of caspase-8 in the DISC inducing apoptosis. However, type II cells respond by only a small amount of FADD and caspase-8 recruitment to the DISC requiring the intrinsic pathway to induce apoptosis. Along this line, in *Bid*$^{-/-}$ mice, hepatocytes behave like type II cells after Fas-L treatment, whereas thymocytes respond to Fas-L in a type I manner (Zheng and Flavell, 2000).

1.1.4 The targets of caspases

The cleavage and subsequent activation of effector caspases (3, 6 and 7) by the initiator caspases 2, 8, 9, 10 and 12 is called the caspase cascade (Degterev et al., 2003). In addition, caspase-8 is able to activate the BH3-only BID protein.

Caspase-3, the predominant effector caspase, activates the signal components that affect the morphological changes associated with apoptosis. These components include the DNA fragmentation factor 45kDa (DFF45) (Liu et al., 1997) involved in DNA degradation (Enari et al., 1998; Mukae et al., 1998) and the subunit of the mitochondrial respiratory complex I (NDUFS) which causes the overproduction of reactive oxygen species (ROS) and the disruption of electron transport (Ricci et al., 2004). The disruption of actin filaments (Kothakota et al., 1997) and cell-to-cell interactions (Ku et al., 1997; Schmeiser et al., 1998) also induced by the effector caspases contribute to the dismantling of the cellular architecture.

The caspases are responsible for the activation of pro-apoptotic protein kinases such as ROCK1 in response to TNF-R activation (Coleman et al., 2001) and MEKK1 through Jun-kinase pathway during Fas-induced apoptosis (Deak et al., 1998). The caspases also cleave anti-apoptotic protein kinases including AKT (Bachelder et al., 2001) and FAK in response to TRAIL signaling leading to a loss of survival signals (Wen et al., 1997).

Caspases have been shown to cleave anti-apoptotic Bcl-2 family members such as BCL-2, BCL-$_{XL}$ suggesting that a positive feedback mechanism is set off by apoptosis (Degterev et al., 2003).

1.1.5 Neuronal apoptosis

Since the pioneering work of Levi-Montalcini on the survival of developing neurons (Hamburger and Levi-Montalcini, 1949), the essential role of apoptosis in the control of neuronal numbers during development of the nervous system has been supported by an increasing number of data (Becker et al., 2004). Neuronal apoptosis not only has a major role in sculpting relationships between neuronal populations in the developing brain, but is also the cardinal cell death process in many neurodegenerative diseases (Yuan and Yankner, 2000).

1.1.5.1 Apoptosis during neuronal development

Transgenic mouse lines over-expressing or knocked-out for the major factors involved in the mitochondrial pathway have been generated, and these models have revealed the importance of intrinsic mitochondrial apoptosis during brain development. For example, knocking-out the pro-apoptotic factor *Bax* gene has been shown to result in the increase of specific neuronal populations, such as peripheral ganglia, motor pools in the spinal cord and trigeminal brainstem nuclear complex (White et al., 1998) and Purkinje cells in the cerebellum (Fan et al., 2001) suggesting that these neurons undergo a period of naturally occurring, BAX-mediated cell death during brain development. This is also in line with the increase of Purkinje cell population observed when BCL-2, the main BAX antagonist is overexpressed (Zanjani et al., 1996).

An interesting study by Krajewska et al (2002) has described the onset of BAX, BAK, BCL-2, BID and BCL-$_{XL}$ expression in the developing central nervous system and outlined the antagonistic functions of these pro and anti-apoptotic factors in the formation of the neuronal tube and in the differentiation of proliferative zones in the developing brain.

1.1.5.2 Apoptosis in neurodegenerative diseases

In ischemic brain tissue, neuronal cell death has been shown to occur by Fas-dependent, (Northington et al., 2001) caspase-3-mediated apoptosis (Namura et al., 1998).

Neuronal death mechanisms have been extensively investigated in a number of neurodegenerative diseases including amyloid neuropathologies. In Alzheimer disease, the β–amyloid peptide is known to induce oxidative stress (Behl et al., 1994) which activates caspase-12-mediated apoptosis (Nakagawa et al., 2000). In prion diseases, apoptosis is detected in the prion-infected brain tissue (Fairbairn et al., 1994; Lucassen et al., 1995). This neuronal loss has been shown to result from the neurotoxicity of the abnormal proteinase-resistant forms PrP^{res} of the prion protein PrP^{C} (Forloni et al., 1996). More recently, *Bax* inactivation has been shown to antagonize apoptosis of infected cerebellar granule cells *in vitro* (Chiesa et al., 2005). However, the neurodegenerative events induced by the cerebellotropic 22L scrapie strain were changed neither in the *Bax*$^{-/-}$ nor in the *Hu-bcl-2* overexpressing mice (Bailly, unpublished). Similar results were obtained with the 6PB1 bovine spongiform encephalopathy (BSE) strain in *Bax*$^{-/-}$ mice

(Coulpier et al., 2006) and with RML scrapie strain in *Bax*$^{-/-}$ and *Hu-bcl-2* overexpressing mice (Steele et al., 2007). In expanded polyglutamine repeats diseases such as Huntington disease, abnormal protein aggregates have been shown to activate caspase-8-mediated apoptosis (Ona et al., 1999; Sanchez et al., 1999).

In Parkinson disease, an augmentation of IFN-γ (Teismann et al., 2003) has been shown to upregulate Fas activating the extrinsic apoptotic pathway (Hayley et al., 2004).

The mutant superoxide dismutase (SOD) responsible for amyotrophic lateral sclerosis forms intra-neuronal aggregates and induces oxidative stress resulting in neuropathological features including apoptosis similar to those observed in Alzheimer and Huntington diseases (Cleveland, 1999).

1.2 ***Autophagy***

Normal cellular development and cellular response to changes in the extra- and intra-cellular media require a balance between synthesis and degradation of protein. Autophagy is a lysosomal pathway involved in the degradation of long-lived cytosolic proteins (De Duve et al., 1955). In the focus of my thesis, autophagy involving the sequestration and subsequent lysosomal degradation of bulk cytosol is macro-autophagy. An alternative cellular-to-vacuole targeting pathway is a specific autophagic process which permits selective degradation without bulk cytosol (Harding et al., 1996). It will not be detailed here although it has led to a better understanding of autophagic mechanisms (Wang and Klionsky, 2003).

1.2.1 Induction and regulation of autophagy

The discovery of TOR (Kunz et al., 1993), a target of the autophagy-inducer rapamycin (Blommaart et al., 1997a), initiated the study of mechanisms regulating autophagy. TOR inhibits autophagy and is activated by amino acids (Luiken et al., 1994), class I phosphoinositide 3-kinase (PI3-K) (Codogno and Meijer, 2005), eIF2a and Ras (Meijer and Codogno, 2004) and is inhibited by class III PI3-K (Blommaart et al., 1997b). The autophagic cascade is mediated by 31 autophagy genes (ATGs) in mammalian cells. Eighteen of these ATGs are involved in the formation of the autophagosome (Kabeya et al., 2007). In the initial phase of the autophagic cascade, ATG13 normally phosphorylated by TOR, is dephosphorylated, thereby allowing it to complex with ATG1 (Fig. 5).

Beclin1 (ATG6) belongs to the class III PI3-K complex (Kihara et al., 2001) and is able to inhibit TOR and trigger the autophagic cascade. Beclin1 and BCL-2 have been shown to interact via their common BH3 domain (Liang et al., 1998). Beclin1 is expressed by cortical, hippocampal and cerebellar neurons (Liang et al., 1998; Yue et al., 2002; Diskin et al., 2005) and its activation is a hallmark of autophagy during neurodegeneration (Shibata et al., 2006).

1.2.2 The autophagic sequence

Our current knowledge about the molecular basis of the autophagic cascade is summarized in the Figure 5.

1.2.2.1 Role of ATGs in the autophagosome formation

The dephosphorylation of ATG13 allows its binding to ATG1. The ATG1-13 complex then recruits ATGs 11-17-20-24 (Kamada et al., 2000) leading to the formation of the autophagosome. The construction of the autophagosome from the pre-autophagosomal membrane depends on 2 conjugation systems (Ohsumi and Mizushima, 2004). The first associates ATG16 to ATGs 5-7-10-12 to form the isolation membrane as a pre-autophagosome (Mizushima et al., 2003) from cytosolic organelles. The second is the ATG8/LC3 system (Mizushima et al., 1998). ATG8/LC3 was first identified as microtubule associated protein1-light chain 3 (Mann and Hammarback, 1994). In the autophagic process, pro-LC3 is processed into a cytosolic form LC3-I (Kabeya et al., 2000). LC3-I is activated by ATG7 (Tanida et al., 2001) and cleaved into a LC3-II membrane-bound form by ATG4 and conjugated with a phosphatidylethanolamine (PE) by ATG3 (Kabeya et al., 2000) (Tanida et al., 2002). Pre-autophagosome membrane-bound LC3-II-PE seems to be required for completion of the autophagosome (Kabeya et al., 2000). Finally, LC3-II delipidation by ATG4 will let it leave the autophagosome (Kirisako et al., 2000).

1.2.2.2 The autophagolysosome

After completion, the autophagosome fuses with lysosomes, a step involving the autophagosome-specific GTPase Rab7 (Gutierrez et al., 2004) and the lysosome-specific proteins Lamp1 and Lamp2 (Eskelinen et al., 2002). After fusion, the autophagosome inner single-membrane vesicle is released inside the vacuole lumen and termed

autophagic body and this autophagic body is then degraded by lysosomal enzymes such as cathepsins B, D and L (Punnonen et al., 1992; Uchiyama, 2001). Cathepsin B is a cysteine protease which belongs to the papain superfamily (Takio et al., 1980), cathepsin D is an aspartine protease which belongs to the pepsin superfamily of proteinases (Ferguson et al., 1973) and cathepsin L is a thiol protease (Kirschke et al., 1977).

Once degradation has been completed, monomeric units are exported to the cytosol for reuse. ATG22 has been identified as a putative amino acid effluxer (Yang et al., 2006) that cooperates with other vacuolar permeases such as the lysosomal amino acid transporters LYAAT 1 and 2 (Sagne et al., 2001)

1.2.3 Physiological autophagy

1.2.3.1 Autophagic degradation of cellular components

Autophagy is an essential mechanism of cell survival. In response to starvation, cells degrade their own cytoplasmic material by an autophagic-dependent mechanism. Autophagy is a survival mechanism of mammalian HeLa cells to serum and amino acids deprivation. Indeed, blockade of autophagy in this case induces apoptotic cell death (Boya et al., 2005). Cultured bone marrow cells deficient for BAX and BAK undergo autophagy if the interleukin 3 growth factor is suppressed, allowing extended survival. RNAi blocking ATG5 or ATG7 rapidly induces cell death (Lum et al., 2005). *In-vivo*, ATG7 deficiency in the central nervous system provokes apoptosis of cortical, hippocampal and cerebellar neurons (Komatsu et al., 2006).

In the developing brain, constitutive activation of autophagy is implicated in neuroprotection and cellular remodeling of neurites and growth cones during neurite extension (Hollenbeck, 1993). Autophagy can also selectively degrade cell-surface receptors. At neuromuscular junction of *Caenorhabditis elegans*, presynaptic terminals can induce clustering of GABA-A receptors leading to their degradation by a LC3 homolog-mediated autophagy (Rowland et al., 2006).

1.2.3.2 Autophagy: the type II programmed cell death

A role for autophagy in regulating cell populations during development of living organisms is increasingly considered as a phylogenetically old process. For example, in *Drosophila*, embryonic salivary glands (Lee and Baehrecke, 2001) and the fat body at the end of larval stage are eliminated by autophagic programmed cell death (Rusten et al.,

2004). During moth development, autophagy may be involved in the elimination of larval intersegmental muscles (Schwartz et al., 1993).

This type II programmed cell death is called autophagic cell death (Cao et al., 2006) and is characterized by a marked proliferation of autophagic vacuoles and the progressive disappearance of cellular organelles (Schweichel and Merker, 1973). In autophagic cell death, cells, such as neurons, destined for elimination internalize cytoplasmic components into autophagic compartments for self-degradation, and death subsequently occurs by hyperactive autophagy (Nixon, 2006).

1.2.4 Autophagy in neuropathologies

Autophagy has been linked to a number of pathologies including neuropathologies (Nishino et al., 2000; Nixon, 2006). Axotomy rapidly induces autophagic activity in mouse central neurons, well before the beginning of axonal remodeling (Matthews, 1973).

In neurodegenerative diseases including Alzheimer, Parkinson, Huntington and prion diseases, mis-aggregated proteins accumulate in spite of autophagic activity and this has lead to the concept of autophagic failure or autophagic stress. The specific recognition of ubiquitin-positive aggregated proteins has been shown to be mediated by p62/SQSTM1 (Rideout et al., 2004; Bjorkoy et al., 2005) an adaptor protein between ubiquitinated proteins and LC3-expressing autophagosomes (Filimonenko et al., 2007; Pankiv et al., 2007). Accumulation of autophagic profiles are features of degenerative neurons in Alzheimer- and prion-diseased brains (Nixon et al., 2005; Liberski et al., 2008). In Alzheimer disease, autophagy at the synaptic level parallels decreased synaptic activity (Sikorska et al., 2004). In Huntington disease too, the abnormal accumulation of autophagic vacuoles containing mutated huntingtin (Ravikumar et al., 2005) is believed to induce neuronal apoptosis (Hickey and Chesselet, 2003). In Parkinson disease, the cell death pattern is complex with features of apoptosis in addition to the accumulation of autophagosome-like structures (Stefanis, 2005). Alpha-synuclein mutations that have been identified in certain cases of Parkinson disease can induce cell death in association with accumulation of autophagic vacuoles that are not completely acidified or missing cathepsin D (Stefanis et al., 2001).

1.3 ***Interplay between autophagic and apoptotic pathways***

1.3.1 Apoptosis blockade induces autophagy

Experiments on *Bax*$^{-/-}$; *Bak*$^{-/-}$ double knock-out MEF and bone marrow cell lines exposed to either DNA damage or stress signals indicate overactivation of autophagy. In MEF cells, massive autophagy delays cell death and can be abrogated by knocking-down Beclin1 or ATG5 gene (Shimizu et al., 2004). Conversely, in bone marrow cells, increased autophagy favors cell survival whereas knock-down of ATG5 or ATG7 genes restores cell death (Lum et al., 2005).

Blockade of lipopolysaccharide-induced apoptosis by inhibition of caspases triggers autophagic cell death of L929 macrophages. This can be abrogated by knocking-down the Beclin1 gene (Yu et al., 2004; Xu et al., 2006). This indicates that deficiency or blockade of apoptosis can switch the cell response from stress to autophagy.

1.3.2 Blockade of autophagy induces apoptosis

Cells with deficient autophagic machinery can undergo apoptosis. Along this line, Lamp2-deficient HeLa cells can not complete autophagic process, and this results in a strong activation of apoptotic cell death (Boya et al., 2005; Gonzalez-Polo et al., 2005). These data support the concept of a dual autophagic and apoptotic cell death mechanism in the above mentioned neurodegenerative diseases (Chu, 2006; Nixon, 2006).

1.3.3 Crosstalk between apoptosis and autophagy

1.3.3.1 Regulation of apoptosis by ATGs

Surprisingly, overexpression of the autophagy gene ATG5 leads to increased apoptosis, but not autophagy. Yousefi et al (2006) demonstrated that calpain can cleave ATG5 into its truncated form, tATG5. This tATG5 induces apoptosis and the release of mitochondrial cytochrome c. Moreover, tATG5 has been shown to directly bind BCL-$_{XL}$. Thus, ATG5 plays an important role in the regulation of apoptosis because tATG5 is able to sequester Bcl-2 anti-apoptotic proteins, thereby allowing the pro-apoptotic homodimerization of BAX (Fig. 6) (Yousefi et al., 2006).

1.3.3.2 Regulation of autophagy by Bcl-2 family members and caspases

Beclin1, a major ATG (see §1.2.1) was initially shown to bind BCL-2 (Liang et al., 1998). This interaction inhibits autophagy induced by starvation. For a presently

unexplained reason, only the ER-bound, but not mitochondria-bound BCL-2 can inhibit autophagy (Pattingre et al., 2005).

Interestingly, caspases are not only used during apoptosis, but also function in the regulation of autophagic cell death as in the case of the *Drosophila* salivary gland (Martin and Baehrecke, 2004). Beclin1 and ATG7-dependent autophagic death has also been shown to be induced by caspase-8 inhibition (Yu et al., 2004).

1.3.3.3 Regulation of autophagy by the apoptotic extrinsic pathway

A functional relationship between autophagy and the extrinsic apoptotic pathway (Jia et al., 1997) is further supported by the absence of autophagic vacuoles in cultured mammary epithelial cells where TRAIL-mediated apoptosis is inhibited (Mills et al., 2004) and by the induction of autophagy by TNFα activation in T-lymphoblastic cells (Jia et al., 1997). In addition, some proteins involved in the extrinsic signaling pathway have recently been implicated in autophagy. For example, down-regulation of ATG5 expression in HeLa cells suppresses cell death and vacuole formation induced by IFNγ and FADD (Pyo et al., 2005).

These data demonstrate that common upstream signals may trigger autophagy and apoptosis, meaning that apoptotic and autophagic machinery share common pathways that either link or polarize the cellular response.

2. The mouse cerebellum

The cerebellum is a highly folded hindbrain structure that lies dorsal to the pons and medulla. Three pairs of cerebellar peduncles, the inferior, middle and superior peduncles connect the cerebellum to the brainstem.

*2.1 **The cerebellar anatomy***

2.1.1 General organization

The cerebellum is bilaterally symmetrical: two lateral hemispheres are separated medially by the central longitudinal vermis (Larsell, 1952). It is divided into anterior and posterior lobes by a transverse primary fissure and a ventral flocullonodular lobe. The cerebellum has a folded appearance with fissures separating its antero-posterior extent into 10 lobules (Fig. 7A). Two major structures can be discerned: a uniformly structured cortex surrounding 3 right and left deep cerebellar nuclei within the central white matter: the fastigial (median), the interposed (intermediate) and the dentate (lateral) nuclei (Fig. 7B) (Larsell, 1952).

2.1.2 The cerebellar cortex

Each single folium is composed of a cortex or superficial grey matter over a central white matter. The cerebellar cortex is histologically homogenous with a uniform microstructure throughout the entire cerebellum and has 4 main neurons: the granule, the Purkinje (Purkinyé, 1877), the Golgi and the basket/stellate cells. Three layers make up the cerebellar cortex (Fig. 7B). The outer molecular layer contains basket/stellate cells (Ramón y Cajal, 1911), the Purkinje cell dendritic tree and parallel fibers emanating from the granule cells. The Purkinje cell somata form a dense monolayer between the internal granular layer and the molecular layer (Palay and Chan-Palay, 1974). The internal granular layer contains the granule cells and the Golgi cells (Ramón y Cajal, 1911). The deep cerebellar nuclei contain deep cerebellar neurons and interneurons.

2.1.2.1 The Purkinje cell

Purkinje cells are the principal neurons of the cerebellar cortex (Fig. 7B) and serve as the sole output from the cerebellar cortex to the deep cerebellar nuclei (Palay and Chan-Palay, 1974; Ito, 1984). Within the Purkinje cell layer, Purkinje cells express

biochemical heterogeneity of certain proteins (such as zebrin I and II) that segregate Purkinje cells into sagitally organized bands (Hawkes et al., 1985; Sotelo and Wassef, 1991; Bailly et al., 1995; Sarna and Hawkes, 2003). The Purkinje cell dendritic tree has a planar shape oriented in the sagittal plane (Fig. 7B) (perpendicular to the long axis of the folium) and is formed from one to two primary branches that further subdivide into secondary and tertiary branches (Palay and Chan-Palay, 1974; Ito, 1984). Two types of spines are located along the dendritic tree: *i*) spines that are restricted to the major dendritic trunks and bear primarily climbing fiber synapses and *i*) spines that stem from distal tertiary branchlets and make synapses with parallel fibers (Ramón y Cajal, 1911; Ito, 1984).

The axon of the Purkinje cell emerges from the basal pole of the soma, descends through the internal granular layer and makes GABAergic synapses mainly on the deep cerebellar neurons (Palay and Chan-Palay, 1974). Some Purkinje cell axons from specific lobules (lobule X and the floculus) make synapses on the neurons of the vestibular nuclei to mediate balance (Ito, 1984). Collaterals are also emitted along the descending Purkinje cell axon and re-enter the Purkinje cell layer in the same sagittal plane as the Purkinje cell dendrites. These collaterals contribute to supra- and infraganglionic plexuses, which form above and below the Purkinje cell layer, thereby inhibiting adjacent Purkinje, Golgi, basket and stellate cells (Palay and Chan-Palay, 1974).

2.1.2.2 The granule cells

The granule cells are found in the internal granular layer (Fig. 7B) and relay inputs from mossy fibers to the Purkinje cells. Granule cell somata are 5 to 8 μm in diameter and their dendrites, which are generally short, receive mossy fiber terminals (rosettes) to form complex synapses (glomeruli) with inhibitory Golgi axon terminals (Palay and Chan-Palay, 1974; Ito, 1984; Voogd and Glickstein, 1998). Granule cell axons ascend through the molecular layer, bifurcate and run parallel to the longitudinal axis of the folium as parallel fibers (Palay and Chan-Palay, 1974; Voogd and Glickstein, 1998) forming *en passant* synapses on the spines of the Purkinje cell tertiary dendrites and on the inhibitory interneurons.

2.1.2.3 The interneurons

There are at least five types of interneurons in the cerebellar cortex (Fig. 7B): the basket, the stellate, the Golgi, the Lugaro and the unipolar brush cells (Palay and Chan-Palay, 1974). All of these interneurons, with the exception of the unipolar brush cells, have been shown to be inhibitory (Eccles et al., 1966c; Aoki et al., 1986). The basket and stellate axons provide lateral inhibition in the parasagittal plane to adjacent Purkinje cell somata and dendrites, respectively (Palay and Chan-Palay, 1974). The Golgi cell dendrites receive input from granule cells as well as from mossy fibers and, provide feed-back inhibition to granule cells (Palay and Chan-Palay, 1974) thereby forming a closed inhibitory circuit in the cerebellar cortex.

2.1.3 The cerebellar afferents

The olivocerebellar fibers (i.e. climbing fibers) and the mossy fiber system constitute the two major cerebellar afferent systems. Both systems carry sensorimotor information directly to the deep cerebellar nuclei and also to the cerebellar cortex to regulate the extent to which the climbing and the mossy fibers activate the deep cerebellar nuclei. The differences that emerge in the synaptic strength within the cerebellar cortex are thought to form the basis of cerebellar plasticity during learning (De Zeeuw and Yeo, 2005).

2.1.3.1 The olivocerebellar system

Climbing fibers are one of the main operational inputs into the cerebellar cortex. Electrophysiological (Eccles et al., 1966b), autoradiographic (Courville and Faraco-Cantin, 1978) and degeneration (Desclin, 1974) techniques have shown that climbing fibers originate exclusively from neurons in the inferior olive of the medulla. The inferior olive sends olivocerebellar axons to the cerebellum, where the final segment of the axon takes the form of a climbing fiber in the cerebellar cortex. The olivocerebellar projection predominantly crosses the medullary midline, terminating in the contralateral hemicerebellum with the addition of very small uncrossed component innervating the ipsilateral cerebellum (Chan-Palay et al., 1977; Sugihara et al., 1999). From the inferior cerebellar peduncle, axons extend into the white matter and branch into the cortex in the parasagittal plane (Azizi and Woodward, 1987) to synapse onto 5 to 7 Purkinje cells (Sugihara et al., 2001).

In the rat, the olivocerebellar projection has an organized topography that is arranged into a pattern of parallel longitudinal zones in the cerebellar cortex (Azizi and Woodward, 1987; Sugihara et al., 2001). In general, each subnucleus of the inferior olive projects contralateral climbing fibers that closely adhere to one or more parasagittal Purkinje cell zones which then projects to a specific part of the deep cerebellar nuclei (Campbell and Armstrong, 1983; Wassef et al., 1992; Sugihara et al., 2001). Furthermore, each olivary subnucleus gives off collaterals to the deep cerebellar nuclei, which receives Purkinje cell input from the same parasagittal zone(s) (Andersson and Armstrong, 1987) and in turn, the deep cerebellar neurons project to the same olivary subnuclei from which they receive collaterals (Ruigrok and Voogd, 2000). In summary, the Purkinje cells in each longitudinal zone receive climbing fibers from a discrete zone within the inferior olive and these Purkinje cells send axons to a specific region in the deep cerebellar nuclei. A salient feature of the adult murine cerebellum is that each Purkinje cell receives input from only one climbing fiber, but every climbing fiber can innervate 5 to 7 Purkinje cells.

In the white matter, the olivocerebellar axons are thick (2 to 3 µm in diameter) and myelinated (Palay and Chan-Palay, 1974; Sugihara et al., 1999). However, as they ascend towards the internal granular layer, the axons become unmyelinated in the Purkinje cell layer and synapse onto the thick dendritic trunks of the Purkinje cells (Palay and Chan-Palay, 1974; Sugihara et al., 1999). In the Purkinje cell dendritic tree, climbing fiber arborisations emit fine beaded tendrils (including varicosities approximately 2 µm thick) that run along the Purkinje cell dendrites and synapse onto spines. These climbing fiber-Purkinje cell synapses are entirely covered by sheets of Bergmann glia (Palay and Chan-Palay, 1974). In addition to climbing fibers projecting onto Purkinje cells, climbing fibers also send collateral branches to the deep cerebellar nuclei (Palay and Chan-Palay, 1974; Sugihara et al., 1999; Ruigrok and Voogd, 2000; Sugihara et al., 2001).

2.1.3.2 The mossy fiber relay system

Mossy fibers are the second main cerebellar afferent and have different morphological and anatomical characteristics than the climbing fibers. They are derived from multiple sensory sources in the brainstem and the spinal cord (e.g. spino-cerebellar, vestibulo-cerebellar and lateral reticular, pontine reticulo-tegmental and external cuneate nuclei) (Palay and Chan-Palay, 1974; Voogd and Glickstein, 1998). Those mossy fibers

that arise from the brainstem or pons enter the cerebellum via the contralateral middle cerebellar peduncle (Mihailoff et al., 1981), while spinal mossy fibers enter the cerebellum through the inferior and superior peduncles (Chan-Palay et al., 1977). Similar to climbing fibers, mossy fibers run a myelinated axon into the white matter and emit collaterals to the deep cerebellar nuclei (Arsenio Nunes et al., 1988). From the white matter, mossy fibers ascend into the internal granular layer to synapse onto granule cell soma and dendrites (Palay and Chan-Palay, 1974). Their synaptic terminals form tapering enlargements called rosettes (Arsenio Nunes et al., 1988) that are enveloped by granule cell dendrites and Golgi cell axons.

Mossy fiber terminals were previously thought to have a very different spatial distribution in the cerebellar cortex compared to climbing fibers. Yet, new anatomical analysis shows the convergence of both afferent systems (Pijpers et al., 2006) such that non-adjacent cerebellar zones that receive the same climbing fiber input also share the same mossy fiber input. Unlike their distribution, the action of mossy fibers on Purkinje cells is different from that of climbing fibers. Firstly, excitatory activation of mossy fibers is exerted onto Purkinje cells indirectly by parallel fibers, the granule cell axons. Secondly, each parallel fiber contacts many Purkinje cells in the translobular plane and has a few synapses with each single Purkinje cell.

2.1.4 The cerebellar efferents

The major input to the deep cerebellar nuclei is made by the Purkinje cell axons (Voogd and Ruigrok, 2004) and the organization of the cortico-nuclear projection gives rise to the three functional zones of the cerebellum. Purkinje cells whose axons terminate onto the fastigial nucleus are located predominantly in the vermis, ventral paraflocculus and flocculus while Purkinje cell axons which terminate in the interposed and dentate nuclei primarily compose the paravermis and hemispheres, respectively. In turn, these three functional zones differ in the type of afferents received and are assumed to be implicated in different aspects of motor activity. For example, the vermis is considered to control balance both in stance and locomotion (Thach and Bastian, 2004) whereas goal-directed and visually-guided movements, in addition to the modulation of rhythmic-synchronized movements are controlled by the paravermis and hemispheres (Cooper et al., 2000).

Furthermore, individual deep cerebellar nuclei have characteristic efferent targets. The fastigial nuclei send both crossed and uncrossed efferent projections to the vestibular nuclei, the inferior olive, the pontine and lateral reticular nuclei. The interposed nuclei project primarily to the contralateral red nucleus. The dentate nuclei project principally to the contralateral thalamus, the prefrontal cortex and the red nucleus.

2.1.5 The cerebellar circuitry

The key neuron of the cerebellar circuit is the Purkinje cell, as it integrates a variety of inputs and is the sole efferent of the cortex. Purkinje cells receive 2 main excitatory inputs (Fig. 8): climbing fibers making synapses directly onto Purkinje cells in a 1 climbing fiber:1 Purkinje cell ratio and mossy fibers, indirectly via granule cells, in an approximately 100, 000 parallel fibers:1 Purkinje cell ratio (Eccles et al., 1966a; Ito, 1984). Climbing fibers stimulate sagitally oriented bands of Purkinje cells, whilst parallel fibers activate a transverse beam. It is presumed that cerebellar function takes place at the intersection of these two afferents. Simultaneously, Purkinje cell activity is modulated by the basket and stellate axons (Fig. 8) (running in a plan perpendicular to parallel fibers) which sharpen the Purkinje cell output by inhibiting sagitally adjacent Purkinje cells. The Golgi cells provide feedback inhibition to the mossy fiber-granule cell synapse and inhibit the transversally activated Purkinje cell beam (Fig. 8) (Palay and Chan-Palay, 1974). Then, the Purkinje cell axons exit the cortex and inhibit the deep cerebellar nuclei. In turn, the axons of the deep cerebellar neurons exit the cerebellum via the superior and inferior peduncles and transmit information to other motor centers (for example, the red nucleus and the thalamus).

2.1.6 Development of the cerebellar neurons and afferents

The first neurons to form are the inferior olivary neurons, followed by the deep cerebellar neurons and the Purkinje cells, which leave the ventricular neuroepithelium and settle in the developing cortical plate before birth (Altman and Bayer, 1997). The inhibitory interneurons also originate from the neuroepithelium of the 4th ventricle, migrate like the Purkinje cells but continue to proliferate during their transit through the deep cerebellar mass (Schilling, 2000). Similarly, a secondary neuroepithelium, the external germinal layer (EGL), is generated prenatally, the precursor cells migrating onto

the surface of the cerebellar plate by birth. This latter epithelium produces the granule cells (Altman and Bayer, 1997).

2.1.6.1 Development of the Purkinje cells

The Purkinje cells originate in the neuroepithelium of the 4th ventricle from embryonic day 13 (E13) (Altman, 1975). They ascend through the zone of the deep cerebellar nuclei to the cortical plate on the surface of the cerebellar primordium anlage by E15 (Altman and Bayer, 1978) leaving their axons behind and establishing synaptic contacts with deep cerebellar neurons as early as E20 (Eisenman et al., 1991). From E20, climbing fibers are present in the Purkinje cell plate (Chedotal and Sotelo, 1992) and make transient contacts with Purkinje cells (Morara et al., 2001), suggesting very early influence of climbing fibers on Purkinje cells. At birth, the morphological features of Purkinje cells are clearly apparent at the inner boundary of the EGL and are arranged in a 6-12 cell-deep plate (Altman and Bayer, 1997). Their somata contain a large clear nucleus and minimal cytoplasm that emit short thin processes across the EGL (Altman and Bayer, 1997).

From birth to postnatal day 2 (P2), transient synapses exist between climbing fiber axon terminals and transient emerging dendrites of the Purkinje cells (Armengol and Sotelo, 1991; Chedotal and Sotelo, 1993). From P3 to P4, Purkinje cells begin to align in a monolayer (Altman and Bayer, 1997) which is thought to be influenced by parallel fiber growth from above and granule cell migration to Purkinje cells from below (Goldowitz and Hamre, 1998). They display fine somatic processes that receive immature climbing fiber arbors (Mason et al., 1990; Armengol and Sotelo, 1991). From P5 to P7, a large apical cone develops increasing the Purkinje cell somatic cytoplasm, and extends into the molecular layer (Altman and Bayer, 1997). At P7, Purkinje cells begin to develop primary dendrites which receive parallel fiber synapses (Scelfo and Strata, 2005) while their perisomatic processes receive climbing fiber synapses (Altman and Bayer, 1997). The morphology of the Purkinje cells has transformed by P10, with somatic processes reabsorbed into the soma and basket cell axons contacting Purkinje cell somata (Berry and Bradley, 1976). By P12, the apical dendrites enlarge forming numerous secondary and tertiary branches (Altman and Bayer, 1997). From P12 to P15, the Purkinje cell dendritic arbor increases in complexity, first laterally and then growing in height in parallel with the molecular layer (Berry and Bradley, 1976) and generation of parallel

fiber synapses (Altman and Bayer, 1997). By P14, the distal branches of the Purkinje cell dendrites and spines located in the lower half of the molecular layer receive parallel fibers (Altman and Bayer, 1997). At P15, the Purkinje cell dendritic tree has formed its full width and the Purkinje cell soma is synaptically mature (Berry and Bradley, 1976). By P21, parallel fibers establish synapses with the distal spines on the dendritic tree in the upper half of the molecular layer (Altman and Bayer, 1997). The growth of the Purkinje cell dendritic tree continues through the upper molecular layer until P30 as the parallel fibers do (Berry and Bradley, 1976).

2.1.6.2 Development of the granule cells

At birth, the EGL has formed 6 to 8 rows of differentiating granule cell precursors. These cells undergo rapid proliferation until P9 and the EGL increases in depth with 8 to 12 rows of cells (Altman and Bayer, 1997). The EGL displays two distinct zones of cells: an outer proliferative zone and an inner premigratory zone of bipolar cells (Altman and Bayer, 1997). The bipolar cells of the premigratory zone grow processes that elongate laterally and run parallel to the developing pial surface to become parallel fibers whilst the bipolar cell somata remain on this plane (Altman and Bayer, 1997). Once the parallel fibers have grown their optimal length, the soma migrates vertically to its position in the internal granular layer while the parallel fiber remains in the upper region of the molecular layer (Altman and Bayer, 1997). By P6, functional synapses exist between parallel fibers and Purkinje cells (Scelfo and Strata, 2005). From P7 to P12, the generation of granule cell numbers increase reaching a peak from P10 to P11 and maximal migration occurs between P9 and P17 (Altman and Bayer, 1997). From P15, the granule cells in the internal granular layer develop short claw-like dendrites that envelope the immature mossy fiber rosettes (Altman and Bayer, 1997).

2.1.6.3 Development of the climbing fibers

The development of the climbing fiber is concurrent with the development of the cerebellar cortex. The inferior olive neurons are generated in the dorsal neuroepithelium of the caudal hindbrain between E12 and E13 (Bourrat and Sotelo, 1991) and their axons extend and invade the cerebellar cortical plate by E17 where they are arranged in broad sagittal zones resembling the adult climbing fiber distribution and contact Purkinje cells (Arsenio Nunes and Sotelo, 1985; Chedotal and Sotelo, 1993). The onset of transient

(non-synaptic) contacts between climbing fiber axon terminals and transient Purkinje cell somatic processes first occur embryonically (Chedotal and Sotelo, 1993; Morara et al., 2001). Postnatally, climbing fibers undergo 4 major developmental stages:

- The "creeper stage" (Fig. 9). By P2 climbing fibers begin to "creep" as mini arbors to associate with immature Purkinje cell somata and their transient somatic processes via attachment plates within the Purkinje cell plate. Although this contact regresses by P3, a large portion of climbing fibers maintain contacts onto several adjacent Purkinje cells (Chedotal and Sotelo, 1993). These early postnatal contacts are functional (Crepel et al., 1976) despite their perisomatic location.
- The "pericellular nest stage" (Fig. 9). By P5, climbing fibers make pericellular nests onto the Purkinje cell somata aligned in a monolayer (Chedotal and Sotelo, 1993). Each Purkinje cell receives inputs from several climbing fibers, with a maximum mean number of 3.5 climbing fibers synapsing onto perisomatic processes of each Purkinje cell (Ramón y Cajal, 1911; Crepel et al., 1976; Mariani and Changeux, 1981; Lohof et al., 1996).
- The "capuchon stage" (Fig. 9). Between P8 and P9 a somatodendritic translocation occurs and climbing fibers terminate onto the main trunk of the developing Purkinje cell dendritic tree (Ramón y Cajal, 1911; Mason et al., 1990). The regression of multi-innervation that reduces the percentage of Purkinje cell innervation by 50% is contemporaneous with the climbing fiber translocation (Crepel et al., 1976; Chedotal and Sotelo, 1992).
- The "young climbing fiber stage" (Fig. 9). From P10 to P15, reorganization of climbing fiber terminals occurs (Chedotal and Sotelo, 1992) so the adult 1:1 relation between climbing fibers and Purkinje cells is reached (Crepel et al., 1981; Mariani and Changeux, 1981).

One salient feature of the developing climbing fiber-Purkinje cell synapse is the elimination of transient climbing fiber multi-innervation. This mechanism is highly dependent on the presence of granule cells and the formation of normal parallel fiber-Purkinje cell synapses (Mariani and Changeux, 1980; Sugihara et al., 2000). Multiple innervations of climbing fibers persists when granule cell numbers are decreased in mutant rodents such as *weaver* and *reeler* (Appendix N°1) (Mariani et al., 1977; Puro and Woodward, 1977). Multiple climbing fiber innervation is also maintained abnormally

when parallel fibers are unable to form normal synapses with Purkinje cells in the *staggerer* (Mariani and Changeux, 1980) and the *hotfoot* mutant mice (§3.5.2).

2.1.6.4 Development of the mossy fibers

Mossy fibers derived from the brainstem nuclei also develop during embryogenesis and emerge into the cerebellar white matter by P3 (Arsenio Nunes and Sotelo, 1985). From P3 to P5, they invade the internal granular layer where they make synapses onto granule cell dendrites and by P7, mossy fibers are organized in topographical arrangement matching that of the adult mossy fiber input (Arsenio Nunes and Sotelo, 1985). By P15, the mossy fibers forming rosettes with granule cell dendritic branches are biochemically mature (Altman and Bayer, 1997). However, the presence of only minimal number of glomeruli prevents mossy fiber-granule cell synapses maturity until P21 (Altman, 1972), even though parallel fiber-Purkinje cell synapses are active at P7 (Scelfo and Strata, 2005).

2.2 ***Functions of the cerebellum***

The anatomical organization of the cerebellum and its afferents and efferents are particularly important when considering the role of the cerebellum in different modes of information processing. However, the relative contributions of each structure as well as the functional changes that occur between these structures and other motor and non-motor centers of the brain during learning and memory are unclear. Overall, in terms of cerebellar efferent flow, there appears to be two close efferent "loops" (Fig. 10): one with the red nucleus and the other with the premotor/motor cortex. In the first case, the deep cerebellar nuclei project axons to the red nucleus which in turn projects to the precerebellar nuclei of the spinal cord, medulla (e.g. external cuneate nucleus and inferior olive) and pons (e.g. pontine gray and vestibular nuclei) that also receive peripheral input. The precerebellar nuclei innervate the cerebellar cortex and a subset of deep cerebellar neurons to form one closed circuit (Altman and Bayer, 1997). As the precerebellar nuclei receive peripheral input (by way of the spinal cord) and cerebellar input (by way of the deep cerebellar nuclei and red nucleus), they may form part of a regulatory or readjustment system between both external and internal input (Palay and Chan-Palay, 1974; Altman and Bayer, 1997).

In the second case, the deep cerebellar nuclei project directly to the thalamus (Fig. 10) that receives input from the motor cortex, and the premotor, parietal and prefrontal cortices. Also, the thalamus and the motor cortex project to the pontine nuclei, which in turn project to the deep cerebellar nuclei forming another closed circuit. This massive descending path from the cerebral cortex to the pontine nuclei is thought to carry information about an extended action (Altman and Bayer, 1997). The structural link between the cerebellar cortex-deep cerebellar nuclei and cerebellar function provides a site where the process of motor learning and non-motor activities may occur, although the mechanisms involved are not fully elucidated.

2.2.1 Motor functions

The importance of the cerebellum and its components in motor functions are demonstrated by studies using cerebellar lesions and mutant mice. The removal of the whole cerebellum causes a lack of sensorimotor coordination and impaired equilibrium in both neonatal and adult rats (Zion et al., 1990; Caston et al., 1995). Comparatively, the removal of half of the cerebellum has a differential effect on adult and neonatal rats (Molinari et al., 1990). In adult rats, the effects include asymmetrical gait, deficits in dynamic postural adjustments and coordination and a side preference contralateral to the lesion (Molinari et al., 1990). However, neonatal hemicerebellectomised rats exhibit a normal gait, but a delay in acquiring dynamic postural adjustment and a permanent impairment of motor skills (Petrosini et al., 1990). The transition from normal motor development to defective motor behavior following neonatal hemicerebellectomy, referred to as “growing into a deficit”, is evidenced by the progressive reduction of hind limb grasping and a directional bias in posture correction (Petrosini et al., 1990). It has been suggested that these motor abnormalities are independent of cerebellar control in early development, but as the rat matures, the retention of these motor skills requires a cerebellar circuit (Petrosini et al., 1990).

Further investigations using mutant mice have provided evidence for the relative roles of the deep cerebellar nuclei and the cortex in cerebellar function. The mutant mice models include *Lurcher* mice which display cerebellar cortical and inferior olive degeneration and *Hu-bcl-2*-overexpressing mice which exhibit an excess of neurons. *Lurcher* mutant mice are ataxic and limited in equilibrium, motor coordination and postural sensorimotor skills in complex motor tasks (Hilber et al., 1998; Hilber and

Caston, 2001). As deep cerebellar neuron output is the only part of the cerebellar circuit remaining in this mutant, the deep cerebellar neuron activity is apparently adequate for simple sensorimotor tasks, but not for more complex tasks. Similarly, transgenic *Hu-bcl-2* mice that possess supernumerary Purkinje, granule and inferior olivary neurons display impairment of complex motor abilities, such as synchronized walking movement, but not in basic motor tasks (Rondi-Reig et al., 1999). This indicates that one function of the deep cerebellar nuclei is to learn and maintain simple sensorimotor behavior, while a correct cerebellar cortical circuit is necessary for complex learning.

2.2.2 Cognitive functions

A role of the cerebellum in cognitive functions is supported by a variety of studies based on neuroanatomical, functional imaging, clinical and experimental approaches (Schmahmann and Sherman, 1997). The extensive interconnexions between the neocortex and lateral cerebellar hemispheres have led to the interpretation that these two structures are functionally related hence implying a cerebellar role in cognition (Middleton and Strick, 2000). Although these studies demonstrate cerebellar processing in non-motor tasks, the specific regions of the vermis and the lateral zone involved in these functions remain poorly defined. Generally, lesions to the lateral zone of the rat cerebellum induce selective deficits in spatial orientation tasks, but not in visuomotor abilities, while lesions to the vermis only impair visuomotor abilities (Joyal et al., 1996). Furthermore, vermal lesions induce autistic-like symptoms, such as limited attention capacity and decreased anxiety (Bobee et al., 2000).

The cerebellum is also involved in associative learning as shown by results of avoidance-condition tasks. In these tasks, cerebellectomised rats learn as fast as intact rats to avoid an electrical shock when given an auditory stimulus. However, retention of the acquired conditioned response does not occur, indicating that the cerebellum is required to maintain learned avoidance behavior (Dahhaoui et al., 1990).

2.2.3 Spatial functions

Spatial functions have been reported to be affected by cerebellar pathologies (Petrosini et al., 1996; Molinari et al., 1997). Indeed, cerebellar connections with structures known to mediate visuospatial abilities such as frontal and parietal regions, limbic system and superior colliculus are consistent with this hypothesis (Schmahmann

and Pandya, 1989; 1997). Patients with Friedreich's ataxia perform spatial tasks deficiently (Fehrenbach et al., 1984) and patients with focal cerebellar lesions develop spatial deficits (Wallesch and Horn, 1990).

Mutant mice such as *Lurcher*, *Pcd*, *nervous* and *staggerer* mice develop different spatial impairments, such as exploratory deficits in mazes and defective spatial memory (Lalonde and Botez, 1990). *Lurcher* mice display deficits in habituation (Lalonde et al., 1986b) and in controlling the direction of swimming (Lalonde et al., 1988a) in aquatic mazes. Selective Purkinje cell degeneration in *Pcd* mice apparently interferes with the acquisition of spatial tasks (Goodlett et al., 1992). *Staggerer* mutant mice that loose Purkinje cells and granule cells, exhibit prolonged exploration of new environments and deficits in maze learning (Lalonde et al., 1987; 1988b). In complex environment, n*ervous* mutant mice which have damaged Purkinje cells and deep cerebellar neurons lack ability to retain the learned behavior compared to controls (Lalonde and Botez, 1985; Lalonde et al., 1986a). This suggests that an intact cerebellar cortical circuit and afferents are needed for learning and retention of spatially demanding tasks.

3. The *hotfoot* and *Lurcher* Grid2 mutant mice

Ionotropic glutamate receptors mediate most of the excitatory synaptic transmission in the central nervous system and are essential for synaptic plasticity (Brockie and Maricq, 2006). These include AMPA receptor subunits (GluR1-GluR4, GluRA-D), Kainate receptor subunits (GluR5-GluR7, KA1 and KA2) and NMDA receptor subunits (NR1, NR2A-NR2D and NR3A-NR3B). Two additional members of this superfamily, termed δ1 and δ2 have been identified in both mouse and rat brain.

3.1 ***The Grid2 gene***

The δ2 glutamate receptor (GluRδ2) has been identified in both mouse and rats by homology screening of mouse cDNA libraries. It is positioned at equal distances in the phylogenic tree from NMDA, AMPA and Kainate receptors (Araki et al., 1993; Lomeli et al., 1993) and is coded by the *Grid2* gene which contains 16 exons over a region of 1.4Mb located on chromosome 6 in mice (see review in Yuzaki, 2003). This may be a reason for the numerous of spontaneous mutations of this locus. Sixteen of these mutations provoke ataxia. The high frequency of *Grid2* mutations reflects its hypermutability as underlined by the occurrence of two independent translocations recently reported in a relatively small colony (Robinson et al., 2005).

Two natural *Grid2* mutant mice have been extensively studied since their discovery in the 60's, *Lurcher* (Phillips, 1960) and *hotfoot* (Dickie, 1966). Indeed, investigating these two mutant mice has brought important insights into the roles of GluRδ2 in Purkinje cell synaptogenesis and programmed cell death (Gounko et al., 2007).

3.2 ***Structure and localization of GluRδ2***

3.2.1 GluRδ2 is an orphan glutamate receptor

The topology of GluRδ2 is predicted to be similar to other ionotropic GluRs with an extracellular N-terminal region containing LIVBP-like and LAOBP-like domains, three transmembrane domains (TM1, TM3 and TM4), an ion channel-forming segment (TM2) and a cytoplasmic C-terminal region (Fig. 11A) (Araki et al., 1993; Lomeli et al., 1993).

GluRδ2 is an ionotropic glutamate receptor because:

- it possesses four hydrophobic segments (Araki et al., 1993),
- it shares amino acid sequence with other GluR channel subunits,

- it is localized at the parallel fiber-Purkinje cell synapses (Araki et al., 1993; Mayat et al., 1995; Takayama et al., 1995) where it is a receptor of the postsynaptic density beard by the Purkinje cell spine (Takayama et al., 1995; Landsend et al., 1997),
- it is co-expressed with AMPA GluR2/3 receptors in the postsynaptic membrane of the Purkinje cell synapses (Takayama et al., 1995; Landsend et al., 1997)
- it has the characteristic ion channel pore of glutamate receptors (Kohda et al., 2000).

However, it does not form functional glutamate-gated ion channels when expressed in transfected cells and does not bind glutamate analogs (Araki et al., 1993; Lomeli et al., 1993). In addition it does not complex with other glutamate receptor subtypes (Mayat et al., 1995). For these reasons it is considered to be an *orphan* glutamate receptor.

3.2.2 The GluRδ2 of Purkinje cells

Similarly to other glutamate receptors (Ajima et al., 1991), the distribution of GluRδ2 differs at the two main types of excitatory synapses of the Purkinje cell dendritic tree, the climbing fiber-Purkinje cell and parallel fiber-Purkinje cell synapses. In the adult cerebellum, GluRδ2 is expressed at the postsynaptic side of the parallel fiber-Purkinje cell synapse, but is absent from the climbing fiber-Purkinje cell synapse (Takayama et al., 1996; Landsend et al., 1997). Nevertheless, in the postnatal rat cerebellum, GluRδ2 is expressed at both climbing fiber-Purkinje cell and parallel fiber-Purkinje cell synapses although it disappears from climbing fiber-Purkinje cell synapse as early as P15 (Zhao et al., 1997). At this time, the multiple innervations of Purkinje cells by climbing fibers have completely regressed while parallel fiber-Purkinje cell synaptogenesis peaks. This specific developmental change in synaptic localization of GluRδ2 suggests that it is involved in parallel and climbing fiber synaptogenesis with Purkinje cell (Mayat et al., 1995).

*3.3 **Molecular partners of GluRδ2***

As illustrated in Figure 11B, a number of proteins involved in the docking and trafficking, as well as in the functional regulation of GluRδ2 have been identified.

The cytoplasmic C-terminal tail of all ionotropic GluRs intervenes in synaptic clustering (Malinow and Malenka, 2002). The post-synaptic anchoring PDZ proteins specifically recognize the C-terminus of their associated receptor (Hung and Sheng, 2002). Along this

line, the sequence of the 4 last amino acids of the GluRδ2 C-terminus has been shown to specifically bind the post-synaptic density (PSD)-93 protein, a member of the PSD-95 family of PDZ proteins, at the parallel fiber-Purkinje cell synapse (Roche et al., 1999). Finally, yeast two hybrid screening revealed that several PDZ-containing proteins interact with GluRδ2 C-terminus. These proteins include:

- the protein-tyrosine phosphatase PTP-MEG (Hironaka et al., 2000) which might play a role in the regulation of the activity of GluRs through tyrosine dephosphorylation
- delphilin which is selectively localized at the post-synaptic side of the parallel fiber-Purkinje cell synapse (Miyagi et al., 2002) and may link GluRδ2 with the actin cytoskeleton
- n-PIST an activator of the autophagy inducer Beclin-1 (Yue et al., 2002)
- scaffold proteins Shank 1 and 2 (Uemura et al., 2004) which allow GluRδ2 interaction with the main post-synaptic actors of long-term depression: the metabotropic glutamate receptor mGluR1, the AMPA-type glutamate receptors and the inositol 1,4,5-triphosphate receptor (IP_3R)
- PICK1, a protein interacting with C kinase 1 (Yawata et al., 2006). PICK1 has been shown to be involved in long term depression (LTD) and interacts with the C-terminus of GluRδ2. This demonstrates the importance of the GluRδ2 C-terminus in cerebellar LTD (Kohda et al., 2007)
- S-SCAM/MAGI-2, a PDZ domain-containing protein localized at post-synaptic site of Purkinje cell synapses. Binding of S-SCAM/MAGI-2 with GluRδ2 is regulated by protein kinase C (PKC)-mediated phosphorylation of the receptor and this may be necessary for the trafficking and clustering of GluRδ2 (Yap et al., 2003a)
- Spectrin, a member of the actin-binding family of proteins. Thus, spectrin may directly act on immobilization and clustering of GluRδ2 at the parallel fiber-Purkinje cell synapse (Hirai and Matsuda, 1999)
- EMAP, a microtubule-associated protein which binds selectively GluRδ2 and spectrin (Ly et al., 2002) indicating the involvement of cytoskeleton in GluRδ2 trafficking.

Finally, new insights into the intracellular movement, anchoring and clustering of GluRδ2 at the post-synaptic membrane have been provided by the discovery of Region A, a region adjacent to the TM4 of GluRδ2 (Fig. 11B) (Matsuda and Mishina, 2000; Matsuda et al.,

2004), and of the Adaptor protein complex-4 AP-4 (Yap et al., 2003b). Indeed, the former has been shown to target GluRδ2 from the endoplasmic reticulum to the plasma membrane and the latter has been shown to be involved in the intracellular trafficking of GluRδ2.

3.4 ***GluRδ2 in LTD***

Data from treatment of Purkinje cells with either antisense oligonucleotides (Hirano et al., 1994) or antibody against GluRδ2 (Hirai et al., 2003) also implicate GluRδ2 as a critical element in AMPA receptor trafficking and the induction of LTD at the parallel fiber-Purkinje cell synapse. Indeed, the C-terminus of GluRδ2 has been shown to be phosphorylated by the PKC *in vitro* and a LTD-inducing signal *in vivo* (Kondo et al., 2005). GluRδ2 devoid of C-terminal PDZ-ligand domains cannot restore the abrogated LTD in *Grid2*$^{-/-}$ mice (Kohda et al., 2007; Kakegawa et al., 2008). Transgenic *Grid2*$^{-/-}$ mice carrying mutant GluRδ2 with specific PDZ-ligand domain deletions have been generated (Uemura et al., 2007; Yasumura et al., 2008). Deletion of the C-terminal PDZ-ligand domain, the T site (Fig. 11B), revealed an impairment of LTD induction at the parallel fiber-Purkinje cell synapse and a distal expansion of climbing fiber territory. However, elimination of surplus climbing fiber innervation at proximal dendrites appeared to proceed normally (Uemura et al., 2007). Nevertheless, when S segment (Fig. 11B), the second PDZ-binding domain of the C-terminus, was deleted, the amount of GluRδ2 in cerebellar PSD fractions was reduced. In addition, mismatched parallel fiber and naked spines emerged and the climbing fiber territory expanded to distal regions of Purkinje cell dendritic tree (Yasumura et al., 2008). This implies a differential involvement of these PDZ-binding domains in diverse GluRδ2 functions.

3.5 **Hotfoot,** ***a natural Grid2 knock-out mouse***

The *hotfoot* mutation was first described in mouse by Dickie (1966) but has only recently been related to GluRδ2 (Lalouette et al., 1998). The *hotfoot* mutation causes a natural GluRδ2 deficiency due to a deletion in the *Grid2* gene. At least, 18 independent *hotfoot* mutations of the *Grid2* gene have been documented causing ataxia in the *hotfoot* mouse (Mouse Genome Informatics, 2000). The recessive loss-of-function mutations carried by the *hotfoot* alleles of *Grid2* result in ataxia without Purkinje cell death. Two specific *hotfoot* mutations *hotfoot*-4J (*ho-4J*) (Lalouette et al., 1998) and *hotfoot*-Nancy (*ho*^{Nancy}) (Lalouette et al., 2001) are of particular interest. The *ho-4J* mutation results in the loss of 170 amino acids from the extracellular N-terminal LIVBP-like domain of GluRδ2 (Lalouette et al., 1998). This deletion

induces the retention of GluRδ2 in the ER (Matsuda and Yuzaki, 2002; Wang et al., 2003). The *ho*^{Nancy} mouse expresses a truncated transcript lacking TM1, 2 and 3. No protein could be detected by Western Blot (Lalouette et al., 2001) indicating that this *hotfoot* mutant is a complete *Grid2* knock-out mutant that does not express any GluRδ2 protein.

3.5.1 The hotfoot behavioral phenotype

Motor coordination is impaired in all *hotfoot* mice as early as P12 (Kashiwabuchi et al., 1995) due to a dysfunction of deficient Purkinje cells. In addition, the GluRδ2$^{ho/ho}$ mouse displays a characteristic involuntary spontaneous eyeblink that can be suppressed by ablation of cerebellar floculi. This suggests an abnormal signal output by the cerebellar cortex (Yoshida et al., 2004). These motor impairments are likely to result from Purkinje cell dysfunction due to the lack of GluRδ2 at parallel fiber synapses.

3.5.2 Development and synaptogenesis of the Purkinje cells in the hotfoot GluRδ2$^{ho/ho}$ *mice*

In the adult cerebellar cortex, GluRδ2 is exclusively expressed in parallel fiber-Purkinje cell synapses (Fig. 12A) (Landsend et al., 1997), although it is transiently expressed in developing climbing fiber-Purkinje cell synapses (Zhao et al., 1997).

GluRδ2$^{ho/ho}$ Purkinje cell dendrites display naked postsynaptic spines which are surrounded by Bergmann glia and are equipped with PSD but are lacking contact with presynaptic parallel fiber boutons (Fig. 12B) (Kashiwabuchi et al., 1995). Quantification of parallel fiber-Purkinje cell synapses in GluRδ2$^{ho/ho}$ cerebellar molecular layer indicated a loss of parallel fiber synapses (Kurihara et al., 1997). In addition, *Grid2* knock-out mice (GluRδ2$^{-/-}$) selectively devoid of GluRδ2 proteins in cerebellar Purkinje cells, also had naked spines (Fig. 12B) (Takeuchi et al., 2005) and mismatching of parallel fiber boutons and Purkinje cell postsynaptic densities on tertiary spines, as previously described in natural *hotfoot* mutant mice (Lalouette et al., 2001). Furthermore, the multiple climbing fiber innervation of Purkinje cells persists abnormally in the *hotfoot* adult (Kashiwabuchi et al., 1995) whereas the normal projection domain of climbing fibers (i.e. the primary and secondary Purkinje cell dendrites) extends distally to tertiary spiny branchlets (Fig. 12B). Here, climbing fibers make aberrant ectopic synapses on the spiny branchlets of the neighboring Purkinje cells (Ichikawa et al., 2002). The blockade of electrical activity in wild-type cerebellum induces the formation of parallel fiber synapses on primary and secondary dendrites which are normally specific for

climbing fiber synapses (Fig. 12C). In this case, the expression of GluRδ2 occurs in both climbing fiber- and parallel fiber-Purkinje cell synapses (Morando et al., 2001) suggesting that climbing fiber activity normally prevents GluRδ2 targeting to proximal dendrites in the mature Purkinje cells. In support of this hypothesis, the blockade of the electrical activity of climbing fibers induced the expression of GluRδ2 at the proximal spines which are reinnervated by parallel fibers in the absence of climbing fiber innervation (Fig. 12C) (Cesa et al., 2003). The reinnervation of these spines by climbing fibers after restoring electrical activity was accompanied by the disappearance of GluRδ2. These data demonstrate that GluRδ2 is downregulated by mature climbing fiber activity.

3.6 The Lurcher *mutation kills Purkinje cells*

The *Lurcher* mutation was first described as a spontaneous mutation in mouse by Phillips (1960) and is attributed to a semi-dominant gain-of-function mutation in the SYTANLAAF motif of the TM3 domain of the *Grid2* gene (Zuo et al., 1997). The perinatal death of the homozygous GluRδ2$^{Lc/Lc}$ mutant mice can be explained by the loss of large trigeminal motor neurons involved in suckling (Cheng and Heintz, 1997).

The heterozygous GluRδ2$^{Lc/+}$ mutant mice display specific ataxia due to postnatal Purkinje cell degeneration which results in an almost total loss of the Purkinje cell population and target death-related loss of granule cells (Swisher and Wilson, 1977; Wetts and Herrup, 1982).

3.6.1 The Lurcher *mutation and glutamate receptors*

The implication of the mutated SYTANLAAF motif in the constitutive activation of GluRδ2^{Lc} has been validated by inducing this mutation in AMPA and kainate receptors which then display similar activity without ligand binding, slower deactivation and desensitization in the presence of an agonist (Kohda et al., 2000; Schwarz et al., 2001). This indicates that the *Lurcher* mutation affects receptor affinity and that this conserved SYTANLAAF motif plays a major role in the ionic channel properties of ionotropic glutamate receptors. This motif seems to play an important role in the gating properties of AMPA and NMDA GluR channels too, because these properties are also altered when the *Lurcher* mutation is introduced in these receptors (Ikeno et al., 2001). The introduction of the *Lurcher* mutation in GluR1 increased the affinity of GluR1^{Lc} for glutamate and reduced the desensitization of GluR1^{Lc} channel at low receptor occupancy. This explains the small and sustained current observed in these

GluR1^{Lc} channels (Klein and Howe, 2004). GluR1^{Lc} and GluR6^{Lc} mutant receptors expressed in HEK cells can form functional heteromeric channels with GluRδ2^{Lc}, which permit glutamate-induced currents. In contrast, the formation of heteromeric wild-type GluRδ2/iGluR channels modifies glutamate signaling by forming non-functional channels, leading to the reduction of glutamate-induced currents (Kohda et al., 2003). The pore of GluRδ2^{Lc} shows functional similarities to Ca^{2+}-permeable AMPA/kainate receptors and is able to form ion channels that are potentiated by extracellular Ca^{2+} at physiological concentrations (Wollmuth et al., 2000). This Ca^{2+} influx or Ca^{2+}-dependent potentiation may contribute to the role of GluRδ2 in cerebellar LTD.

3.6.2 The molecular basis of Purkinje cell death in the Lurcher *mouse*

The massive neurodegeneration observed in the cerebellar cortex of the GluRδ2$^{Lc/+}$ mouse has been shown to involve apoptosis (Phillips, 1960; Norman et al., 1995; Wullner et al., 1995; Selimi et al., 2000c). Purkinje cell death is believed to result from excitotoxic effects of the *Lurcher* mutation in the GluRδ2 receptor (Zuo et al., 1997) even in the absence of any ligand binding (Kohda et al., 2000).

Investigation of the apoptotic mechanisms leading to Purkinje cell death in the *Lurcher* mouse revealed increased BAX and BCL-$_{XL}$ expression by Purkinje cells (Wullner et al., 1998). Nevertheless, overexpressing *Bcl-2* (Zanjani et al., 1998a) and knocking-out *Bax* only delayed Purkinje cell death (Doughty et al., 2000; Selimi et al., 2000c). Overexpressing *Bcl-2* prevented target-related cell death of inferior olivary neurons (Zanjani et al., 1998b), whereas inactivation of *Bax* rescued granule cells from target-related apoptotic cell death (Selimi et al., 2000c). The involvement of mitochondrial apoptotic factors in *Lurcher*-induced cell death is supported by the translocation of cytochrome c from mitochondria to the Apaf1 complex during the period of maximal neuronal death in the cerebellum of the GluRδ2$^{Lc/+}$ mouse (Frischmuth et al., 2006). The effector protein caspase-3, but not the apoptotic factor p53, was shown to be involved in apoptosis of GluRδ2$^{Lc/+}$ Purkinje cells and granule cells (Doughty et al., 2000; Selimi et al., 2000a; 2000b). As both *Bax* inactivation and *Bcl-2* overexpression only delayed GluRδ2$^{Lc/+}$ Purkinje cell death, Bcl-2 family members seem to contribute to Purkinje cell apoptosis, but additional extracellular apoptotic signaling cannot be ruled out. Indeed, the elimination of tissue plasminogen activator, a serine protease known to activate caspase-8 also delayed GluRδ2$^{Lc/+}$ Purkinje cell and granule cell apoptosis (Lu and Tsirka, 2002). Caspase-3 was not detected in many cerebellar neurons stained with fluoro-Jade B, a

specific marker for dying neurons (Baurle et al., 2006). These data suggest that more than one neuronal death mechanism operates in the GluRδ2$^{Lc/+}$ Purkinje cells. Further investigations should help to elucidate complex neuronal responses elicited by excitotoxicity in the *Lurcher* Purkinje cells.

4. The Nagasaki prion protein-deficient mice

Prion diseases or transmissible spongiform encephalopathies (TSEs) are infectious, fatal neurodegenerative diseases that affect humans and various animals. These include Creutzfeld Jacob disease, scrapie and BSE (appendix N°2). In humans, prion diseases manifest as rapid progressive dementias with clinical visual or cerebellar signs and akinetic mutism.

The agents that cause TSEs are known as prions (Prusiner, 1982). One hypothesis postulates that prions are PrP^{res} (prion protein resistant to proteinase K) (Bolton et al., 1982; Prusiner et al., 1984) a conformational isoform of the host cellular prion protein, PrP^{C} (Caughey and Raymond, 1991). Although many different functions have been attributed to PrP^{C}, its physiological function remains unclear.

4.1 ***The prion protein family***

The prion gene family consists in three known members: the prion protein gene *Prnp*, the Doppel gene *Prnd* and the Shadoo gene *Sprn*.

4.1.1 The prion protein gene Prnp

The prion protein gene *Prnp* is located on chromosome 2 in mice (Horiuchi et al., 1998) and encodes PrP^{C} (Oesch et al., 1985; Basler et al., 1986). PrP^{C} is the cardinal protein involved in prion diseases (Bueler et al., 1993) including the genetic forms of TSEs caused by mutations in *Prnp*. These are hereditary Creutzfeld-Jacob disease, fatal familial insomnia and Gertsmann-Straüssler-Scheinker syndrome.

4.1.2 The Doppel gene Prnd

The Doppel gene *Prnd* is located 16kB downstream of the murine *Prnp* locus and encodes a 179 amino acid-long prion protein-like Doppel (Dpl) (Moore et al., 1999; Li et al., 2000a) which shares 25% identity with PrP^{C}. Detailed analysis of the toxicity of abnormal overexpression of Dpl in central neurons in some $Prnp^{0/0}$ mouse lines is one of the research axis of my thesis (see below).

4.1.3 The Shadoo gene Sprn

Shadoo, a short protein homologous for the central hydrophobic region of PrP^{C} was discovered while looking for nucleotide sequences homologous to the *Prnp* sequence. The

Shadoo coding gene *Sprn* is located on chromosome 7 in mouse and its expression is restricted to the brain (Premzl et al., 2003).

4.1.4 The prion proteins

4.1.4.1 The cellular prion protein

The cellular prion protein is a glycosyl phosphatidyl inositol (GPI)-anchored glycoprotein that contains a long, flexible N-terminal tail, three α-helices, and a two-stranded antiparallel β-sheet that flanks the first α-helix. PrP^C displays a single disulfide bond which links helices 2 and 3 thereby stabilizing the C-terminus (Fig. 13). The N-terminus contains two specific regions, a copper-binding N-terminal octapeptide regions and a hydrophobic core (Fig. 13) (Locht et al., 1986; Riek et al., 1997). Mutations within this hydrophobic region favor the formation of C- and N-terminal ^{ctm}PrP and ^{Ntm}PrP transmembrane topological variants of PrP^C. The ^{Ctm}PrP causes neurodegeneration (Hegde et al., 1998). Although it does not appear to be infectious when inoculated in reporter mice, transgenic mice expressing ^{Ctm}PrP develop neurological illness and neuronal death that resembles certain prion diseases (Stewart et al., 2005).

4.1.4.2 Doppel

Dpl, like PrP^C, is an α-helical protein with a C-terminal GPI anchor (Fig. 13) (Silverman et al., 2000). Nevertheless Dpl resembles a truncated form of PrP^C with approximately 25% sequence identity and an additional outer disulfide bond (Lu et al., 2000). Disruption of the helix B separates it into B and B′ regions, and the two β-strands have opposing orientations (Fig. 13). Interestingly, no evidence of disease association with Dpl could be disclosed (Mead et al., 2000) and Dpl does not have any scrapie isoform (Settanni et al., 2002; Peoc'h et al., 2003).

4.1.4.3 Shadoo

Murine Shadoo is a protein with N- and C-terminal signal sequences similar to PrP^C and Doppel (Fig. 13) (Premzl et al., 2003). Shadoo shares homology to the PrP^C N-terminal domain with a series of N-terminal charged tetrarepeats. As mentioned, the bulk of the homology between Shadoo and PrP^C is found within the hydrophobic tract. Additionally, Shadoo is devoid of cysteine residues, preventing the formation of stabilizing disulfide bonds.

Shadoo has recently been shown to counteract neurotoxic activity of Dpl in a PrP^C-like neuroprotective activity (Watts et al., 2007)

4.2 ***The cellular prion protein PrP^C***

4.2.1 Expression of PrP^C in the central nervous system

Prion protein gene expression is detected as early as E13.5 in the developing mouse brain (Manson et al., 1992). It further increases in a region-specific manner during the postnatal period (Mobley et al., 1988). In addition, glial cells express prion protein mRNAs throughout postnatal development in rodent brain (Moser et al., 1995). In the adult, high levels of PrP^C are found in brain and spinal cord neurons (Manson et al., 1992; Harris et al., 1993), as well as in neurons and Schwann cells of the peripheral nervous system (Ford et al., 2002). In the brain, PrP^C has been detected in neurons of the olfactory bulb, the neocortex, the amygdala, the putamen, the hippocampus, the brainstem and the cerebellum (Fournier et al., 1995; Sales et al., 1998; Haeberle et al., 2000; Laine et al., 2001; Mironov et al., 2003; Bailly et al., 2004; Galvan et al., 2005). PrP^C is a neuronal cell surface protein (Caughey and Raymond, 1991) distributed in patches on the surface of the cerebellar Purkinje cells, granule cells and deep cerebellar neurons (Bailly et al., 2004). The preferential synaptic distribution of PrP^C has been described at pre- and post-synaptic elements of excitatory synapses on Purkinje cells (Haeberle et al., 2000; Bailly et al., 2004), as in other central synapses (Fournier et al., 1995; Bailly et al., 2004). Taking into account the synaptic localization of PrP^C and electrophysiological studies on hippocampal slices devoid of PrP^C strongly suggest that PrP^C has important synaptic functions (Collinge et al., 1994; Fournier et al., 1995; Herms et al., 1999). Indeed, in these hippocampal slices, long term potentiation is impaired (Collinge et al., 1994) and the expression level of PrP^C is correlated with glutamatergic synaptic transmission (Carleton et al., 2001). Moreover, Ca^{2+}-activated K^+ currents are decreased in Purkinje cells from *Prnp*-ablated mice (Herms et al., 2001) and the whole cell current of granule cells incubated with a recombinant PrP is reduced (Korte et al., 2003). This and the fact that hippocampal neurons depleted in cholesterol have an abnormal cell membrane expression of PrP^C (Galvan et al., 2005) suggest an important role for PrP^C in the regulation of synaptic transmission.

In cultured fetal hippocampal neurons, expression of a recombinant form of PrP results in the elaboration of axons and dendrites and increases the number of synaptic contacts (Kanaani et al., 2005). Neuritogenesis decreases when neurons or astrocytes do not express PrP^C in

neuron-astrocyte cocultures (Lima et al., 2007) and it has been demonstrated *in vivo* that PrP^C also plays an important role in cellular differentiation during neural development (Steele et al., 2006). Taken together, these data indicate that PrP^C can function as a growth factor involved in neurogenesis and the development of neuronal polarity.

4.2.2 Cell trafficking of PrP^C

PrP^C biosynthesis is achieved by translocation to the ER due to the presence of an N-terminal signal peptide. This signal peptide is then cleaved in the ER lumen and the C-terminal GPI anchor is added (Caughey et al., 1989). The secreted PrP^C form is then transported to the cell surface where it is expressed for 1 hour before undergoing endocytosis in early endosomes (Fig. 14). The important intracellular amount of PrP^C (35 to 50%) indicates that integral PrP^C is continuously cycled between the cell surface and the endosomes (Fig. 14) (Shyng et al., 1993). In this way, endocytosis of raft-associated PrP^C is likely to involve a clathrin-dependent mechanism. The copper-binding N-terminal region of PrP^C is implicated in this mechanism since deletions within the N-terminal region of PrP^C reduces internalization of the protein (Shyng et al., 1994; 1995). Furthermore, when copper ions bind to the N-terminal octapeptide repeats, PrP^C is liberated from lipid rafts prior to its internalization by clathrin-mediated endocytosis (Fig. 14) (Taylor et al., 2005). In addition, this mechanism of endocytosis depends on the intervention of the low-density-lipid receptor 1 (Taylor and Hooper, 2007).

Inhibition of the proteasome induces a cytosolic accumulation of PrP^C indicating that PrP^C can be catabolized through the ER-associated degradation (ERAD) pathway (Fig. 14) (Yedidia et al., 2001).

4.2.3 Neuronal effects of PrP^C deficiency

Less than a decade after the discovery of PrP^C, mice homozygous for disrupted *Prnp* were generated in order to characterize its physiological functions. Two *Prnp* knock-out ($Prnp^{0/0}$) mutant mouse lines Zurich I (Bueler et al., 1992) and Edinburgh (Manson et al., 1994) were generated and developed normally. Only subtle deficiencies related to neuronal functions were detected. Alteration of sleep rhythms characterized by enhanced sleep fragmentation including short waking episodes was observed in these *Prnp*-deficient mice (Tobler et al., 1996; 1997). In addition, several alterations were observed including impairment of long-term potentiation, weakened GABA receptor-mediated inhibition (Collinge et al., 1994) and

disruption of K+ currents (Colling et al., 1996) in hippocampal slices of these *Prnp*-deficient mice. Interestingly, these neurophysiological phenotypes were rescued by the introduction of a high number of transgenic copies of *Prnp* confirming that this phenotype is a consequence of the loss of PrP^C (Whittington et al., 1995). Moreover, loss of PrP^C alters both the intracellular calcium homeostasis of cultured cerebellar granule cells (Herms et al., 2000) and the maximal increase of intracellular calcium concentration provoked by depolarization in Purkinje cells. These data provide strong evidence that Ca2+-activated K+ currents in mice are reduced due to an altered intracellular calcium homeostasis. Furthermore, a profound but transient impairment of synaptic excitation and plasticity has been evidenced in granule cells of 3 week-old $Prnp^{0/0}$ mice suggesting that PrP^C plays an important role in granule cell development (Prestori et al., 2008).

In addition, PrP^C deficiency also impairs the anti-apoptotic PI3-kinase/Akt pathway, leading to apoptotic caspase-3 activation (Weise et al., 2006) (see below).

4.2.4 Molecular partners of PrP^C

Identification of molecular partners of PrP^C is one way to elucidate its potential physiological functions. The trafficking of PrP^C implies that it is exposed to a large number of molecular partners in various cell compartments, including the extracellular matrix, plasma membrane and vesicular compartments. The N-terminal domain of PrP^C has been shown to be critical for the internalization of the protein (Gauczynski et al., 2001) and several molecules bind PrP^C *in vitro* although the physiological relevance of these interactions remains to be determined.

High concentrations of PrP^C have been purified with caveolae-like domains (Vey et al., 1996) supporting an interaction with caveolin, a transmembrane adaptor of caveolae-like domains (Harmey et al., 1995). Such a direct interaction might explain the functional coupling of PrP^C that has been demonstrated with Fyn (Mouillet-Richard et al., 2000; Toni et al., 2006), a tyrosine kinase involved in mediating semaphorin function (reviewed in Ahmed and Eickholt, 2007). A number of intracellular proteins involved in neuronal signaling processes are able to bind PrP^C. They include synapsin Ib, Grb2, and prion interactor protein Pint-1 (Spielhaupter and Schatzl, 2001) although the significance of their interaction with PrP^C remains obscure.

At the cell surface, N-CAM (Schmitt-Ulms et al., 2001), glycosaminoglycans (Pan et al., 2002) and clathrin (Shyng et al., 1994; 1995) are plasmalemma-bound molecular partners of

PrP^C. Glycosaminoglycans could intervene in the pathogenic conversion of PrP^C (Pan et al., 2002) and clathrin is involved in the endocytosis of PrP^C (see above) (Shyng et al., 1994; 1995). Interaction of N-CAM with PrP^C permits the recruitment of N-CAM to lipid rafts and activation of Fyn (Santuccione et al., 2005).

Prior to its endocytosis, PrP^C can also bind the 37/67 kDa laminin receptor (Rieger et al., 1997; Gauczynski et al., 2001; Hundt et al., 2001). A binding site in both PrP^C and laminin receptor may provide direct interaction, and a second one depends on heparan sulfate proteoglycan (HSPG)-mediated interaction (Rieger et al., 1997; Gauczynski et al., 2001; Hundt et al., 2001). In turn, laminin receptor binds both PrP^C and laminin (Rieger et al., 1999)

and PrP^C binds laminin itself (Graner et al., 2000; Coitinho et al., 2006). These interactions may have important consequences during neuritogenesis (Graner et al., 2000) and memory processing (Coitinho et al., 2006).

The stress inducible protein 1 (STI1) was discovered as an additional PrP^C-associated membrane protein (Zanata et al., 2002). STI1 is a co-chaperone protein organizing Heat Shock proteins 70 and 90 complexes. STI1 binds to the hydrophobic core of PrP^C to induce neuroprotection by preventing protein kinase A-dependent cell death (Lopes et al., 2005). Binding of ST1 to PrP^C is also able to induce neuritogenesis (Lopes et al., 2005). This mechanism requires endocytosis of STI1-bound PrP^C (Caetano et al., 2008). In addition, interaction between STI1 and PrP^C also affects short- and long-term memory (Coitinho et al., 2007).

Na^+/K^+ ATPase, β-actin, α-spectrin and creatine kinase-β have been identified as potential molecular partners of PrP^C in PrP/affinity chromatography fractions (Petrakis and Sklaviadis, 2006). PrP^C, in association with Na^+/K^+-ATPase and cytoskeletal proteins may cluster and stabilize receptors in the cell membrane, while creatine kinase-β might regulate vesicle transport and neurotransmitter release.

4.2.5 Copper-binding and anti-oxidative properties of PrP^C

The well-established copper-binding and anti-oxidant properties of PrP^C are thought to be important in the regulation of synaptic copper concentration and its anti-apoptotic functions.

Copper is an essential cofactor of many cellular redox reactions and it is now widely accepted that PrP^C is a copper-binding protein (Brown et al., 1997a). Binding of a copper ion to PrP^C is achieved by two of the N-terminus octapeptide repeats (Fig. 13) (Stockel et al., 1998; Garnett and Viles, 2003) and by two histidines close to the hydrophobic core (Jones et

al., 2004). The octapeptide repeats specifically bind up to 4 Cu^{2+} ions copper in a pH-dependent and negatively cooperative manner (Walter et al., 2006). Indeed, at physiological pH, Cu^{2+} initially binds to PrP^C at the two histidines with a high affinity. Subsequently Cu^{2+} ions bind to single histidine residues within the octarepeat region with a lower affinity (Wells et al., 2006a; Wells et al., 2006b; Klewpatinond et al., 2008). In addition, there is evidence that Cu^{2+} can facilitate PrP^C self association (Wells et al., 2006a; Wells et al., 2006b) and that zinc significantly alters the distribution of copper among the available binding modes (Walter et al., 2007).

Copper binding alters PrP^C biochemical and biological properties by switching the α-helical tertiary structure into a β-sheet configuration that is different from the β-sheet-rich pathological PrP^{res} (Qin et al., 2000; Quaglio et al., 2001; Leclerc et al., 2006). Copper-bound PrP^C has increased protease resistance (Quaglio et al., 2001). PrP-deficient mice are hyper sensitive to copper toxicity and oxidative stress (Brown et al., 1998) which can be explained by the reduction in the activity of Cu/Zn superoxide dismutase (Brown et al., 1997b; Brown et al., 2002) and glutathione reductase (White et al., 1999) in neurons. Thus, PrP^C is proposed to have a proper superoxide dismutase activity (Brown et al., 1999) which directly detoxifies reactive oxygen species (ROS), responsible for oxidative stress-induced apoptosis (Brown et al., 2002). Alternatively, but not exclusively, PrP^C may indirectly up-regulate distinct Cu/Zn superoxide dismutases depending on the level of its copper charge (Fig. 15) (Brown et al., 2001; Sakudo et al., 2005a).

4.2.6 Anti-apoptotic activity of PrP^C

Neuronal apoptosis occurs in the brain infected by scrapie (Lucassen et al., 1995) and in fatal familial insomnia (Dorandeu et al., 1998) suggesting that either prions are pro-apoptotic agents or PrP^C has an anti-apoptotic cytoprotective function that is absent in prion-infected brains due to its conversion in pathological PrP^{res}. This important question remains to be answered.

Using a yeast two-hybrid system, Kurschner and Morgan (1995; 1996) demonstrated that PrP^C binds Bcl-2 family members through a common BH domain. This suggests that PrP^C could have BCL-2-like properties. In PrP-deficient neurons, serum-free culture conditions induced apoptosis that was abrogated by either PrP^C or BCL-2 (Kuwahara et al., 1999). This suggests that PrP^C is an anti-apoptotic BCL-2-like protein. This is supported by the suppression of BAX-dependent apoptosis in human neurons by PrP^C. This anti-BAX effect

has been shown to be specific for the octapeptide repeat domain of PrP^C (Bounhar et al., 2001). Furthermore, BAX-mediated neuronal death is counteracted by overproduction of cytosolic PrP^C induced by ERAD reverse translocation (Roucou et al., 2003). The anti-apoptotic properties of PrP^C have been challenged because induction of PrP^C expression in HEK cells provokes p53-mediated caspase-3 activation (Paitel et al., 2002; 2003; 2004). However, this was subsequently shown to be a cell line-specific effect since PrP^C upregulation has no neurotoxic effect on N2A cells. Moreover, antibody-induced down-regulation of PrP^C increased BAX expression, caspase-3 induction and apoptosis (Zhang et al., 2006). Also, PrP-deficient neuronal cell lines underwent down-regulation of BAX, caspase-3 and cytochrome c after PrP^C expression was restored (Kim et al., 2004). Recently, PrP^C anti-apoptotic activity was shown to be independent of BCL-2 in yeast (Bounhar et al., 2006). In addition, the anti-BAX properties of PrP^C have been confirmed *in vivo* as PrP^C rescued neurons from ethanol-induced BAX-mediated apoptosis (Gains et al., 2006). On the other hand, PrP^C does not prevent BAK- , BID- and staurosporine-induced apoptosis indicating that anti-apoptotic properties of PrP^C are BAX-specific. Indeed, PrP^C prevents the initial conformational change of BAX necessary for its pro-apoptotic activation (Roucou et al., 2005).

4.3 ***The prion protein paralogue Doppel***

Research efforts to determine the functions of PrP^C in knock-out mutant mice (Fig. 16) have revealed that large deletions in *Prnp* result in the ectopic neuronal expression of Dpl. Such disruptions of *Prnp* extend into the upstream region of intron 2 resulting in cis-activation of *Prnd*. This allows an abnormal intergenic splicing between the *Prnp* promoter and the *Prnd* ORF (Moore et al., 1999) and prevents cleavage of *Prnp* pre-mRNA which is elongated up to the last exon of *Prnd*. The production of chimerical mRNAs made up of *Prnp* exons 1 and 2 and *Prnd* coding exons results in abnormal regulation of *Prnd* under the control of the *Prnp* promoter (Fig.17) (Moore et al., 1999; Li et al., 2000a; 2000b; Yoshikawa et al., 2007). The resulting ectopic Dpl neuronal expression in $Prnp^{0/0}$ mouse lines (Fig. 16) such as Ngsk (Sakaguchi et al., 1995; 1996), Rcm0 (Moore et al., 1999), ZrchII (Rossi et al., 2001) and Rikn (Kuwahara et al., 1999) leads to late onset Purkinje cell degeneration and ataxia.

4.3.1 Somatic and germinal expression of Dpl

Dpl is highly expressed in Sertoli cells and spermatozoa of the testis (Peoc'h et al., 2002) (Tranulis et al., 2001) during adulthood. During embryogenesis, Dpl is transiently expressed under the control of Brn-3a and Brn-3b transcription factors in dorsal root ganglia and spinal cord neurons as early as E13.5 (Calissano et al., 2004). Lower levels of Dpl mRNA are detected in spleen, heart, bone marrow, skeletal muscles and neocortex of the neonatal hamster, but not in kidney, liver and lung (Li et al., 2008). Significant levels of Dpl expression have never been detected in the brain of any adult mammalian species.

4.3.2 Physiological functions of Dpl

The only obvious phenotypic feature of Dpl-deficient *Prnd*$^{0/0}$ mice is male, but not female sterility. *Prnd*$^{0/0}$ spermatid numbers are strongly reduced and spermatozoid heads are misoriented (Behrens et al., 2002). This deficit impairs the capacity of the spermatozoid to cross the *zona pellucida* of the egg (Behrens et al., 2002). The important oxidative DNA damages resulting from Dpl deficiency in male gametes suggests that Dpl is involved in spermatogenesis through its anti-oxidant protective functions (Paisley et al., 2004). In addition, the subcellular localization of Dpl appears to evolve with the different stages of male gametogenesis with a strong early nuclear localization before concentrating in the cytoplasm (Kocer et al., 2007).

The structural homology between PrP^{C} and Dpl (Fig. 13) suggests a subcellular colocalization. Indeed, the immunohistochemical distribution of Dpl in the brain of *NP*$^{0/0}$ mouse and of PrP^{C} in the brain of wild-type mouse are similar (Al Bersaoui et al., 2005). In neuroblastoma cell cultures, Dpl and PrP^{C} get internalized together from patches on the plasma membrane (Massimino et al., 2004). In addition, like PrP^{C} (Zidar et al., 2008), Dpl is a copper-binding protein (Qin et al., 2003). Since copper-binding is a prerequisite for PrP^{C} through interaction with the precursor of laminin receptor, the binding of Dpl to this precursor suggests that PrP^{C} and Dpl are internalized together (Yin et al., 2004). Nevertheless, Dpl does not interact with all the molecular partners of PrP^{C}. It does not interact with Grb2 (Azzalin et al., 2005), but interacts with RACK1 (Azzalin et al., 2006), an intracellular adaptor protein involved in the regulation of intracellular calcium (Sklan et al., 2006). Whereas PrP^{C} increases the concentration of subplasmalemmal calcium pools in CHO cells, Dpl has a depleting effect which can be abolished by PrP^{C}. This is consistent with a functional interplay

and an antagonistic role of the two proteins, whereby PrP^C protects and Doppel sensitizes cells toward stress conditions (Brini et al., 2005).

4.3.3 The neurodegenerative phenotype of the Nagasaki mouse

When the first $NP^{0/0}$ mouse line overexpressing Dpl was observed to display premature Purkinje cell loss (Sakaguchi et al., 1996), it was postulated that PrP^C was needed to ensure Purkinje cell survival during aging (Nishida et al., 1999). In other words, the resistance to prion disease conferred by PrP^C deficiency should result in late onset ataxia. Several neuropathological signs have been reported in the brain of the Dpl-overexpressing mice, including necrosis of pyramidal cells in the hippocampus (Moore et al., 2001), torpedo-like varicosities of Purkinje cell axons projecting into the granular layer, abnormal myelinisation in the spinal cord and peripheral nervous system (Nishida et al., 1999), and abnormal astrocytic and microglial activation in the forebrain and cerebellum (Atarashi et al., 2001).

Subsequently, Dpl was shown to be expressed in $NP^{0/0}$ Purkinje cells and hippocampal pyramidal cells and to be responsible for Purkinje cell neurodegeneration (Li et al., 2000a; 2000b). Furthermore, overexpression of Dpl in the non-ataxic ZrchI $Prnp^{0/0}$ mice induced severe granule and Purkinje cell loss in the cerebellum (Moore et al., 2001). Conversely, Purkinje cell neurodegeneration did not occur when Dpl overexpression was suppressed by knocking-out *Prnd* in ZrchII $Prnp^{0/0}$ mutant mice. These data confirm that Dpl is responsible for Purkinje cell death in the Dpl-overexpressing $Prnp^{0/0}$ mutant mouse lines (Genoud et al., 2004). Furthermore, decreased Dpl expression in ZrchI $Prnp^{0/0}$; $NP^{0/0}$ and ZrchI $Prnp^{0/0}$; ZrchII $Prnp^{0/0}$ double mutant mice displayed a delayed neurological effect of Dpl in proportions suggesting that the onset of ataxia and Purkinje cell loss is Dpl gene dose-dependent (Moore et al., 2001; Rossi et al., 2001; Valenti et al., 2001).

4.3.4 Neurotoxicity of Doppel

4.3.4.1 Doppel can be considered as a N–terminal truncated PrP (ΔPrP)

To identify regions of the PrP^C sequence involved in prion disease, ZrchI mice expressing N-terminal truncated PrP^C have been generated. Among these transgenic mouse lines, the ΔPrP (lacking aa32-134) transgenic mouse display severe granule cell loss and severe ataxia that is rescued by re-expression of a full-length PrP (Shmerling et al., 1998). When targeted to Purkinje cells, the ΔPrP transgene induces Purkinje cell loss and ataxia, as observed in Dpl-overexpressing mouse lines, suggesting that Dpl and ΔPrP cause Purkinje cell death by the

same mechanism (Flechsig et al., 2003). Indeed, Dpl fused with the N-terminal segment lacking in ΔPrP has anti-apoptotic activity *in vitro* (Lee et al., 2006).

4.3.4.2 Pro-apoptotic properties of Doppel

Pro-apoptotic properties of Dpl were suggested by TUNEL-identification of apoptotic granule cells in the cerebellar cortex of ZrchI mice transgenically expressing Dpl (Moore et al., 2001). Indeed, oxidative stress was observed in Dpl overexpressing central neurons of *Rcm0* mice with increased activity of heme oxygenase 1, as well as neuronal and inducible nitric oxide synthase (Wong et al., 2001). Furthermore, abnormal activation of nitric oxide synthase by exogenous Dpl peptide was antagonized by exogenous PrP^C in cultured cerebellar cells (Cui et al., 2003). Removing serum from culture medium of Dpl-overexpressing Rikn hippocampal neurons (Kuwahara et al., 1999) induced apoptosis which could be abolished by reexpression of PrP^C, suggesting that PrP^C-Dpl interaction can regulate cell survival (Sakudo et al., 2005b). Dpl-induced apoptosis involves a caspase-dependent apoptotic pathway since N2A cells transfected with a plasmid coding for Dpl display activation of the apoptotic effector caspase-3, DNA fragmentation and apoptosis (Qin et al., 2006). In addition, overexpression of BCL-2 in ΔPrP mice reduced caspase-3 activation (Nicolas et al., 2007).

4.3.4.3 Doppel and PrP^C antagonism

Two hypothetical mechanisms have been proposed to explain the interaction between PrP^C and Dpl: the competition mechanism and the sensitization mechanism (Fig. 18).

1.1.1.1.1 The competition model

In the competition model (Fig. 18A), PrP^C and a still unknown low affinity molecule called π compete to bind an unidentified ligand L_{PrP} to elicit an anti-apoptotic activity (Shmerling et al., 1998; Kuwahara et al., 1999). Dpl as well as ΔPrP are able to bind L_{PrP} in the absence of PrP^C and, this would hijack its cell survival-promoting properties into apoptosis (Shmerling et al., 1998; Flechsig et al., 2003; Lee et al., 2006). Along this line, the laminin receptor precursor protein has been proposed to be a potential L_{PrP} because it binds both PrP^C and Dpl (Gauczynski et al., 2001; Hundt et al., 2001; Yin et al., 2004).

1.1.1.1.2 The sensitization model

Serum withdrawal increases apoptosis of *Prnp*-deficient neurons (Kuwahara et al., 1999), suggesting that PrP^C has a neuroprotective role itself (Fig. 18B). Indeed, PrP^C SOD activity

(Brown et al., 1999) can antagonize Dpl-induced oxidative stress (Wong et al., 2001; Cui et al., 2003) and N-terminal truncated PrP^C abrogates its capacity to rescue neurons from Dpl toxicity (Atarashi et al., 2003). In the sensitization hypothesis, the apoptogenic oxidative damage caused by Dpl would not be detoxified in neurons because lacking PrP^C.

Results

1. Purkinje cell death mechanisms induced by mutations of the glutamatergic GluRδ2 receptor in mouse

1.1 *Publication 1.* Lurcher GRID2-induced death and depolarization can be dissociated in cerebellar Purkinje cells. Selimi F, Lohof AM, Heitz S, Lalouette A, Jarvis CI, Bailly Y, Mariani J. Neuron (2003) 37:813-9.

Based on data showing that the C-terminus region of GluRδ2^{Lc} activates the autophagic promoter Beclin1 (Liang et al., 1998) via nPIST (Yue et al., 2002), the first research theme of my thesis was focused on the mechanisms controlling the onset of Purkinje cell death induced by GluRδ2^{Lc}. I contributed to a first study in which heteroallelic mutant mice expressing only one copy of the *Lurcher* allele and no copy of the wild-type allele were generated. In these mice, Purkinje cell death occurred already at P5, when the cerebellum is not yet obviously affected and preceded chronic depolarization in GluRδ2$^{Lc/+}$ mutant mice. In the GluRδ2$^{ho/Lc}$ Purkinje cells, autophagic profiles occurred in correlation with massive Purkinje cell death, when GluRδ2^{Lc} could not induce depolarization of Purkinje cells. These results suggest that GluRδ2^{Lc} receptors activate a neuronal death pathway independent of depolarization.

1.2 GluRδ2^{Lc}-induced excitotoxicity kills Purkinje cells

Although the *Lurcher* mutation has been known to be responsible for excitotoxic apoptotic Purkinje cell death for a long time, clear evidence for autophagy has only been recently obtained showing autophagosomes accumulation in axonal swellings as well as in somato-dendritic compartments of the *Lurcher* Purkinje cells (Wang et al., 2006). The increased density of mitochondria and oxidative stress in the GluRδ2$^{Lc/+}$ Purkinje cells suggests that an apoptotic pathway is activated in these neurons (McFarland et al., 2007). Nevertheless, autophagic profiles appear in GluRδ2$^{Lc/+}$ Purkinje cells before ion flux-mediated excitotoxicity induces neuronal death suggesting the early activation of a non-ionotropic cell death mechanism. The putative dissociation between non-ionotropic (reflected by autophagy) and ionotropic neurotoxic cascade may be achieved by experimentally blocking GluRδ2^{Lc}-mediated ion flux. For this reason, I treated GluRδ2$^{Lc/+}$ organotypic cerebellar cultures with 1-Naphtyl-acetyl-spermine (NASP), an open channel blocker known to considerably reduce Ca^{2+} entry (Koike et al., 1997) and GluRδ2$^{Lc/+}$-mediated currents (Kohda et al., 2000). If autophagy is independent from GluRδ2$^{Lc/+}$-induced excitotoxicity, NASP-blockade of excitotoxic ion flux is predicted to not affect autophagy in the treated GluRδ2$^{Lc/+}$ Purkinje cells.

1.2.1 Blockade of ion flux excitotoxicity rescues GluRδ2$^{Lc/+}$ Purkinje cells

Organotypic cerebellar cultures obtained from P0 GluRδ2$^{Lc/+}$ and wild-type cerebellum were maintained for 6 and 12 days *in vitro* (DIV) (Fig. 19A). NASP treatment during the 12 day-long culture period restored GluRδ2$^{Lc/+}$ Purkinje cell numbers to wild-type levels (P = 0.97) while this treatment had no effect on wild-type Purkinje cells (P = 0.97) (Fig. 19B). When the culture period was restricted to 6 days, equivalent Purkinje cell populations survive in GluRδ2$^{Lc/+}$ and wild-type cultures whereas 6 day-long NASP treatment had no effect on either culture (P = 0.95) (Fig. 19B). In addition, GluRδ2$^{Lc/+}$ Purkinje cells did not survive when NASP treatment was applied during the 6 first days of the 12 DIV period (P = 1.00). On the contrary, when these 12 DIV GluRδ2$^{Lc/+}$ cerebellar organotypic cultures were treated with NASP for either the 4 or the 2 last days, there was an increased Purkinje cell survival (P < 0.05 and P < 0001) (Fig. 19B). GluRδ2$^{Lc/+}$ Purkinje cell numbers were restored to wild-type level when NASP treatment was applied for the last 6 days of the 12 DIV period (P = 0.29) (Fig. 19B). This indicates that Purkinje cells of 12 DIV cerebellar organotypic cultures from P0 GluRδ2$^{Lc/+}$ mice have a differential sensitivity to GluRδ2^{Lc} toxicity after 6 DIV.

1.2.2 Blockade of ionotropic excitotoxicity suppresses autophagy and rescues dendritic development of GluRδ2$^{Lc/+}$ Purkinje cells.

Immunofluorescent staining for the autophagic marker LC3B disclosed autophagic corpuscles in the soma of CaBP-positive GluRδ2$^{Lc/+}$ Purkinje cells which display severe dendritic atrophy after 12 DIV in organotypic cultures (Fig. 20 A-C). Blocking the excitotoxicity of GluRδ2$^{Lc/+}$ by NASP treatment for 12 DIV suppressed LC3B immunoreactivity (Fig. 20 E, F)of GluRδ2$^{Lc/+}$ Purkinje cells (Fig. 20 D, F) and rescued wild-type-like morphology of the dendritic arborisation (Fig. 20 G-I).

NASP treatment during the 12 day-long culture period (Fig. 21A) restored GluRδ2$^{Lc/+}$ Purkinje cell dendritic area and maximum dendritic length to the same extent as wild-type (P = 0.87 and P = 0.91) while this treatment had no effect on wild-type Purkinje cells (P = 0.91 and P = 1.00) (Fig. 21B). The dendritic area and maximum dendritic length of GluRδ2$^{Lc/+}$ Purkinje cells were not restored by a NASP treatment applied during the 6 first days of the 12 DIV period (P = 0.57 and P = 0.76) (Fig. 21B). On the contrary, in these 12 DIV GluRδ2$^{Lc/+}$ cerebellar organotypic cultures, NASP treatment during the last 4 days induced a significant increase in the maximum dendritic length of Purkinje cells (P < 0.05) (Fig. 21B). GluRδ2$^{Lc/+}$ Purkinje cell dendritic area and maximum dendritic length were restored to the same extent as wild-type Purkinje cells when NASP treatment was applied for the last 6 days of the 12 DIV period (P = 1.00 and P = 0.77) (Fig. 21B). As observed for Purkinje cell survival, this indicates that growth of Purkinje cells in cerebellar organotypic cultures during 12 DIV from P0 GluRδ2$^{Lc/+}$ mice is only sensitive to GluRδ2^{Lc} toxicity after 6 DIV.

*1.3 **Impaired survival and dendritic development of* **hotfoot** *Purkinje cells ex vivo.***

The effect of a GluRδ2-deficiency on Purkinje cell survival and growth was investigated in cerebellar organotypic slice cultures derived from natural *Grid2* knock-out *hotfoot* mice (Table 1). The GluRδ2$^{ho/ho}$ Purkinje cell population was significantly decreased compared to the wild-type Purkinje cell population (T = 2.89, P < 0.05) (Fig. 22A). No significant differences between the two genotypes were evident by a quantitative measurement of dendritic tree area (W = 16.0, P = 1.00) and maximum dendritic length (W = 10.0, P = 0.058). Nevertheless, estimation of branching points disclosed a significant difference between GluRδ2$^{ho/ho}$ and wild-type secondary (P < 0.01) and tertiary (P < 0.0001) dendritic

ramifications whereas the GluRδ2$^{ho/ho}$ and wild-type proximal dendrites did not differ (P = 0.97) (Fig. 22B, C).

1.4 ***Delayed climbing fiber translocation in the developing*** **hotfoot** ***cerebellar cortex***

During the normal postnatal period, pruning of multiple climbing fiber innervation of Purkinje cells establishes the mono-innervation of each Purkinje cell by a single climbing fiber (Lohof et al., 1996). This regressive phenomenon is contemporaneous of climbing fiber somato-dendritic translocation (Chedotal and Sotelo, 1992). Whereas in the adult GluRδ2$^{ho/ho}$ cerebellar cortex, immature multiple climbing fiber innervation of Purkinje cells is abnormally maintained and the climbing projection domain on Purkinje cells is aberrantly extended (Ichikawa et al., 2002), little is known about the developmental sequence of climbing fiber-Purkinje cell relationships during the postnatal period. Using specific immunolabelling of climbing fibers by VGlut2, the somato-dendritic translocation of the climbing fibers was found to be delayed in the GluRδ2$^{ho/ho}$ cerebellar cortex as soon as the end of the pericellular nest stage at P10 (Fig. 23).

2. Doppel-induced cell death mechanism(s) in prion protein-deficient Purkinje cells of the Nagasaki mutant mouse

In PrP-deficient mice of the Nagasaki strain ($NP^{0/0}$), ectopic expression of PrP-like protein Doppel (Dpl) in central neurons induces significant Purkinje cell death resulting in late-onset ataxia. In an initial set of experiments, I have found that $NP^{0/0}$ Purkinje cell death can be partly prevented by either knocking-out the apoptotic factor BAX or overexpressing the anti-apoptotic factor BCL-2 suggesting that apoptosis may be involved in Dpl-induced death of Purkinje cells. In the next set of experiments using Western blotting and immunohistofluorescence, I showed that before (3-4 months) and during significant PC loss (6-8 months), the $NP^{0/0}$ Purkinje cells displayed increased expression of the scrapie responsive gene 1 (Scrg1) potentially associated with autophagy and of the autophagic markers LC3B and p62. At the ultrastructural level, autophagic-like profiles accumulated in somato-dendritic and axonal compartments of the $NP^{0/0}$, but not in those of the wild-type Purkinje cells. Interestingly, the most robust autophagy was observed in $NP^{0/0}$ Purkinje cell axon compartments in the deep cerebellar nuclei suggesting that autophagy is initiated in the axons. Taken together, these results indicate that Dpl may trigger both autophagic and apoptotic

processes in $NP^{0/0}$ Purkinje cells. The increased autophagic features observed in the Purkinje cells of the $NP^{0/0}$ mice may result from either an upregulation of autophagy or an extensive accumulation of autophagosomes due to progressive dysfunctioning of autophagy as observed in amyloid neurodegenerative diseases. In this latter case, impaired autophagic proteolysis may trigger apoptotic cascades.

2.1 ***Publication 2.*** **Bax contributes to Doppel-induced apoptosis of prion protein-deficient Purkinje cells. Heitz S, Zanjani H, Lutz Y, Gautheron V, Bombarde G, Richard F, Fuchs JP, Vogel M, Mariani J, Bailly Y. Dev Neurobiol, (2007) 67:670-686.**

2.2 ***Publication 3.*** **BCL-2 counteracts Dpl-induced apoptosis of prion protein-deficient Purkinje cells in the Ngsk Prnp$^{0/0}$ mouse. Heitz S, Gautheron V, Lutz Y, Rodeau J-L, Zanjani HS, Sugihara I, Bombarde G, Richard F, Fuchs J-P, Vogel MW, Mariani J, Bailly Y. Dev Neurobiol, (2008) 68:332-348**

2.3 ***Publication 4.*** **Autophagy and cell death of Purkinje cells overexpressing Doppel in Ngsk Prnp-deficient mice. Heitz S, Leschiera R, Haeberlé A-M, Demais V, Grant N, Bombarde G, Bailly Y. Brain Pathol, in review.**

Discussion

I will briefly summarize the main results of my thesis and discuss them in the specific context of the *Lurcher* and the Nagasaki mutant mice and other murine models of neurodegeneration. The importance of apoptotic and autophagic processes in the degeneration of Purkinje cells in *Lurcher* and Nagasaki mice will be then compared in light of the mechanisms provoking neuronal death in the two models. Finally, future experimental research axes will be described that could further improve our knowledge of neuronal death mechanisms in the central nervous system.

1. Excitotoxicity and autophagy are related during *Lurcher* Purkinje cell death

Young postnatal GluRδ2$^{Lc/+}$ Purkinje cells have been reported to display autophagy before they undergo excitotoxic apoptotic cell death (Yue et al., 2002). This autophagy has been suggested to occur independently from GluRδ2^{Lc}-induced ionotropic excitotoxicity since I contributed to show that Purkinje cell death and autophagy occur in the heteroallelic GluRδ2$^{Lc/ho}$ double mutant mouse well before ionotropic excitotoxic cell death could operate in GluRδ2$^{Lc/+}$ Purkinje cells (Selimi et al., 2003: publication 1). In most cases, autophagy is believed to constitute a major neuroprotective response to cellular stress and metabolic deficiencies, but autophagy may also be a response to an early, GluRδ2^{Lc}-mediated injury distinct from ionotropic excitotoxicity.

A clue for the mechanism underlying Purkinje cell death in this model comes from my recent experiments on organotypic cerebellar cultures of GluRδ2$^{Lc/+}$ mice. The massive death of GluRδ2$^{Lc/+}$ Purkinje cells which occurs in this model was rescued by treatment with NASP, a cationic open channel blocker. This indicates that excitotoxicity is the main cause of GluRδ2^{Lc}-induced Purkinje cell death. Furthermore, NASP treatment downregulated the

expression of the autophagic marker LC3B-II in the GluRδ2$^{Lc/+}$ Purkinje cells as shown by immunocytofluorescence of organotypic cerebellar cultures. This suggested that autophagy depends on the excitotoxic mechanism induced by the aberrant functioning of mutated GluRδ2^{Lc}. One open question is whether this excitotoxicity-related autophagy is a neuroprotective response or a true cell death pathway activated by excitotoxic stress in parallel with apoptosis. The possible rescue of some GluRδ2$^{Lc/+}$ Purkinje cells when autophagy is inhibited with 3-methyl-adenine (3-MA) in *Lurcher* organotypic cerebellar cultures may support the latter hypothesis.

In addition, NASP treatment alone was sufficient to completely restore arborisation of the GluRδ2$^{Lc/+}$ Purkinje cell dendritic tree to the same extent as the wild-type suggesting that GluRδ2^{Lc} also mediates dendritic atrophy of the GluRδ2$^{Lc/+}$ Purkinje cells. Indeed, dendritic growth seems to proceed normally until 6 DIV in organotypic culture and until P6 *in vivo* (Bailly et al., 1996). The remaining question is whether GluRδ2^{Lc}-mediated autophagy is involved in Purkinje cell dendritic atrophy. Insight into this question will come from determining the effect of the autophagy inhibitor 3-MA on the dendritic growth of the GluRδ2$^{Lc/+}$ Purkinje cells in organotypic cerebellar cultures.

My investigations indicate that Purkinje cell death also occurs in the absence of GluRδ2 in the deficient *hotfoot* mutant mice, even though Purkinje cell loss could not be evidenced *in vivo* (Kurihara et al., 1997), suggesting that firstly, GluRδ2$^{ho/ho}$ Purkinje cells are more sensitive to cell death conditions and secondly, that these conditions may be counteracted *in situ*, but exacerbated in organotypic cerebellar cultures. In addition, the Purkinje cell dendritic tree of GluRδ2$^{ho/ho}$ mutant mice is not as developed as the wild-type mice in organotypic culture indicating that GluRδ2$^{ho/ho}$ Purkinje cell growth is impaired in organotypic cultures. On the other hand, the extent of the Purkinje cell dendritic tree is similar in wild-type and GluRδ2$^{ho/ho}$ mice *in situ* (Ichikawa et al., 2002). My data in organotypic cerebellar cultures confirm that GluRδ2 plays an essential role in the survival and dendritic development of Purkinje cells in this model. Restoring GluRδ2 expression in GluRδ2$^{ho/ho}$ and GluRδ2$^{-/-}$ mutant mice re-establishes the normal synaptic configuration of parallel fiber- and climbing fiber-Purkinje cell innervation (Kohda et al., 2007). The numerous naked spines on GluRδ2$^{ho/ho}$ Purkinje cell dendrites are reinnervated by homologous afferences. This is unlikely to occur in organotypic cultures of GluRδ2$^{ho/ho}$ Purkinje cells since both sources of homologous afferents are lacking. In such conditions, restoring GluRδ2 expression in these

neurons in the presence or absence of 3-MA might provide insight into the potential role of this receptor in the autophagic recycling of naked spines lacking afferent inputs. Furthermore, treating GluRδ2$^{ho/ho}$ Purkinje cells in organotypic cerebellar cultures with the autophagic activator rapamycin should indicate whether the naked spines of the GluRδ2$^{ho/ho}$ Purkinje cells can be recycled by autophagy.

Since Purkinje cell dendritic growth has not yet been investigated in the postnatal cerebellum of the *hotfoot* mouse, retardation in postnatal dendritic development as a potential factor of synaptogenesis impairment cannot be ruled out. This is in line with the deficiency in climbing fiber translocation observed in the cerebellar cortex of the 10 to 15 day-old postnatal GluRδ2$^{ho/ho}$ mutant mice. At this age, GluRδ2 is expressed at the climbing fiber-Purkinje cell synapses in the wild-type cerebellum (Zhao et al., 1997). Thus, the receptor is likely to promote climbing fiber translocation since this process is delayed when GluRδ2 is lacking in the GluRδ2$^{ho/ho}$ Purkinje cells. An important role in Purkinje cell excitatory synaptogenesis has been assigned to GluRδ2 based on its expression in climbing fiber-Purkinje cell synapses during translocation and pruning periods, impaired synaptogenesis in GluRδ2-lacking Purkinje cells, and the observed shift in the partition of Purkinje cell projection domain between parallel and climbing fibers. Whether expression of GluRδ2 is altered in the abnormal development of parallel and climbing fiber synapses in other experimental and mutant models of impaired Purkinje cell excitatory synaptogenesis is an interesting, but still unanswered question. In addition, the involvement of autophagy in the climbing fiber synaptogenesis needs to be examined during the main elimination period of climbing fiber synapse using immunocytochemistry for autophagic markers at the electron microscopy scale.

More than one single mechanism probably accounts for the death of Purkinje cells in GluRδ2$^{Lc/+}$ mutants. The deletion of *Bax* expression alters the pattern of caspase-3 activation, but only delays GluRδ2$^{Lc/+}$ Purkinje cell death (Selimi et al., 2000c) implying that BAX is involved in the caspase-3-dependent death mechanism (Selimi et al., 2000b). Also delayed death of Purkinje cells in GluRδ2$^{Lc/+}$ mutant mice overexpressing *Bcl-2* supports the involvement of pro-apoptotic members of the Bcl-2 family in GluRδ2$^{Lc/+}$ Purkinje cell apoptosis (Zanjani et al., 1998a; 1998b). On the other hand, autophagy has been demonstrated in GluRδ2$^{Lc/+}$ Purkinje cells by LC3 immuncytochemistry and by ultrastructural analysis suggesting that autophagy also plays a central role in GluRδ2^{Lc}-induced Purkinje cell death. In addition, GluRδ2^{Lc} promotes activation of the major autophagic factor Beclin1 in

GluRδ2$^{Lc/+}$ Purkinje cells (Yue et al., 2002). The participation of multiple cell death pathways is further evidenced in GluRδ2$^{Lc/+}$ Purkinje cells by caspase-3 immunohistochemistry and FluoroJade-B labelling which indicates that the cellular overload produced by the initial *Grid2* defect in *Lurcher* mutants apparently activates a variety of apoptotic and non-apoptotic pathways in the Purkinje cell population (Baurle et al., 2006). In addition, nitric oxide-induced oxidative stress occurs in dying GluRδ2$^{Lc/+}$ Purkinje cells (McFarland et al., 2007) and the resulting production of ROS is known to induce autophagy (Djavaheri-Mergny et al., 2006) as well as apoptosis (reviewed in Orrenius, 2007). Nevertheless, treating GluRδ2$^{Lc/+}$ organotypic cerebellar Purkinje cells with NASP, believed to block excitotoxicity, not only significantly reduces Purkinje cell death and dendritic atrophy, but also abrogates the induction of autophagy. This supposes that the induction of autophagy in GluRδ2$^{Lc/+}$ Purkinje cells is not uniquely mediated by the property of GluRδ2^{Lc} channels to promote Beclin1 activity, but is also a cellular response to metabolic stress. Taking into account the downregulation of autophagy and the rescue of GluRδ2$^{Lc/+}$ Purkinje cells by NASP, GluRδ2^{Lc} is likely to induce autophagy in Purkinje cells by a ROS-mediated pathway rather than through its nPIST-mediated interaction with Beclin1. Altogether, these data suggest that the functional significance of autophagy in GluRδ2$^{Lc/+}$ Purkinje cells is likely to be related to a neuroprotective mechanism in response to excitotoxic insult.

2. Multiple death mechanisms induced by Dpl in Nagasaki Purkinje cells

Activation of the neurotoxic mechanism triggered by Dpl in prion-protein deficient *NP*$^{0/0}$ Purkinje cells had previously been shown to provoke the premature death of these neurons (Li et al., 2000b). Our analysis of the impact of *Bax* knock-out as well as of overexpression of *Bcl-2* on Dpl-induced Purkinje cell death revealed a contribution of Bcl-2 family members to Dpl-mediated apoptosis. Although the Purkinje cell population is partially rescued in the *NP*$^{0/0}$*:Bax*$^{-/-}$ and *NP*$^{0/0}$*-Hu-bcl-2* double mutant mice indicating that BAX is involved in Dpl-induced apoptosis, numerous Purkinje cells died by a BAX-independent mechanism in the double mutants (Heitz et al., 2007; 2008: publications 2 and 3). Such a mechanism may be either apoptotic or non-apoptotic. This question has led my further investigations to determine a possible role of autophagy in Dpl-induced Purkinje cell death (Dron et al., 2005; Heitz et al.,

in review: publication 4). Robust autophagy was observed in *NP*$^{0/0}$ Purkinje cells well before significant neuronal loss. Ultrastructural examination showed an abnormal accumulation of autophagic vacuoles and autophagolysosomes in *NP*$^{0/0}$ Purkinje cells which, in addition to the upregulation of the autophagic markers LC3B-II and p62 in these neurons, suggested that autophagic flux was either upregulated or impaired. At the current state of my research, the most plausible hypothesis is that abnormal upregulation of autophagy occurs in Purkinje cells at a maximal rate that reaches a plateau as early as 3 months and continues until 8 months as indicated by the persistence of p62 and LC3B-II upregulation. In this case, autophagy is likely to be a neuroprotective response to Dpl-induced stress, but ultimately is insufficient to prevent neuronal death. Another possibility could be that autophagy precedes and is causally linked to the subsequent onset of programmed Purkinje cell death (Canu et al., 2005; Djavaheri-Mergny et al., 2006; 2007). Alternatively, robust accumulation of autophagic organelles and p62 upregulation could reflect an impairment of the autophagic flux in Dpl-diseased Purkinje cells as it has been proposed in Alzheimer-diseased neurons. Indeed, the accumulation of autophagosomes, late autophagic vacuoles, lysosomes and autophagolysosomes in dystrophic neurites has also been reported in Alzheimer-diseased brain (Nixon et al., 2005; Yu et al., 2005) and other neurodegenerative pathologies such as prion diseases (Liberski et al., 2008). A deficiency in the autophagic process has also been proposed to occur in Huntington disease to explain the abnormal accumulation of p62-positive aggregates (Filimonenko et al., 2007) that are normally degraded through autophagy (Ravikumar et al., 2004). In this case, autophagic stress would be induced by the incapacity of the diseased neurons to clear p62-bound misfolded protein aggregates (Chu, 2006). The robust accumulation of autophagic organelles and the upregulation of p62 argue in favor of the impairment of autophagy in *NP*$^{0/0}$ Purkinje cells.

The contribution of autophagy to Dpl-induced Purkinje cell death needs to be further analyzed *in vitro* using autophagy antagonist 3-MA and agonist rapamycin in *NP*$^{0/0}$ organotypic cerebellar cultures. If autophagy contributes directly to Dpl-induced Purkinje cell death, neuronal death should be increased by rapamycin and decreased by 3-MA. On the other hand, if autophagy is a neuroprotective reaction to Dpl toxicity, rapamycin should rescue Purkinje cells and 3-MA should increase Purkinje cell death. Furthermore, investigating autophagy in the *NP*$^{0/0}$*:Bax*$^{-/-}$ and *NP*$^{0/0}$*-Hu-bcl-2* double mutant mice will provide novel insights into the relationships between intrinsic pathway of apoptosis and autophagy in the mechanism of Dpl-induced Purkinje cell death. Indeed, it is important to determine the

mechanism by which *NP$^{0/0}$:Bax$^{-/-}$* and *NP$^{0/0}$-Hu-bcl-2* Purkinje cells die. This mechanism could be autophagic independently of Bcl-2 family-related apoptosis or non-autophagic by an extrinsic apoptotic pathway (Fig. 24).

3. Differential combination of apoptosis and autophagy in Nagasaki and *Lurcher* cerebellar Purkinje cells

In light of previous reports in the literature, my results support the concept that both apoptosis and autophagy occur in Nagasaki and *Lurcher* cerebellar Purkinje cells (Table 2) (Selimi et al., 2003: publication 1; Heitz et al., 2007, 2008, in review: publications 2, 3 and 4). Although several apoptotic pathways including intrinsic and extrinsic pathways are likely to contribute to *Lurcher* Purkinje cell death (Selimi et al., 2000a; Lu and Tsirka, 2002; Selimi et al., 2003: publication 1), much less is known about the apoptotic mechanism triggered by Dpl in *NP$^{0/0}$* Purkinje cells. Certainly, the BAX-dependent intrinsic pathway is responsible for part of the *NP$^{0/0}$* Purkinje cell loss (Heitz et al., 2007, 2008: publications 2 and 3) but the results reported above argue for a second still unknown and likely extrinsic pathway. On the other hand, autophagy has recently been reported to mediate GluRδ2^{Lc}–induced Purkinje cell degeneration (Yue et al., 2002; Selimi et al., 2003: publication 1). These data show that LC3-positive autophagy occurs abundantly before Purkinje cell death without any signs of autophagic flux impairment such as autophagosome accumulation and p62 upregulation (Wang et al., 2006). The axonal origin of autophagosomes in GluRδ2$^{Lc/+}$ Purkinje cells was clearly evidenced and the incidence of autophagosomes is much less within the cell bodies and dendrites of GluRδ2$^{Lc/+}$ Purkinje cells (Wang et al., 2006). Such a limited autophagy probably cannot rescue GluRδ2$^{Lc/+}$ Purkinje cells from death, but might activate or enhance apoptotic pathways triggered by the massive Ca^{2+} entry through the constitutively activated GluRδ2^{Lc} channels (McFarland et al., 2007). Very interestingly, the autophagic features displayed by the *NP$^{0/0}$* Purkinje cells are strikingly different. Most of the autophagic organelles accumulate within Purkinje cell axon terminals and dystrophic swellings suggesting the axonal initiation of autophagy, but also autophagic flux impairment. This deficiency is supported by the upregulation of p62 in the *NP$^{0/0}$* Purkinje cells (Heitz et al., in review: publication 4). These differences indicate that Dpl and *Grid2* trigger specific cascades of programmed cell death in the same type of central neuron, the cerebellar Purkinje cell.

Autophagic deficiency has been reported in several neurodegenerative diseases including Alzheimer and prion diseases (Nixon, 2006; Liberski et al., 2008) where it is believed to contribute to neurodegeneration. Thus, the $NP^{0/0}$ Purkinje cell provides an attractive model to the cellular and molecular mechanisms of neuronal death in these pathologies in perspective of designing new therapeutic approaches.

Material and Methods

1. Animals and genotyping

1.1 ***Animals***

1.1.1 The Grid2$^{Lc/+}$(Lurcher) and the Grid2$^{ho/ho}$ (hotfoot) mice

Grid2$^{Lc/+}$ mice (gift from J. Mariani) were intercrossed to obtain *Grid2*$^{Lc/Lc}$, *Grid*$^{+/+}$, *Grid2*$^{Lc/+}$ mice in the same litter. *Grid2*$^{ho/ho}$ mice (gift from J. Mariani) were crossed with *Grid2*$^{+/+}$ mice to get *Grid2*$^{ho/+}$ mice. Littermates were intercrossed to obtain *Grid2*$^{ho/ho}$, *Grid2*$^{ho/+}$ and *Grid2*$^{+/+}$ mice in the same litter. The generation of *Grid2*$^{ho/Lc}$ mice is described in Selimi et al., 2003 (Publication 1).

Grid2^{Lc}, *Grid2*$^{+}$ and *Grid2*^{ho} alleles were identified using the further mentioned probes and primer sets.

1.1.2 The NP$^{0/0}$, the NP$^{0/0}$:Bax$^{-/-}$ and the NP$^{0/0}$-Hu-bcl-2 mice

Bax$^{-/-}$ mice were generated by deleting exons 2 to 5 (Knudson et al., 1995) and *Hu-bcl-2* mice from the NSE73a strain were generated by injecting embryos with the EB-2 construct in the cloning vector pSK+ containing the human BCL-2 coding region (Tsujimoto and Croce, 1986; Martinou et al., 1994). Further breeding strategies were designed taking into account that *Bax*$^{-/-}$ males and *Hu-bcl-2* females are sterile. *NP*$^{0/0}$ mice were generated by deleting the entire open reading frame (ORF) of the *Prnp* gene, located in exon 3, as well as 5' and 3' non-coding flanking regions (Sakaguchi et al., 1995) and the deleted sequence was replaced by a Neo cassette. For this study, founding mice have been first backcrossed with C57BL/6 mice for at least 10 generations.

For the generation of the *NP*$^{0/0}$:*Bax*$^{-/-}$ double mutant mouse line, *NP*$^{0/0}$ males and females (gift from S. Katamine) were then crossed with *Bax*$^{+/-}$ males and females (gift from S. Korsmeyer) and offspring were identified by PCR genotyping using the further mentioned

primer sets. $NP^{+/0}$:$Bax^{+/-}$ mice were further intercrossed, generating $NP^{0/0}$:$Bax^{-/-}$, $NP^{0/0}$:$Bax^{+/-}$, $NP^{0/0}$:$Bax^{+/+}$, $NP^{+/0}$:$Bax^{-/-}$, $NP^{+/0}$:$Bax^{+/-}$, $NP^{+/0}$:$Bax^{+/+}$, $NP^{+/+}$:$Bax^{+/+}$, $NP^{+/+}$:$Bax^{+/-}$ and $NP^{+/+}$:$Bax^{-/-}$ genotypes. The $NP^{0/0}$:$Bax^{-/-}$ double mutants were obtained by crossing $NP^{0/0}$:$Bax^{+/-}$ males with $NP^{0/0}$:$Bax^{-/-}$ or $NP^{0/0}$:$Bax^{+/-}$ females.

$NP^{0/0}$ females were crossed with *Hu-bcl-2* males in order to generate the $NP^{0/0}$-*Hu-bcl-2* double mutant mouse line, and offspring were identified using the further mentioned probes and primer sets. $NP^{+/0}$-*Hu-bcl-2* males were further crossed with $NP^{0/0}$ females to generate $NP^{0/0}$-*Hu-bcl-2* offspring. Strict littermates of the different genotypes have been used throughout the study.

Mice were bred at the animal facilities of Anatomisches Institut in Basel and of Neurosciences IFR37 in Strasbourg. They were maintained and submitted to experiments according to the NIH guidelines (NIH Publication 80-23, revised 1996), the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by the Swiss authorities. A minimal number of animals was used and handled with maximum care to minimize their suffering.

1.2 ***Genotyping***

1.2.1 DNA extraction

Tail samples from weanling pups were used to extract DNA. Tails were transferred in a mix of 615µl Tris-SDS-EDTA buffer (TSE, Tris 25mM pH8, SDS 1%, EDTA 25mM pH8, NaCl 75mM) and 15µl Proteinase K diluted 10mg/ml (Sigma Aldrich) for 4 hours at 55°C before centrifugation at 13000 rotations per minute (rpm) for 4 min. Supernatant was collected in 500µl isopropanol and centrifuged as previously described. The pellet was washed in 500µl ethanol 100% and centrifuged. Ethanol was removed and 100µl water was added to the pellet.

1.2.2 Genotyping

Genotyping for $Grid2^{Lc}$ and $Grid2^{ho}$ alleles was performed by PCR followed by single-strand conformation polymorphism (SSCP) using the *Grid2* forward 5'TAAAAGCATATTGATGTTGTTG3' and reverse 5'CAGCATTTGTCAGGTTTGGTGAC3' primers. Cycling parameters for PCR were 5 min at 94°C for one cycle, and 1 min at 94°C , 1 min at 60°C and 1 min at 72°C for a total of 35 cycles before 7 min at 72°C for one last cycle. A solution of 10µl of blue bromophenol

(0.05%), xylene cyanol (0.05%) and 20mM EDTA pH8 in formamide was added to 10µl of each sample before electrophoresis on a 15% acrylamide gel (Biorad) over-night at 4°C using a DCode Universal Mutation Detection System (Biorad). The gel was further stained with the Silver Staining kit (Amersham) and the *Lurcher* and wild-type alleles were detected by 2 different bands and the *hotfoot* allele by the absence of any band.

Genotyping for *Bax* was performed by PCR by using a set of three primers: *Bax* exon 5 forward primer (5'GAGCTGATCAGAACCATCATG3'), *Bax* intron 5 reverse primer 5'GTTGACCAGAGTGGCGTAGG3') and Neo reverse primer (5'CCGCTTCCATTGCT-CAGCGG3'). Cycling parameters were 2 min at 94°C for one cycle, and 45 sec at 94°C , 45 sec at 55°C and 1.5 min at 72°C for a total of 40 cycles before 7 min at 72°C for one last cycle. Four µl of DNA loading buffer (Sigma-Aldrich) was added to 8µl of each sample before an electrophoresis on a 2% agarose gel (Sigma-Aldrich) at 130V for 10 min. Bands were revealed using the UV Appligene equipment (Oncor). The Bax^{-} allele was determined by a band of 507 base pairs (Bp) and the Bax^{+} allele by a band of 304 Bp.

Genotyping for *Hu-bcl-2* was performed by PCR by using a set of two primers: *Hu-bcl-2* transgenic forward 5'GAA-GAC-TCT-GCT-CAG-TTT-GG3' and reverse 5'ATG-AGC-CTT-GGG-ACT-GTG-AA3'. Cycling, electrophoresis and identification of bands parameters were the same as for *Bax* genotyping. The presence of the *Hu-bcl-2* transgene was determined by a band of 496 Bp.

Genotyping for Dpl was performed by PCR. The *Prnp* ORF was identified using the following primers: forward 5'CCGCTACCCTAACCAAGTGT3' and reverse 5'CCTAGACCACGAGAATGCGA3', both located within the *Prnp* ORF; $NP^{0/0}$ mutants were identified using the following primers: forward 5'TGCCGCACTTCTTTGTGAAT3' and reverse 5'CGGTGGATGTGGAATGTGT3' (within Neo cassette). Cycling parameters were 5 min at 94 °C for one cycle, and 45 sec at 94 °C, 30 sec at 53 °C and 1.5 min at 72 °C for a total of 30 cycles before 10 min at 72 °C for one last cycle. Electrophoresis and identification of bands parameters were the same as for *Bax* genotyping. The $Prnp^{+}$ allele was determined by a band of 347 Bp and the $Prnp^{0}$ allele by a band of 228 Bp.

2. Methods

2.1 ***Organotypic cerebellar culture***

Organotypic cerebellar cultures were realized using the static culture method. This method, first described by Yamamoto et al (1989) and further developed by Stoppini et al (1991), allows maintaining postnatal 350µm-thick cerebellar slices in culture. Due to the sagittal orientation of the Purkinje cell in the cerebellar folia, it is possible to preserve almost the complete Purkinje cell within a slice. New-born (P0) *Grid*$^{+/+}$, *Grid2*$^{Lc/+}$ mice and P8 *Grid2*$^{ho/ho}$, *Grid2*$^{ho/+}$ and *Grid2*$^{+/+}$ mice were decapitated and their brain aseptically removed and placed into ice-cold preparation medium made of minimal essential medium (MEM, Gibco) and 2mM glutamax I (Gibco) pH 7.3. The cerebellum was dissected, the meninges removed and 350 µm-thick sagittal sections were cut using a McIllwain tissue cutter under aseptic conditions. Tissue slices were separated and transferred onto permeable membranes (Millicell, Millipore) and incubated on a layer of serum-containing culture medium (25% Basal Eagle's medium, 25% horse serum, 2mM glutamax I, 3.6mM glucose in MEM, Gibco) in a humidified atmosphere with 5% CO_2 at 37°C. The medium was changed every 2-3 days for a total of 12 days. 1-Naphtyl-acetyl-spermine NASP (Sigma-Aldrich) was added at a concentration of 50µM at medium changes.

2.2 ***Histology***

Mice were anaesthetized with sodium pentobarbital (0.15 mL per 100g i.p.; Sanofi) after which brain was dissected and immersed overnight in Carnoy's fixative (60% ethanol, 30% chloroform, 10% acetic acid). After washes in iso-butanol (Carlo Erba), the brain was embedded in paraffin and sectioned 10 µm-thick in the sagittal plane. Sections were deparaffinised and rehydrated in decreasing concentrations of ethanol. They were then immersed in a solution of cresyl violet-thionine (25mg each in 100 ml of water) for 7 minutes, submitted to differentiation in 95% acidic ethanol and mounted in Eukitt before examination with a light microscope (Axioskop-II, Zeiss, Jena, Germany).

2.3 ***Immunohistochemitry***

2.3.1 Tissue sections

Mice were anaesthetized with a mixture of 5% ketamine and 5% xylazine (0.1 mL of the mix per 30g i.p.) and transcardiacally perfused with 4% paraformaldehyde in 0.1M phosphate

buffer (PB) pH 7.3. Brains were then immersed for 4 hours in the same fixative at 4°C before either cryoprotection in 0.44M sucrose in PB at 4°C over-night and freezing in liquid nitrogen (wild-type, *NP$^{0/0}$*, *NP$^{0/0}$:Bax$^{-/-}$* and *NP$^{0/0}$-Hu-bcl-2* mice) or immersion in iso-butanol for 4 days before embedding in paraffin (wild-type, *NP$^{0/0}$*, *NP$^{0/0}$:Bax$^{-/-}$* and *NP$^{0/0}$-Hu-bcl-2* mice). Frozen sections were cut (10µm-thick) in the cerebellum with a cryostat (Leica) and paraffin sections were cut (10µm-thick) with a microtome (Leica). Paraformaldehyde-fixed brains of wild-type and *Grid2$^{ho/ho}$* mice was dissected and sagittal and transversal cerebellar sections (50µm-thick) were obtained with a vibratome (Leica).

2.3.2 Immunohistochemistry

Frozen sections and vibratome floating sections were rinsed in 0.1M phosphate buffer saline (PBS) pH 7.3. Paraffin sections were deparaffinised in toluene and rehydrated in decreasing concentrations of alcohol. All sections were pre-incubation for 45 min in a blocking solution at room temperature. This was made of PBS containing 3% normal goat serum (NGS) and 0.5% Triton X-100 for immunohistofluorescence on frozen and paraffin sections, 2% normal horse serum (NHS) and 0.05% Triton X-100 for immunoperoxidase on paraffin sections and, 0.02% gelatin (D^{r} Oetker), 0.25% Triton-X 100 and 0.1M lysine for immunohistofluorescence on vibratome sections. The sections were then incubated overnight at 4°C in the blocking solution containing specific primary antibodies (Table 3).

Then, sections were rinsed in PBS (2x10 min) and incubated with secondary antibodies (Table 3) in blocking solution. Immunoperoxidase detection was achieved using the avidin biotin complex (ABC) method with 3,3'-diamino-benzidine tetra-hydrochloride (Fast-DAB, Sigma Aldrich). After rinsing in PBS, the fluorescent sections were mounted in Mowiol and the immunoperoxidase sections were mounted in Eukitt. The frozen and the paraffin sections were examined with a light microscope equipped with fluorescence and differential interference contrast illumination (Axioskop-II, Zeiss, Jena, Germany). The vibratome fluorescent sections were analyzed with a Zeiss confocal microscope LSM 510.

2.3.3 Immunohistofluorescence in organotypic cerebellar cultures

Cultures were fixed in a 4% paraformaldehyde in PB overnight at 4°C before rinsing in PB. The cultures were pre-incubated for 45 min in blocking solution made of 3% NGS and 0.1% Triton X-100 in PB (PBT). The sections were then incubated overnight at 4°C with either rabbit polyclonal antibodies against CaBP (Swant and gift from Dr Thomasset) diluted

1/1000 and mouse monoclonal antibody against LC3B (Nanotools) diluted 1/20 in PBT containing 0.3% NGS.

Sections were then rinsed in PB (2x10 min) and incubated with fluorescent secondary antibodies (table 3). After rinsing in PB, the cultures were mounted in Mowiol before examination with a fluorescence microscope (Axioskop-II, Zeiss, Jena, Germany).

2.4 ***Transmission electron microscopy***

Mice were anaesthetized as described above (§2.2) and transcardially perfused with fixative in PB (Table 4). Vibratome sections (60µm-thick) were submitted to Scrg1- and GABA-immunocytochemistry using respectively pre-embedding and post-embedding immunogold as previously described (Heitz et al., in review: publication 4). The samples from cerebellar sections destined to ultrastructural analysis were prepared according to a classical protocol for transmission electron microscopy (Selimi et al., 2003: publication 1). The ultrathin sections were examined with a Hitachi 7500 transmission electron microscope equipped with an AMT Hamamatsu digital camera.

2.5 ***Western blotting***

Western blotting was performed to evaluate the expression of LC3B-I and –II, p62 and Lamp1 in wild-type and $NP^{0/0}$ cerebella according to the protocol described in Heitz et al., in review (publication 4).

2.6 ***Quantitative analysis***

In all Purkinje cell quantitative analysis performed in these studies, Purkinje cell counts were performed by me with the genetic identity of the animal masked.

2.6.1 Morphometric analysis of Purkinje cell dendritic tree in organotypic cerebellar cultures

The size and the ramification degree of the Purkinje cell dendritic tree were evaluated by measuring the following morphometric features: the dendritic area, the distance between the tip of the longest dendrite and the center of the soma (maximum dendritic length) and the number of dendrite branching points per Purkinje cell.

At least 15 Purkinje cells were selected in each mouse studied. These neurons displayed well visible axon and dendrites because isolated from the surroundings. These cells were

photographed with a CoolSnap Pro digital camera (Photometrics) and Image Pro plus software (Media Cybernetics) was used to measure the dendritic area and the maximum dendritic length. The dendrite branching points were counted visually. All morphometric data were submitted to statistical analysis. The non-parametric test of Mann-Witnney was used to compare wild-type and *hotfoot* Purkinje cell dendritic extent and the non-parametric test of Kruskall-Wallis followed, when justified, by *post-hoc* tests for multiple comparisons was used to compare the effects of NASP treatments on wild-type and *Lurcher* Purkinje cell dendritic extent. The significance threshold was set at $P = 0.05$.

2.6.2 Quantitative analysis of Purkinje cells in organotypic cerebellar cultures

Only CaBP-immunopositive Purkinje cells with neurites were counted in 4 organoypic cultures obtained from each mouse in these studies.

Comparison of Purkinje cell numbers between untreated and NASP-treated wild-type and *Lurcher* organotypic cerebellar cultures was submitted to two-way analysis of variance (factor genotype, factor treatment) followed, when justified, by *post hoc* Tukey test for multiple comparisons.

Student test was performed to compare wild-type and *hotfoot* Purkinje cell numbers in organotypic cerebellar cultures. The significance threshold was set at $P = 0.05$.

2.6.3 Size of the cerebellar vermis

The size of the cerebellar vermis was estimated by measuring the mean area of seven sagittal cresyl-violet-stained sections (Image J) separated from each other by 400µm.

2.6.4 Quantitative analysis of Purkinje cells in tissue sections

The Purkinje cell population was quantified and statistically evaluated as previously described (Heitz et al., 2007; 2008: publications 2 and 3) by a profile-based sampling method (Guillery and Herrup, 1997) using the Hendry correction factor (Hendry, 1976).

Appendix

1. Models of Purkinje cell degeneration

Purkinje cell death manifests itself by ataxia and tremor. In addition to the *Grid2* mutant mice and Nagasaki mutant mice, several other mutant mice are featured by Purkinje cell death.

1.1 ***Purkinje cell death and murine mutations***

1.1.1 The nervous mutant mouse

In the cerebellum of *nervous* mutant mice, some Purkinje cells initially develop an alteration of their mitochondria at P9 and further degenerate by P19 when most of their cytoplasmic organelles lyses and the cytoplasmic matrix abruptly condenses followed by cell death (Landis, 1973). The observation of dying Purkinje cells in 12 month-old *nervous* mice suggests a continuous progression of Purkinje cell death process (Sotelo and Triller, 1979) throughout lifespan. Interestingly, Purkinje cell death is not random with 60% of Purkinje cells dying in the vermis and 90% in the hemispheres (Wassef et al., 1987). Purkinje cell death was more important in the anterior lobe than in the posterior one, with zebrin-positive cells exhibiting an enhanced sensitivity to the *nervous* mutation. Although the molecular target of this mutation remains unknown, the selective alteration of the mitochondria seems to indicate that oxidative stress may be at the origin of the Purkinje cell death in this mutant. The late cell death process is probably necrosis since ultrastructural features characterizing apoptosis have not been described (Landis, 1973).

1.1.2 The toppler mutant mouse

This autosomal mutation is characterized by a specific gait alteration (Duchala et al., 2004) and a severe loss of Purkinje cells between P14 and P30. The degenerating Purkinje cells have nuclear DNA double-strand breaks and are TUNEL-positive, suggesting that they die by apoptosis.

1.1.3 The Purkinje cell degeneration (pcd) mutant mouse

Mice homozygous for this mutation lose their Purkinje cells during the third and fourth postnatal weeks. In 4 month-old *pcd/pcd* cerebellum, some Purkinje cells survive, only in the lobule X (Wassef et al., 1986). Degenerating Purkinje cells show nuclear condensation and shrinkage of the cytoplasm suggesting apoptotic cell death (Landis and Mullen, 1978). Characterization of the *pcd* mutant alleles revealed mutations in the Nna1 gene. Nna1 encodes a putative nuclear protein containing a zinc carboxypeptidase domain initially identified by its induction in spinal motor neurons during axonal regeneration (Fernandez-Gonzalez et al., 2002). As degenerating Purkinje cells are TUNEL-positive and have a decreased expression of BCL-2, Purkinje cell death in this mutant occurs essentially by apoptosis (Gillardon et al., 1995a; 1995b).

1.1.4 The woozy mutant mouse

The *woozy* mutation affects Sil1 a protein of the HSP70 family which is present in the lumen of the ER. It is characterized by a late Purkinje cell death which starts after 10 weeks of age and continues until 18 months (Zhao et al., 2005). This progressive death process affects essentially the anterior lobe. Dying Purkinje cells are TUNEL- and caspase-3-positive suggesting that apoptosis is the death mechanism involved. Nevertheless, electron microscope analysis has also disclosed the occurrence of autophagy (Zhao et al., 2005). The abnormal accumulation of proteins resulting from the inactivation of Sil1 may provoke ER stress with subsequent alteration of calcium homeostasis and thus may also contribute to the death of Purkinje cells (Zhao et al., 2005).

1.1.5 The Niemann Pick disease type C

Niemann-Pick disease type C (NPC) is an early onset autosomal recessive disorder characterized by accumulation of cholesterol and other lipids in late endosomes/lysosomes (Loftus et al., 1997). About 95% of the cases are caused by mutations in the *NPC1* gene, whereas the remaining 5% are due to mutations in the *NPC2* gene. The Purkinje cells are preferentially affected with less than 10% neurons remaining in 2 month-old *npc1* mutant mice, but surprisingly glia in the corpus callosum is affected earlier (German et al., 2001). Investigations of the basis of neurodegeneration in these mice have shown that dying Purkinje cells have features of autophagic cell death (Ko et al., 2005) with increased levels of LC3-II

(Bi and Liao, 2007). Furthermore, increases in autophagic activity are closely associated with alterations in lysosomal function and protein ubiquitination (Bi and Liao, 2007).

1.1.6 The leaner mutant mouse

The *leaner* mutant (tg^{la}) mouse is severely ataxic due to cerebellar atrophy, resulting from gradual degeneration of granule, Purkinje, and Golgi cells (Herrup and Wilczynski, 1982; Frank et al., 2003). Pycnotic granule cells are numerous in the internal granular layer as early as P10 but significant Purkinje cell loss is not observed until the end of the first postnatal month and continues at a low rate throughout the life of the animal (Herrup and Wilczynski, 1982). Molecular analysis has shown a mutation in the gene coding for the $Ca_v2.1$ voltage-gated calcium channel which is highly expressed in the cerebellum (Volsen et al., 1995) and hippocampus (Day et al., 1996). The tg^{la} mutation results in alteration of calcium homeostasis of cerebellar Purkinje cells which can be attributed to reduced uptake by the ER (Dove et al., 2000). In addition, an apoptotic process kills cerebellar granule cells with a peak at P20 (Lau et al., 2004). This is accompanied by decreased expression of nNOS (Frank-Cannon et al., 2007). Altogether, these data suggest that a major dysfunction of calcium homeostasis is responsible for granule and Purkinje cell death in this mutant.

1.1.7 The hyperspiny mouse

The *hyperspiny* Purkinje cell mutant mice show cerebellar symptoms from P10. The cerebellum is slightly hypoplastic and histological examination reveals that Purkinje cells have reduced dendritic arbors. As the name of the mutant implies, the proximal dendrites and soma of the Purkinje cells are studded with spines (Guenet et al., 1983). Focal axonal swellings are also found in nearly all axons (Sotelo, 1990b). Axonal pathology leads to a retrograde degeneration resulting in a 15% Purkinje cell loss (Guenet et al., 1983; Sotelo, 1990b; 1990a).

1.1.8 The tambaleante mouse

The *tambaleante* spontaneous mutation particularly affects Purkinje cells, because these neurons suffer from a late, slow and progressive degenerative process starting at about 2 months of age (Wassef et al., 1987). In one year-old mice, the degeneration is almost complete with less that 1% surviving neurons (Wassef et al., 1987; Rossi et al., 1995). Pathological signs are characterized by moderate thickening of the main axonal stems. The

recurrent axon collaterals and varicose axonal enlargements or torpedoes commonly accompany these alterations (Rossi et al., 1995). Dense somatic material, autophagic vacuoles and autophagolysosomes are observed in the soma and dendrites of these Purkinje cells (Dusart et al., 2006) suggesting that Purkinje cells undergo autophagic degeneration. Nevertheless, some Purkinje cells exhibit apoptotic-like condensation of the nucleus, and dendritic debris of the dying Purkinje cells can be seen in the molecular layer of 6 to 12 month old *tambaleante* cerebella. Therefore, it is likely that after an initial intense autophagic phase, the affected Purkinje cells follow a different death pathway, probably involving apoptosis (Dusart et al., 2006).

1.1.9 The weaver mutant mouse

The autosomal recessive *weaver* mutation is a single amino acid exchange in a G-protein coupled, inwardly rectifying K^+ channel (GIRK2) (Patil et al., 1995) and generates chronic depolarization of the affected neurons (Patil et al., 1995; Liss et al., 1999). This leads to massive death of granule cells (80%) during the 2 first postnatal weeks (Smeyne and Goldowitz, 1989). Dying granule cells display typical apoptotic morphology, DNA fragmentation (Smeyne and Goldowitz, 1989; Gillardon et al., 1995a), increased Bax expression (Wullner et al., 1995) and successive activation of caspase-9 and caspase-3 (Peng et al., 2002). In addition, about 40% of Purkinje cells and 25% of the deep cerebellar neurons also die postnatally (Maricich et al., 1997).

1.1.10 The staggerer mutant mouse

The autosomal recessive *staggerer* (*Rora*^{sg}) mutation causes a deletion of the *Rora* gene (Hamilton et al., 1996) which encodes a retinoid-like nuclear receptor involved in neuronal differentiation and maturation, with high expression in Purkinje cells (Hamilton et al., 1996; Ino, 2004). In homozygotes, Purkinje cells declined in number before P5 and, at the end of the first postnatal month, only 25% of them remained (Herrup and Mullen, 1979). The remaining *Rora*^{sg} Purkinje cells are reduced in size, lack tertiary dendritic spines receiving synaptic contacts from parallel fibers (Sotelo, 1975) and are multiply innervated by climbing fibers, a sign of developmental arrest (Mariani, 1982).

1.1.11 The reeler mutant mouse

The autosomal recessive *reeler* mutation disrupts the *Reln* gene (D'Arcangelo et al., 1995) which encodes reelin, an extracellular matrix protein involved in neural adhesion and migration at critical stages of development (D'Arcangelo et al., 1995; Hack et al., 2002). *Reeler* mutants display essentially architectonic disorganization in the cerebellum (Mariani et al., 1977) and the inferior olive (Goffinet, 1983). In the *reeler* cerebellum 90% of granule cells (Caviness and Rakic, 1978), 50% loss of Purkinje cells (Heckroth et al., 1989) and 20% of inferior olivary neurons (Blatt and Eisenman, 1985) are loss. In addition, adult *reeler* Purkinje cells are innervated by more than one climbing fiber (Mariani et al., 1977; Mariani, 1982).

1.2 ***Purkinje cell death in neurological disorders***

1.2.1 Brain ischemia

Brain ischemia induces increases in glutamate, intracellular calcium, and release of free radicals leading to neuronal death (Lipton, 1999; Welsh et al., 2002). A massive excitotoxic loss of Purkinje cells is correlated with ataxia in patients recovering from global ischemic stroke (Sarna and Hawkes, 2003). The origin of this excitotoxic neuronal death is complex, probably, resulting from the intensive innervation of Purkinje cells by glutamatergic climbing/parallel fibers (Sarna and Hawkes, 2003), deficiency of glutamate reuptake (Welsh et al., 2002; Yamashita et al., 2006) and persistent activation of AMPA receptors (Hamann et al., 2005).

1.2.2 Alzheimer disease

Purkinje cell loss was originally demonstrated in brains of patients deceased from familial and sporadic Alzheimer diseases (Fukutani et al., 1996). Significant cerebellar atrophy was related to a 35% loss of Purkinje cells (Wegiel et al., 1999). This loss was accompanied by a loss of inferior olivary neurons and a massive cerebellar gliosis (Sjobeck and Englund, 2001). This is manifested by some of the symptoms and signs observed in Alzheimer disease such as spatial disorientation.

1.2.3 Huntington disease

In the Huntington disease, Purkinje cell loss has been reported (Rodda, 1981), but the death mechanisms causing neuronal death is unknown. Dying Purkinje cells characteristically

exhibit neuronal intranuclear inclusions, condensation of both cytoplasm and nucleus, and ruffling of the plasma membrane while maintaining ultrastructure of cellular organelles (Turmaine et al., 2000). These cells do not develop any sign of nuclear or cytoplasmic blebbing, apoptotic bodies, or DNA fragmentation (Turmaine et al., 2000) and this suggests that Purkinje cell death mechanism is neither apoptosis nor necrosis.

1.2.4 Prion diseases

Creutzfeld-Jacob disease induces a virtually complete disappearance of parallel fibers (Berciano et al., 1990) caused by massive loss of granule cells which is total in the cerebellar vermis (Hauw et al., 1981; Tiller-Borcich and Urich, 1986; Berciano et al., 1990; Kovacs et al., 2001). This is accompanied by hypertrophy of primary and secondary Purkinje cell dendrites (Tiller-Borcich and Urich, 1986) which express PrP^C (Lemaire-Vieille et al., 2000). Interestingly, Fas, Fas-L, and Bax expression are increased and caspase-3 immunoreactivity is enhanced in the cytoplasm of surviving Purkinje cells in CJD (Puig and Ferrer, 2001) suggesting either impairment of apoptosis or non-apoptotic roles for these proteins. In addition, the heat shock protein Hsp72 accumulates in TUNEL-negative Purkinje cells and is absent from TUNEL-positive granule cells suggesting that this protein helps to rescue Purkinje cells from the TSE (Kovacs et al., 2001). This suggests that accumulation of the inducible Hsp-72 in Purkinje cells may be part of a cytoprotective mechanism in prion-diseased cerebellum.

2. Prion diseases

Prion diseases are infectious, genetic, or sporadic TSEs, characterized by prion protein aggregation and neurodegeneration. These diseases include kuru and CJD in humans, scrapie in sheep and BSE in animals. These diseases are incurable, with a variety of motor or cognitive symptoms and fatal issue (Knight and Will, 2004).

Current understanding of TSEs have evolved from the concept of the "prion" that is a proteinaceous, nucleic acid-free, infectious particle (Prusiner, 1998). The pathogenesis of prion diseases is attributed to major changes in the metabolism of PrP^C (Aguzzi et al., 2008).

2.1 The Prion Concept: a protein-only hypothesis of infection

The infectious agent responsible for the transmission of the disease is probably not constituted of nucleic acids since this agent is resistant to doses of radiation that inactivate viruses and bacteria (Alper, 1985). Also, its sensitivity profile to various chemicals differs from viruses and viroids (Behrens and Aguzzi, 2002).

A protein unusually resistant to proteolysis has been evidenced and is required for infectivity without the intervention of any other component (Prusiner et al., 1984). Therefore, a single protease-resistant protein (PrP^{res}), component of the infectious agent (Prusiner, 1998), may be responsible for the transmission of TSEs. The infectious particle shares an identical amino acid sequence with PrP^{C} (Oesch et al., 1985; Basler et al., 1986) suggesting that PrP^{res} is an abnomal conformer of PrP^{C} with distinctive properties (Prusiner et al., 1984).

Several hypotheses postulate the nature of the infectious particle. In the virino hypothesis, (Kimberlin and Wilesmith, 1994) the infectious agent consists of an essential scrapie-specific nucleic acid associated with PrP^{C}. Nevertheless, no evidence for TSE-specific nucleic acids has yet been obtained (Safar et al., 2005). The protein-only hypothesis (Griffith, 1967; Weissmann, 1991; Prusiner, 1998) is currently the most widely accepted. In this hypothesis, posttranslational modifications of the α-helical structure of PrP^{C} into a β-sheet structure results in the production of PrP^{res} from host PrP^{C} (Baldwin et al., 1994). This explains the capacity of PrP^{res} to form protease-resistant aggregates which in turn accumulate within the brain (Ross and Poirier, 2004). *Prnp* knockout mice, devoid of prion protein, are resistant (Sailer et al., 1994) to infection with pathogenic PrP^{res} and reintroduction of *Prnp* restores infectibility and prion pathogenesis in these mice (Aguzzi and Polymenidou, 2004). This suggests that the presence of endogenous PrP^{C} is essential for the development of the disease.

The mechanism of infection and propagation might involve a catalytic cascade where infection with PrP^{res} or conversion of PrP^{C} into PrP^{res} leads to further conversion of PrP^{C} into PrP^{res}. The newly formed PrP^{res} will in turn convert a new PrP^{C} molecule into a proteinase K-resistant entity.

2.2 Molecular and cellular basis of neurodegeneration in prion diseases

2.2.1 Apoptosis

Hypothalamic cell lines infected by scrapie prions develop features of apoptosis such as DNA fragmentation and activation of caspases (Schatzl et al., 1997; Unterberger et al., 2005). In addition, activation of the JNK pathway has been demonstrated (Carimalo et al., 2005),

suggesting that this pathway may be involved in prion-induced neuronal death. In addition, the expression of Fas-L, Bcl-2, BAX, and active caspase-3 are modified in Purkinje cells of human CJD cerebellum (Puig and Ferrer, 2001). An immunohistochemical study shows overexpression of BAX, but not caspase-3 in scrapie-infected sheep (Lyahyai et al., 2006). This suggests that although apoptosis is a relevant cell death pathway in prion disease, it is probably not the exclusive pathway.

2.2.2 Autophagy

The accumulation autophagic vacuoles in TSEs has been described (Liberski et al., 2004; 2008) and may result from intraneuronal accumulation of PrP^{res}. This suggests that accumulation of PrP^{res} may lead to localized sequestration and phagocytosis of neuronal cytoplasm and ultimately to neuronal loss (Jeffrey et al., 1992).

2.2.3 Synaptic and dendritic pathology

A progressive loss of dendritic spines has been reported in addition to dendritic atrophy in CJD brains (Fraser, 2002). Notch-1, an inhibitor of dendritic growth and maturation, is increased and correlates with regressive dendritic changes, suggesting that Notch-1 could be a mediator of this process (Ishikura et al., 2005). The resulting loss of synapses and dendritic spines may isolate neurons from electrical stimuli and trophic factors, which could trigger self-destructive mechanisms (Fraser, 2002; Unterberger et al., 2005) and is probably BAX-independent (Chiesa et al., 2005). This loss of dendritic spines starts with the emergence of dendritic varicosities at sites where spines protrude from the dendrite (Fuhrmann et al., 2007).

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Communications

Scientific articles

2008 Heitz S, Leschiera R, Haeberlé A-M, Demais V, Grant N, Bombarde G, Bailly Y. Autophagy and cell death of Purkinje cells overexpressing Doppel in Ngsk Prnp-deficient mice. **Brain Pathol,** *in review*

Heitz S, Gautheron V, Lutz Y, Rodeau J-L, Zanjani HS, Sugihara I, Bombarde G, Richard F, Fuchs J-P, Vogel MW, Mariani J, Bailly Y
BCL-2 counteracts Dpl-induced apoptosis of prion protein-deficient Purkinje cells in the Ngsk $Prnp^{0/0}$ mouse. **Dev Neurobiol**, (2008) 68:332-348

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Bax contributes to Doppel-induced apoptosis of prion protein-deficient Purkinje cells. **Dev Neurobiol** (2007) 67:670-686.

2003 Selimi F, Lohof AM, Heitz S, Lalouette A, Jarvis CI, Bailly Y, Mariani J.
Lurcher GRID2-induced death and depolarization can be dissociated in cerebellar Purkinje cells. **Neuron** (2003) 37:813-9.

Oral communications

2005 Communication at « II international symposium on the new prion biology » (Venice, avril 2005)
Heitz S, Bailly Y. Bax mediated neurotoxicity of Doppel in the Purkinje cells of the *Ngsk*Prnp KO mouse line.

Posters

2008

Poster at the FENS 6th forum (Geneva, July 2008)
Bailly Y, Haeberle AM, Demais V, Grant N, Bombarde G, Heitz S.
Autophagic cell death of Purkinje cells in Doppel-expressing prion protein-deficient mice.

Poster at « first European synapse meeting » (Bordeaux, march 2008)
Gambino F, Pavlowsky A, Heitz S, Billuart P, Poulain B, Chelly J and Humeau Y. Cerebellar synaptic transmission in a mouse model of mental retardation.

2007

Poster at Abcam meeting « Molecular mechanisms of neurodegeneration » (Antigua, december 2007)
Heitz S, Haeberlé AM, Bombarde G, Richard F, Dron M, Dandoy-Dron F, Bailly Y. Doppel-induced cell death mechanism(s) in prion protein deficient Purkinje cells: apoptosis or autophagy?

Poster at the Colloque de la Société des Neurosciences (Montpellier, mai 2007)
Heitz S, Kapfhammer J, Bailly Y. Synaptic and dendritic development of the cerebellar Purkinje cells in GluRδ2 (GRID2) mutant mice: focus on autophagy and glutamate receptors.

2006

Poster at the FENS 5th forum (Vienna, july 2006)
Heitz S, Zanjani H, Mariani J, Bailly Y. *Bax* knock-out as well as HuBcl-2 save Purkinje cells from Doppel-induced apoptosis *in vivo*.

2005

Poster at the « Society for Neurosciences » 35th meeting (Washington, november 2005)
Bailly Y, Heitz S, Zanjani H, Mariani J. *Bax* knock-out as well as HuBcl-2 save Purkinje cells from Doppel-induced apoptosis *in vivo*.

Poster at the Colloque de la société des Neurosciences (Lille, mai 2005)
Bailly Y, Zanjani H, Mariani J, Heitz S. HuBcl-2 saves Purkinje cells from Dpl-induced apoptosis in the cerebellum of the NgskPrnp$^{0/0}$ mouse.

Poster at the « II international symposium on the new prion biology » (Venice, april 2005)
Poster at the Neurex meeting (Strasbourg, april 2005)
Heitz S, Bailly Y. Bax mediated neurotoxicity of Doppel in the Purkinje cells of the *NgskPrnp*KO mouse line.

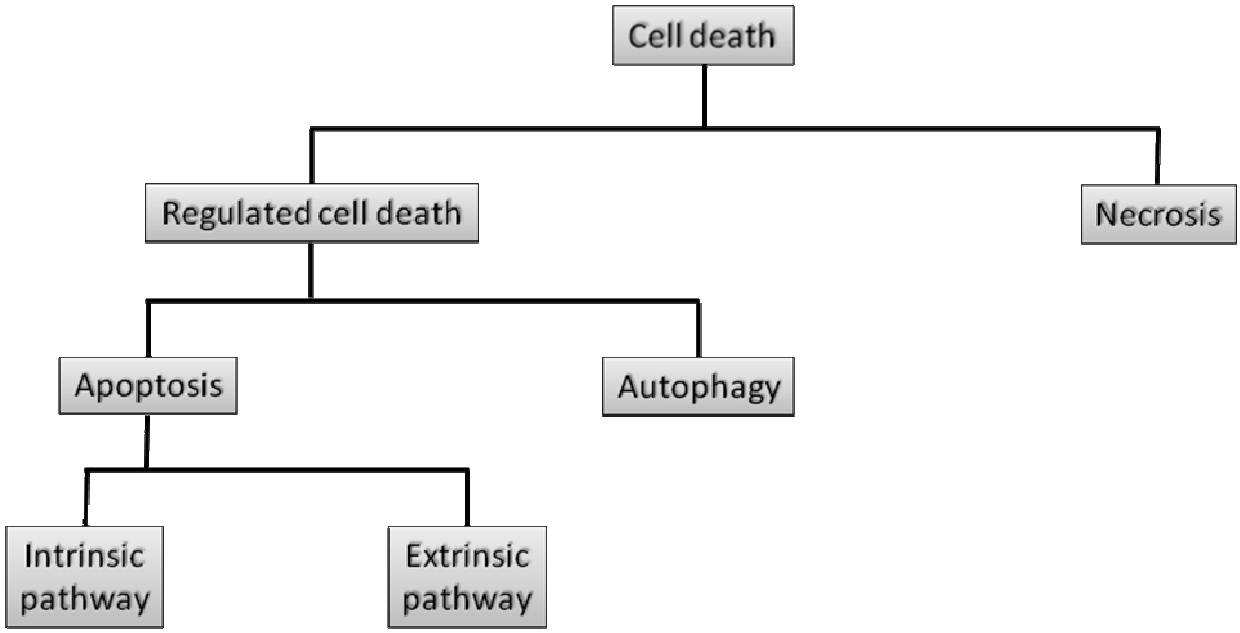

Figure 1. Cell death pathways. (Modified from Chowdhury et al., 2006)

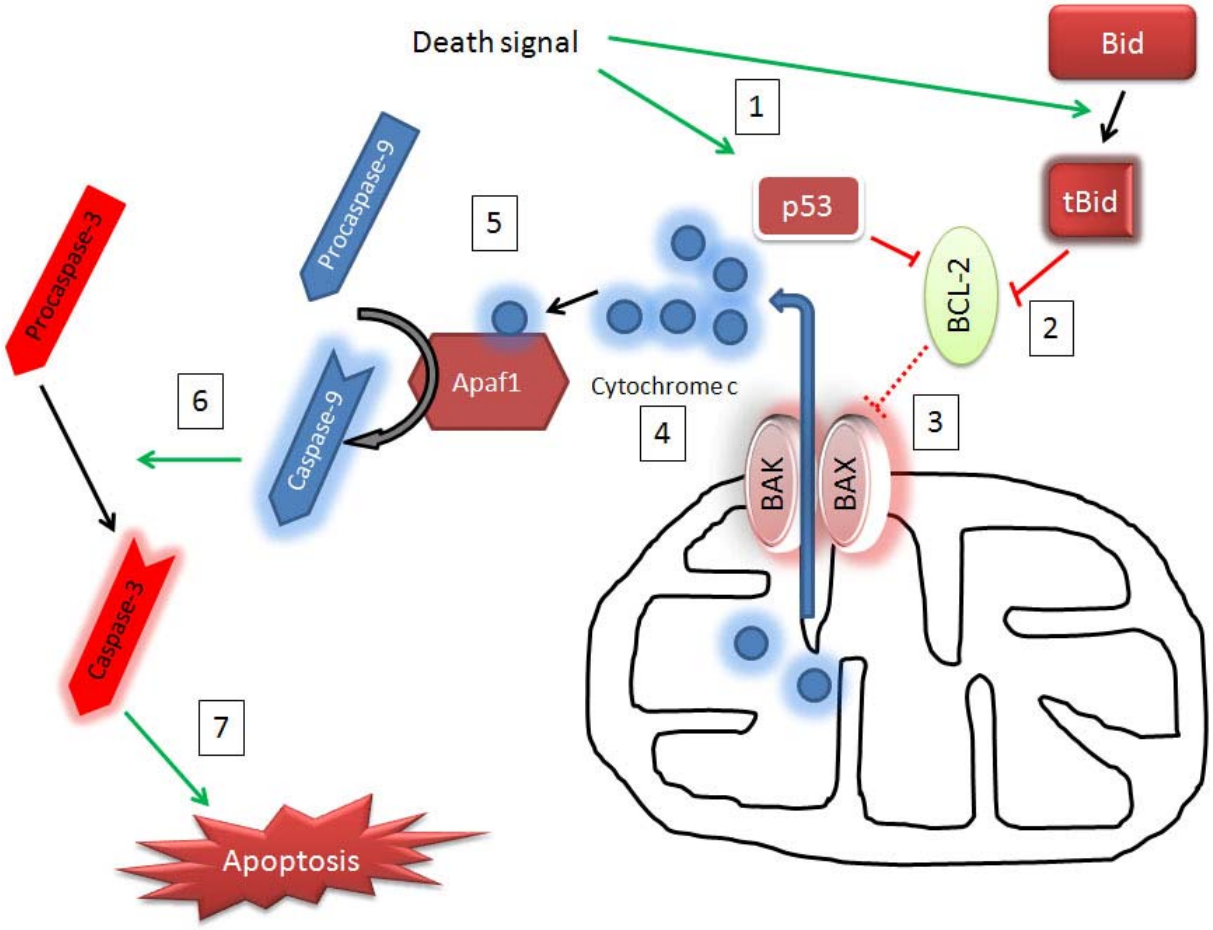

Figure 2. The intrinsic apoptotic pathway.

A death signal (1) will either activate p53 or induce the cleavage of the BH3-only pro-apoptotic factor Bid, resulting in the inhibition of BCL-2. The inhibitor effect of BCL-2 on BAX or BAK (3) will be suppressed, allowing BAX and BAK to form heterodimers and a pore in the mitochondrial outer membrane, resulting in the massive liberation of cytochrome c (4). Cytochrome c-bound Apaf1 will cleave the procaspase-9 into an active initiator caspase-9 (5) which in turn will cleave the effector procaspase-3 (6) into active caspase-3. (7) Active caspase-3 will induce DNA damages, dismantling of the cellular architecture and cell death.

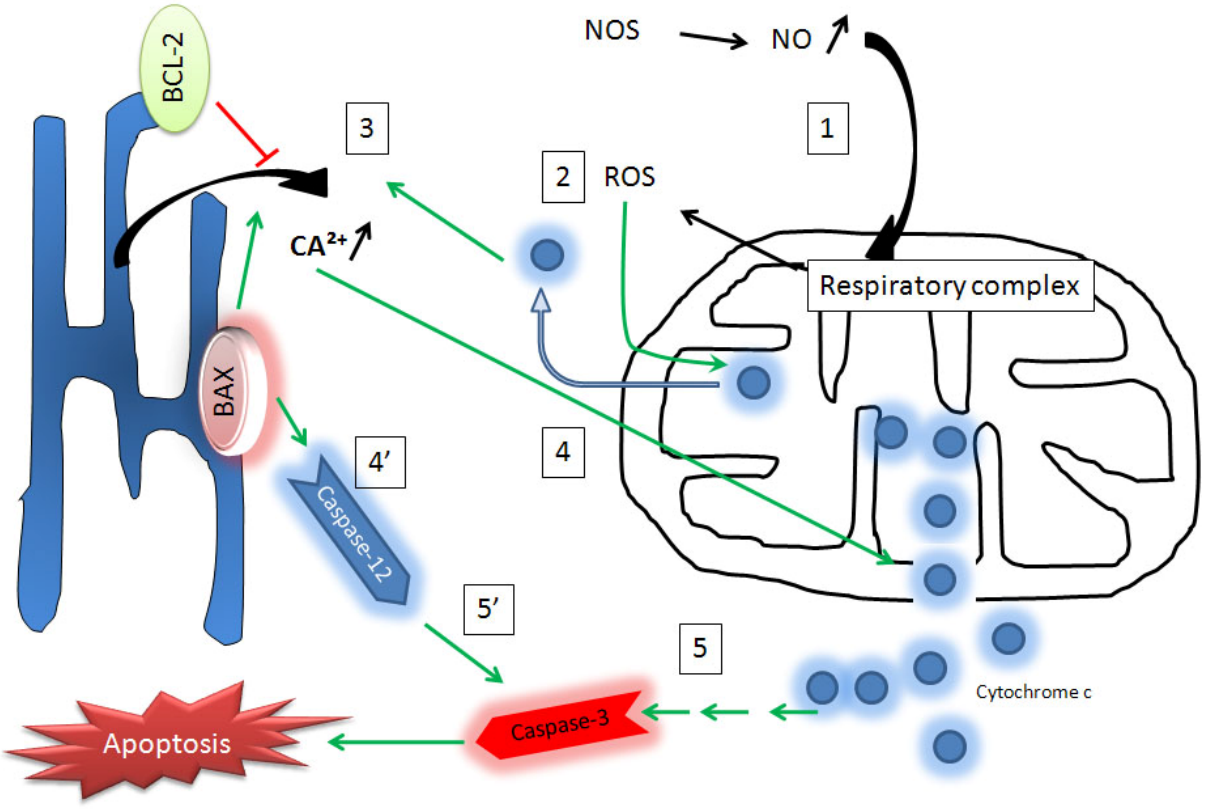

Figure 3. ROS induces ER stress and a caspases-dependent apoptotis.

Nitric oxide activates the mitochondrial respiratory complex (1) and the production of ROS. ROS-mediated MOMP will induce cytochrome c efflux to the cytoplasm (2) resulting in the massive liberation of Ca^{2+} from the ER (3). This Ca^{2+} liberation is regulated by BCL-2 and BAX. The massive liberation of Ca^{2+} will potentiate the MOMP resulting in a massive liberation of mitochondrial cytochrome c (4) and in caspase-3 dependent apoptosis (5). Activated BAX in the ER membrane can itself induce the caspase 12 activation (4') and subsequent caspase 3 activation (5').

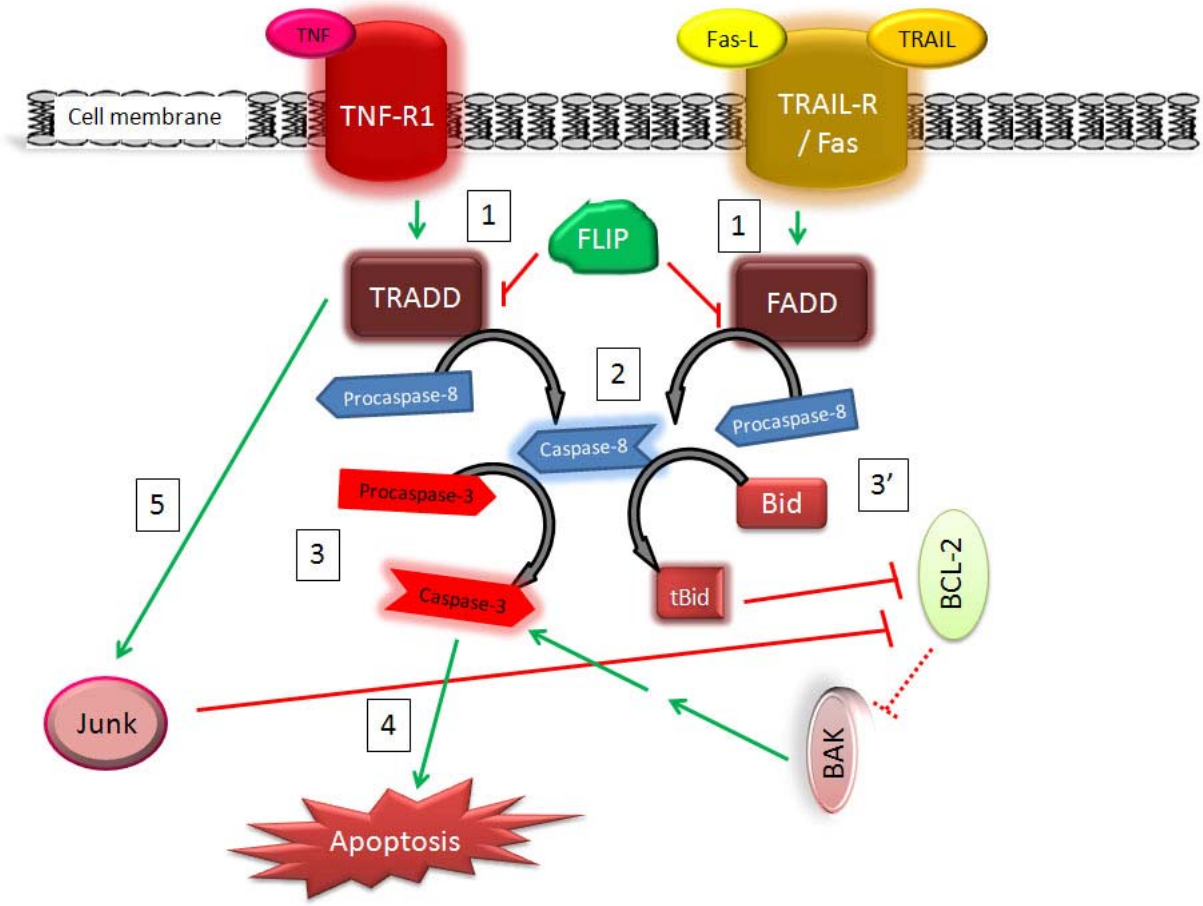

Figure 4. The apoptotic extrinsic pathways.

Death receptors TNF-R1, TRAIL-R and Fas are activated by their ligands and activate TRADD or FADD (1) via their death domain. TRADD and FADD will activate caspase-8 (2). The activate caspase-8 will directly activate caspase-3 (3). Caspase-8 can also cleave Bid into truncated Bid (3'), which inhibits BCL-2. This activates the intrinsic pathway and ultimately to caspase-3-dependent apoptosis (4). Potentiation of the apoptosis can also result from BCL-2 inhibition by TRADD-activated JUNK (5).

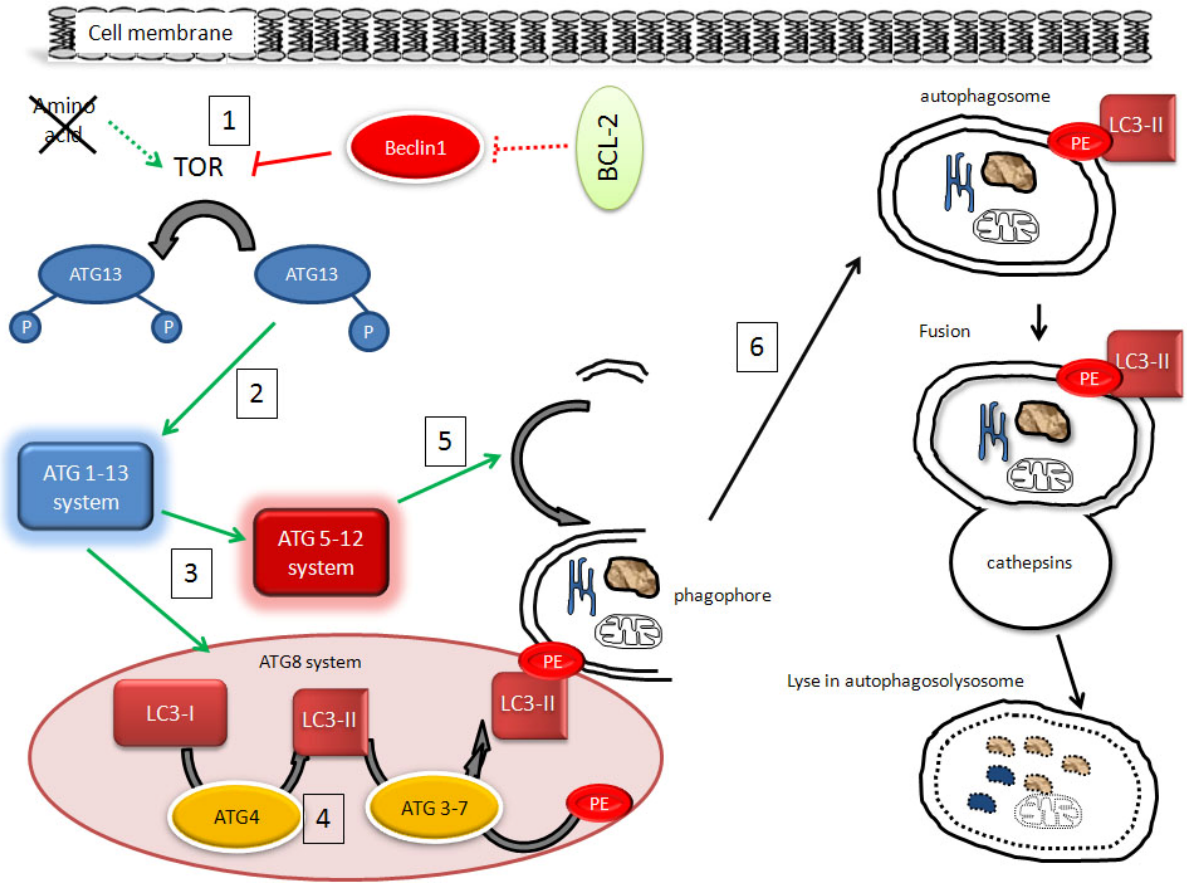

Figure 5. Induction and completion of the autophagic cascade.

(1) In the absence of nutrients and under Beclin1-dependent inhibition, TOR cannot maintain ATG13 phosphorylation. The dephosphorylated ATG13 will form an ATG 1-13 complex (2) which activates the ATG 5-12 and the ATG8 conjugation systems (3). ATG 5-12 will recruit an isolation membrane (5) while LC3-I is cleaved by ATG4 into its LC3-II form (4) in the ATG8 conjugation system. A phosphatidylethanolamine (PE) will be bound to LC3-II by ATG3 and 7. This allows the sequestration of cytoplasm in a double-membraned autophagic vacuole or autophagosome (6) The autophagosome resulting from the isolation membrane elongation will fuse with a lysosome to form an autophagolysosome. The lysosomal cathepsins will finally degrade the autophagic body.

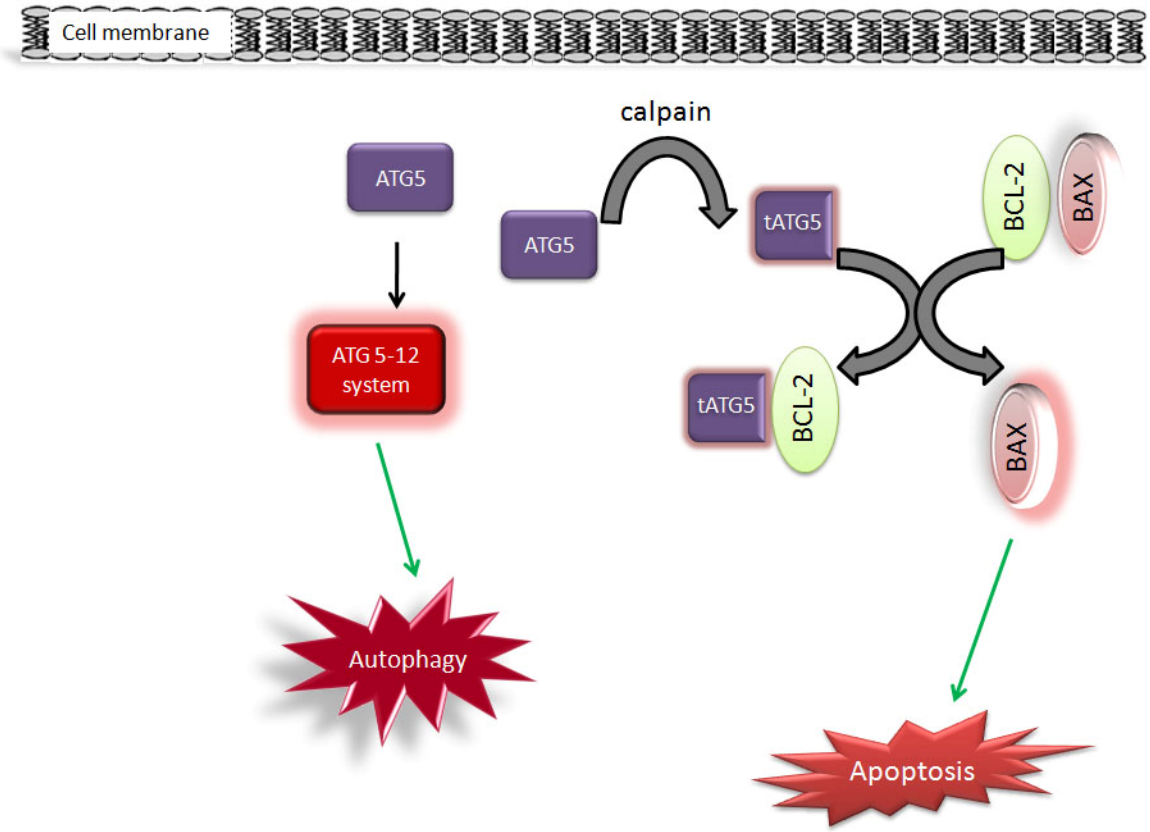

Figure 6. ATG5 mediates interplay between autophagic and intrinsic apoptotic pathways.

Truncated tATG5 resulting from the cleavage of ATG5 by calpains is able to dissociate the BCL-2/BAX complex. This allows tATG5 to bind BCL-2 via a BH3-like domain and BAX to exert pro-apoptotic activity.

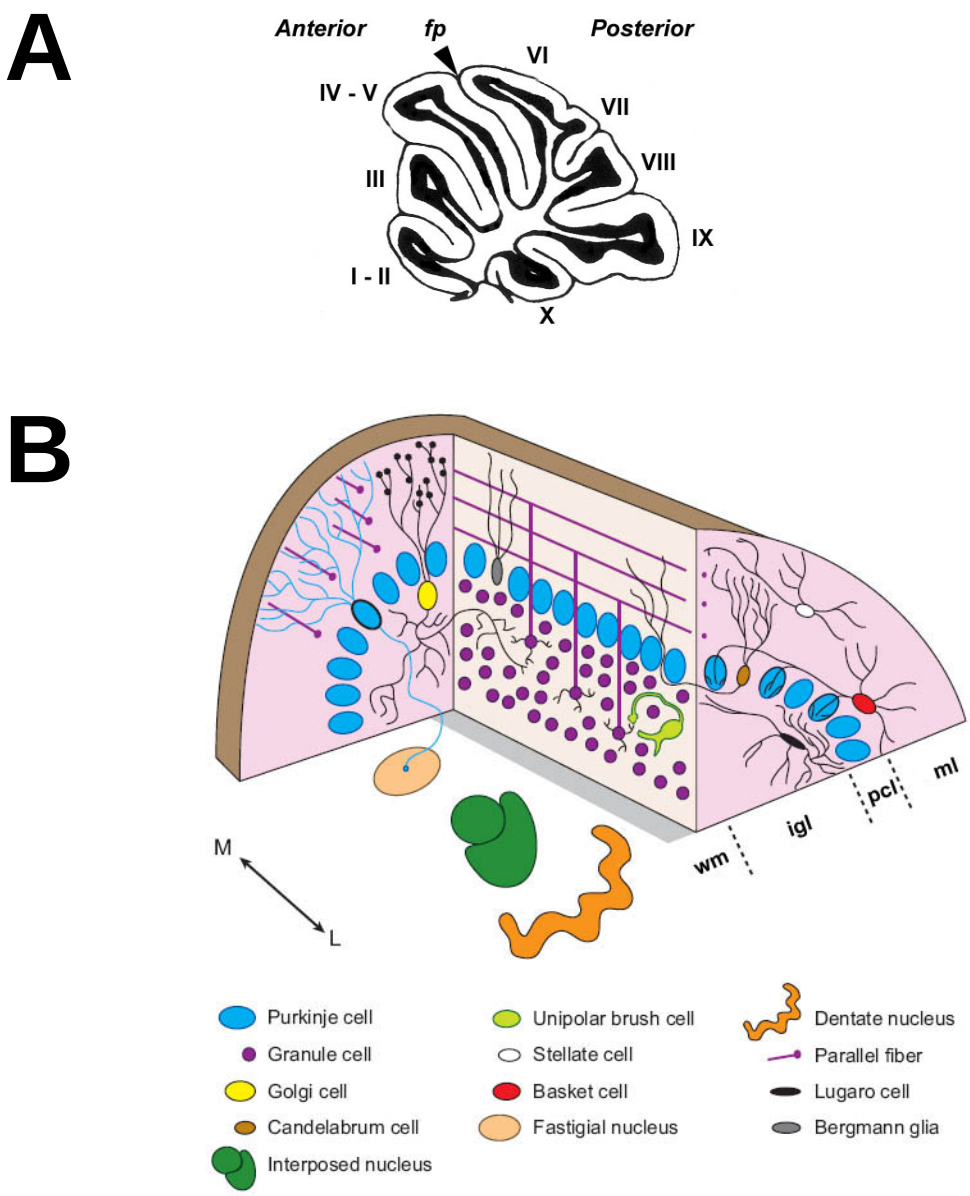

Figure 7. Anatomical organization of the cerebellar cortex.

A. Foliation of the C57Bl6 mouse cerebellum. The cerebellum is divided into anterior and posterior lobes by the fissura principalis (fp). Supplemental fissures further subdivide the anterior and posterior lobes into transversally oriented lobules I to X (Modified from Inouye and Oda, 1980).

B. Neuronal composition of the cerebellar cortex. ml, molecular layer. pcl, Purkinje cell layer. igl, internal granular layer. wm, white matter (Modified from Sillitoe and Joyner, 2007).

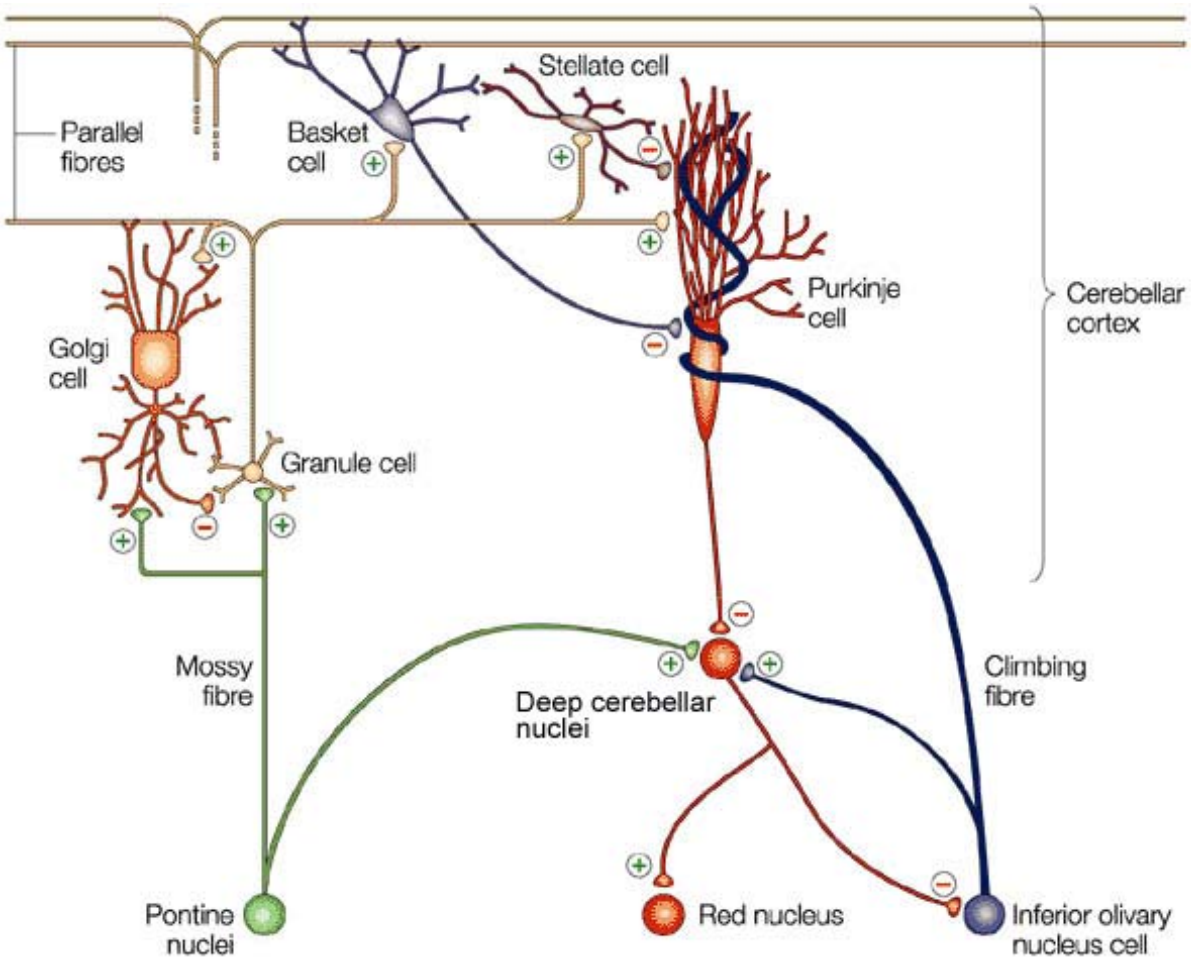

Figure 8. Schematic diagram of the cerebellar circuit with marked excitatory (+) and inhibitory (-) innervation (Modified from Medina et al., 2002)

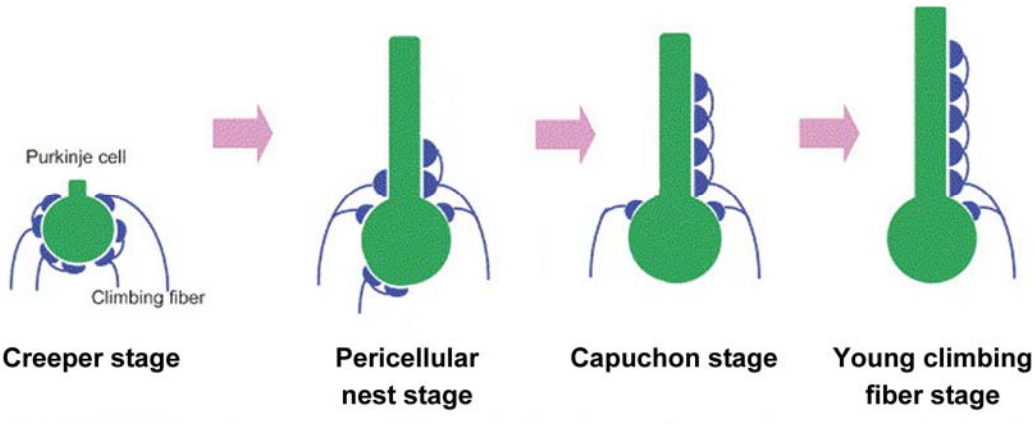

Figure 9. Climbing fiber differentiation during postnatal development. (Modified from Hashimoto and Kano, 2005)

At the "creeper stage" (P3), most Purkinje cells are innervated by 3 or more climbing fibers. Around P5, the peak of the "nest stage" corresponds to the peak of multiple innervation of Purkinje cells by climbing fibers. During the "capuchon stage" (P7-P10), the climbing fiber somato-dendritic translocation correlates with an abrupt decline of the multiple innervation. Then, the adult numerical relationship of one climbing fiber per one Purkinje cell is finally established.

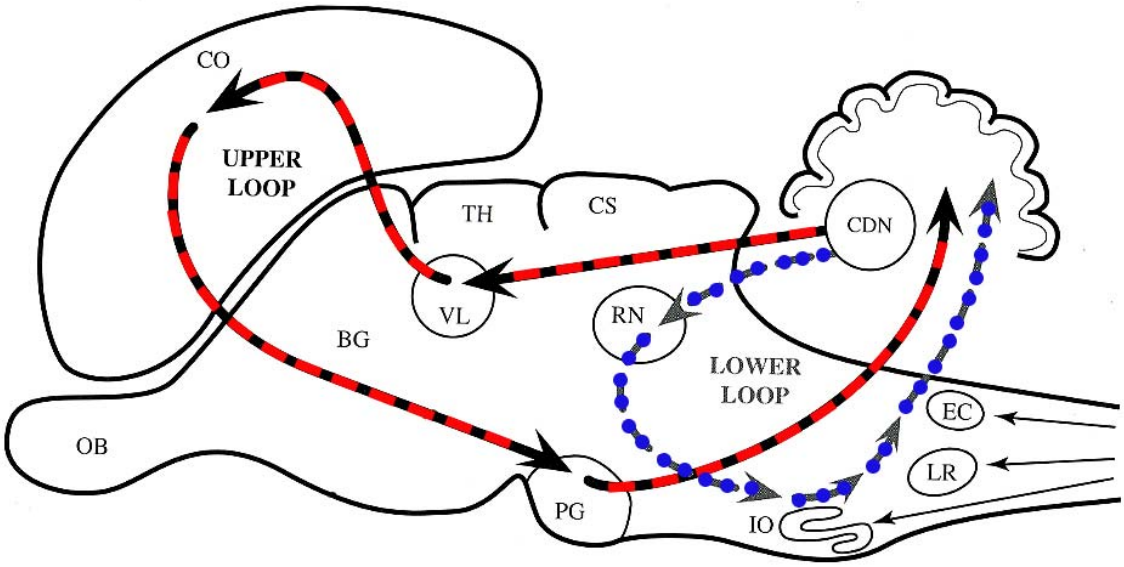

Figure 10. Schematic diagram outlining the major efferent pathways of the deep cerebellar nuclei. (Modified from Altman and Bayer, 1997)

Within the lower closed loop (blue), there are also projections from the red nucleus to the deep cerebellar nuclei (direct) or via the precerebellar nuclei to the deep cerebellar nuclei (indirect). The upper closed loop (red) predominantly involves the thalamus, cerebral cortex and pontine grey nuclei.

CO=cerebral cortex, BG=basal ganglia, TH=Thalamus, VL=ventrolateral thalamus, CS=superior colliculus, RN=red nucleus, PG=pontine grey nucleus, IO=inferior olive, DCN=deep cerebellar nuclei, LR=lateral reticular nucleus, EC=external cuneate nucleus.

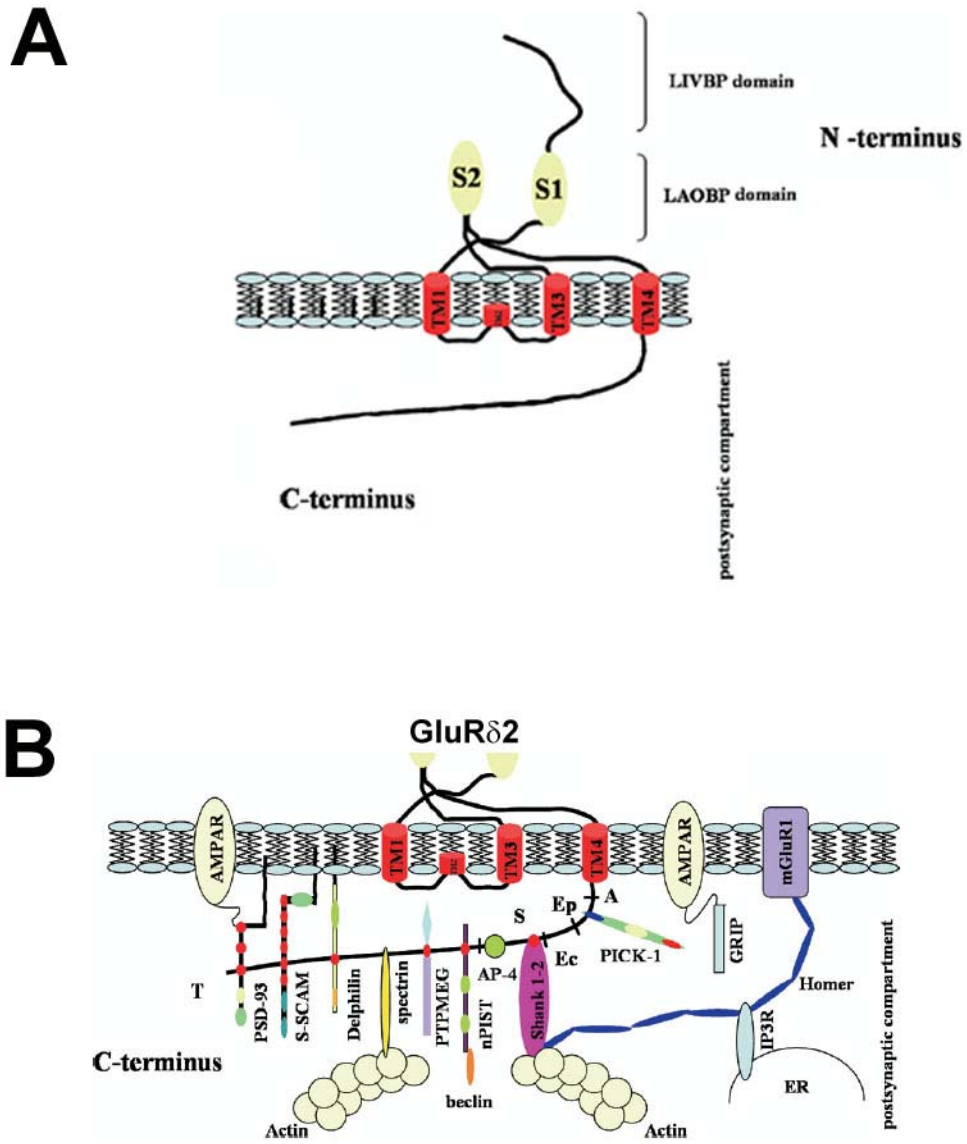

Figure 11. Structure of GluRδ2 and C-terminal partners. (Modified from Mandolesi et al., 2008)

A. The N-terminus region of GluRδ2 contains a LIVBP-like domain, a LAOBP-like domain which is divided in two parts by transmembrane domains (TM) 1 to 3 and a TM4. TM2 forms the ion-channel re-entrant loop segment.

B. The intracellular C-terminus of GluRδ2 interacts with PICK-1, AP-4, spectrin and PDZ domain-containing proteins PSD93, PTP-MEG, delphilin, S-SCAM, nPIST and Shank.

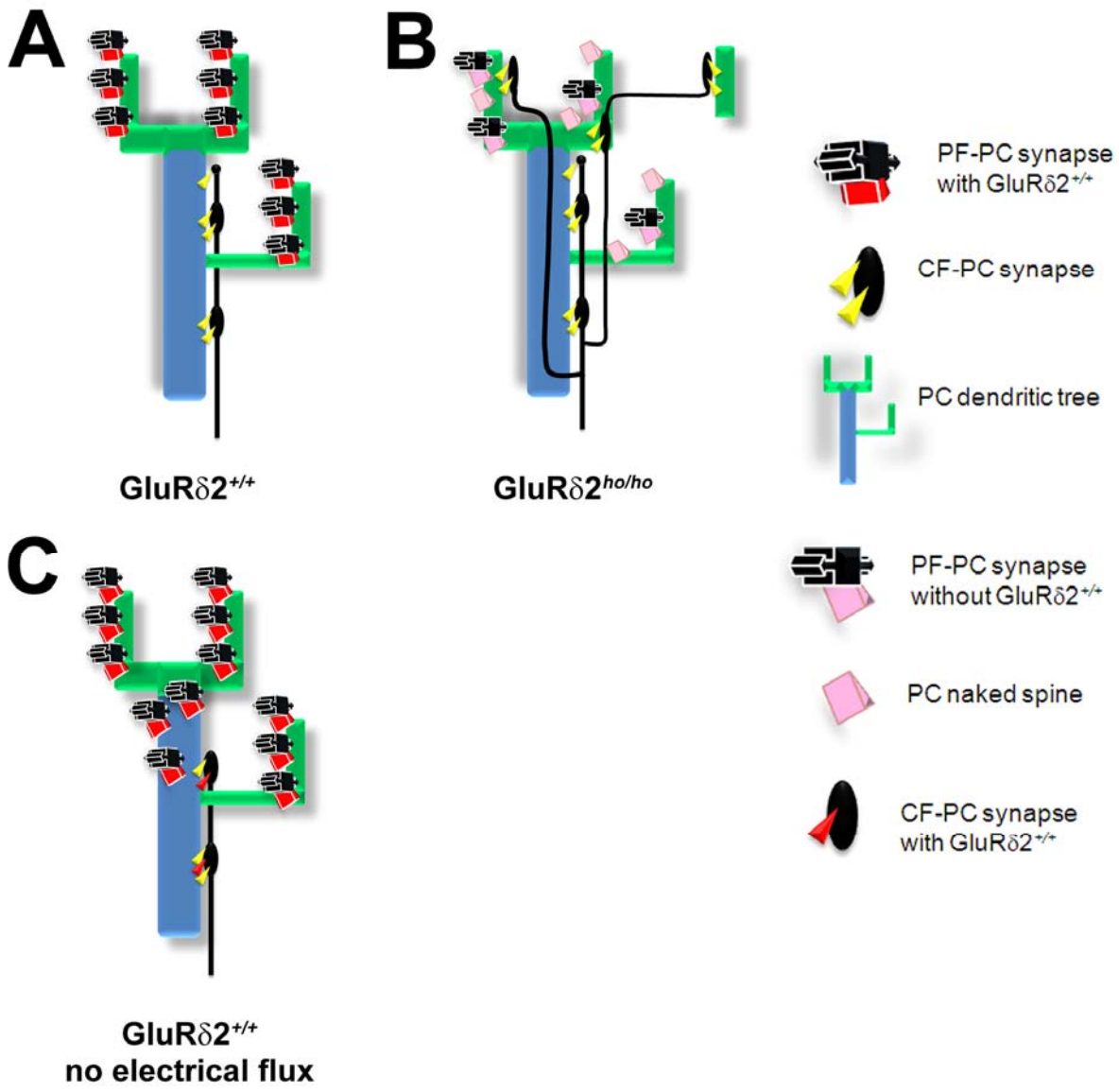

Figure 12. Parallel fiber and climbing fiber projection domains on Purkinje cell dendrites of the wild-type and GluRδ2$^{ho/ho}$ Purkinje cells (Modified from Ichikawa et al., 2002).

A. In the adult wild-type (GluRδ2$^{+/+}$) mouse, climbing fibers innervate proximal primary and secondary dendrites (blue) while parallel fibers innervate tertiary branchlet spines (green). GluRδ2 receptors are expressed at the parallel fiber synapses.

B. In the adult GluRδ2-deficient *hotfoot* (GluRδ2$^{ho/ho}$) mouse, climbing fibers emit collaterals distally extending to abnormally innervate tertiary branchlets. Numerous Purkinje cell dendritic spines remain devoid of innervation. Climbing fibers often jump to form synapses on spiny branchlets of adjacent Purkinje cells.

C. blocking the electrical activity afferent to wild-type Purkinje cells allows the parallel fibers to extend their territory to the proximal dendrite. This leads to the climbing fiber atrophy and restoration of GluRδ2 expression in the adult climbing fiber synapses.

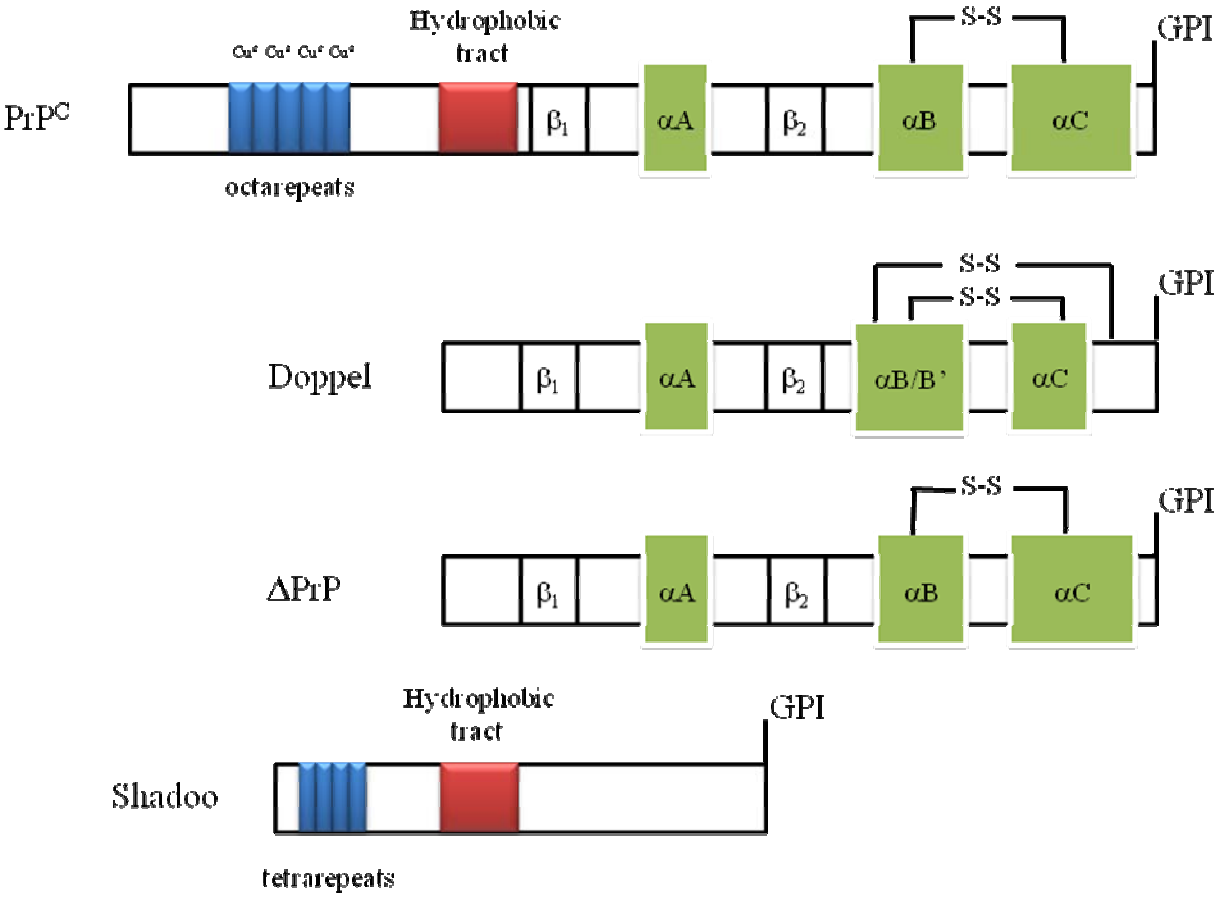

Figure 13: Structure of PrP^C, Dpl, ΔPrP and Shadoo. (Modified from Watts and Westaway, 2007).

PrP^C, Dpl and ΔPrP have a structured C-terminal domain including 3 α-helices and 2 β-sheets which are absent in Shadoo. Dpl has two disulfide bonds (S-S) when only one is present in PrP^C and ΔPrP. Hydrophobic tract and repetitive regions are conserved in PrP^C (octarepeats) and Shadoo (tetrarepeats).

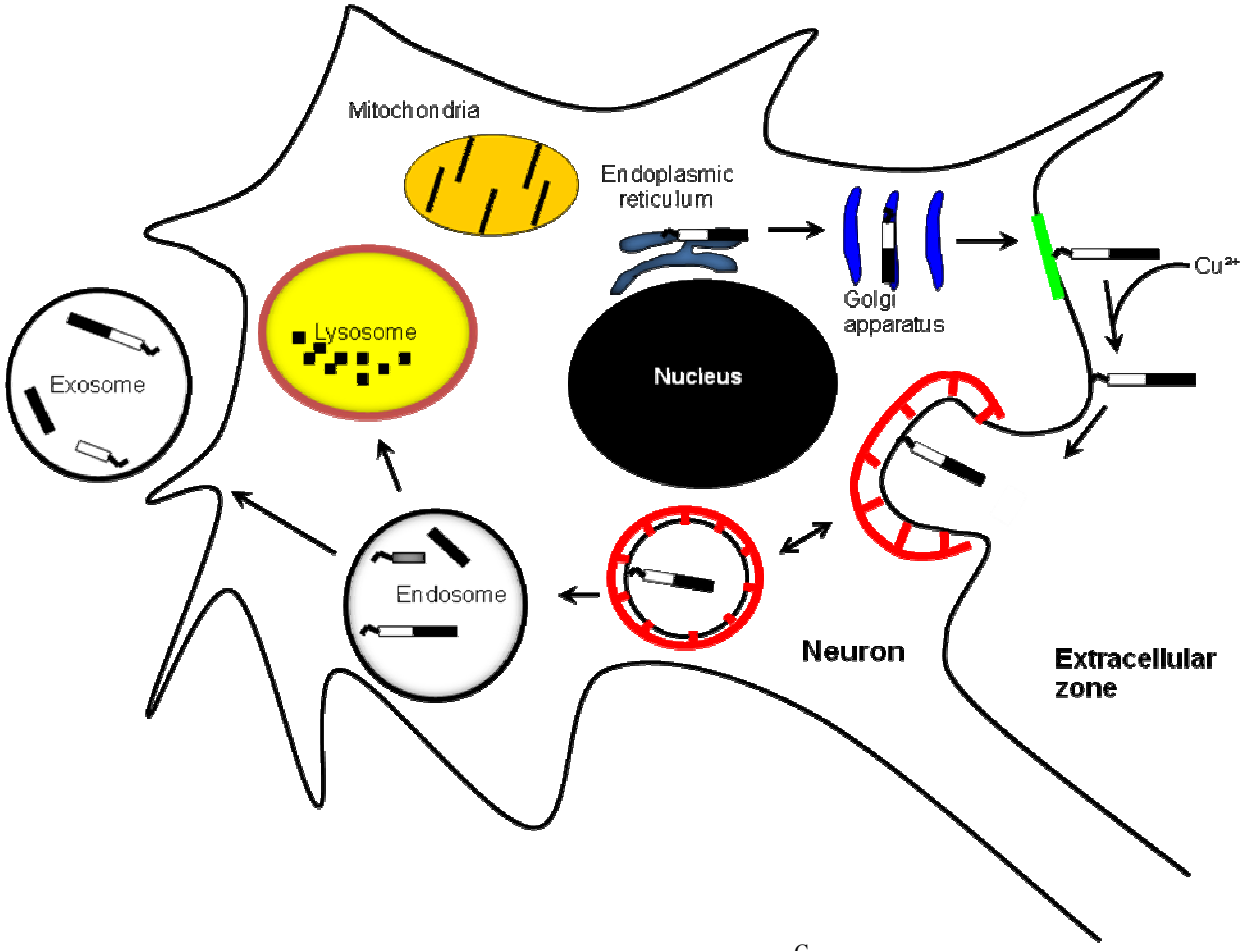

Figure 14: Intracellular and plasma membrane trafficking of PrP^C. (Modified from Shyng et al., 1993)

After ER synthesis, PrP^C is post-translationally modified in the ER and the Golgi apparatus before trafficked to the cell surface in a lipid raft microdomain (green). Copper-binding makes PrP^C to exit the lipid raft to be internalized by clathrin-mediated endocytosis and submitted to constitutive cycling. A part of the internalized molecules are cleaved within endosomes and either externalized via exosomes or degraded by lysosomes.

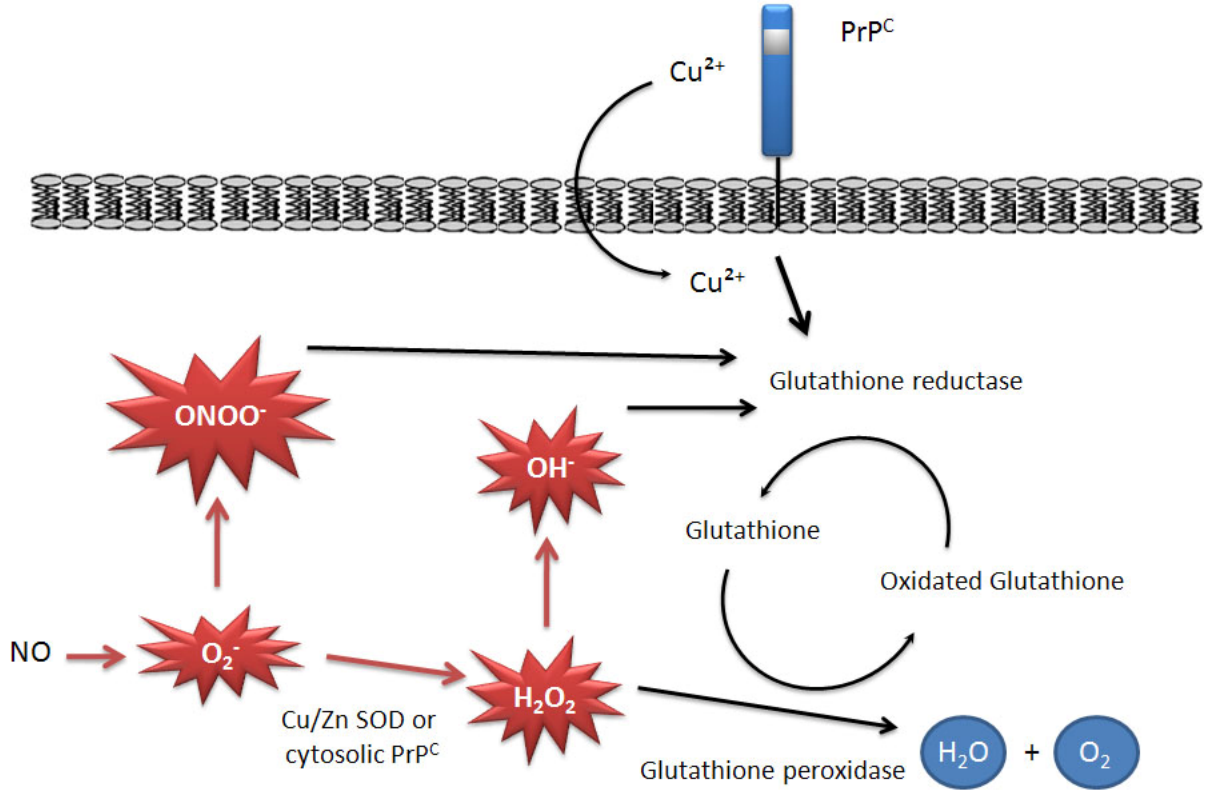

Figure 15: Schematic representation of Cu/ZnSOD and glutathione anti-oxidant pathways and putative interaction with PrP^{C}-Cu^{2+}. (Modified from White et al., 1999)

PrP^{C} might facilitate the reduction of glutathione by the glutathione reductase which ultimately reduces reactive oxygen species. In the absence of PrP^{C}, the reduction of oxidative species is impaired leading to a less efficient protection against oxidative stress.

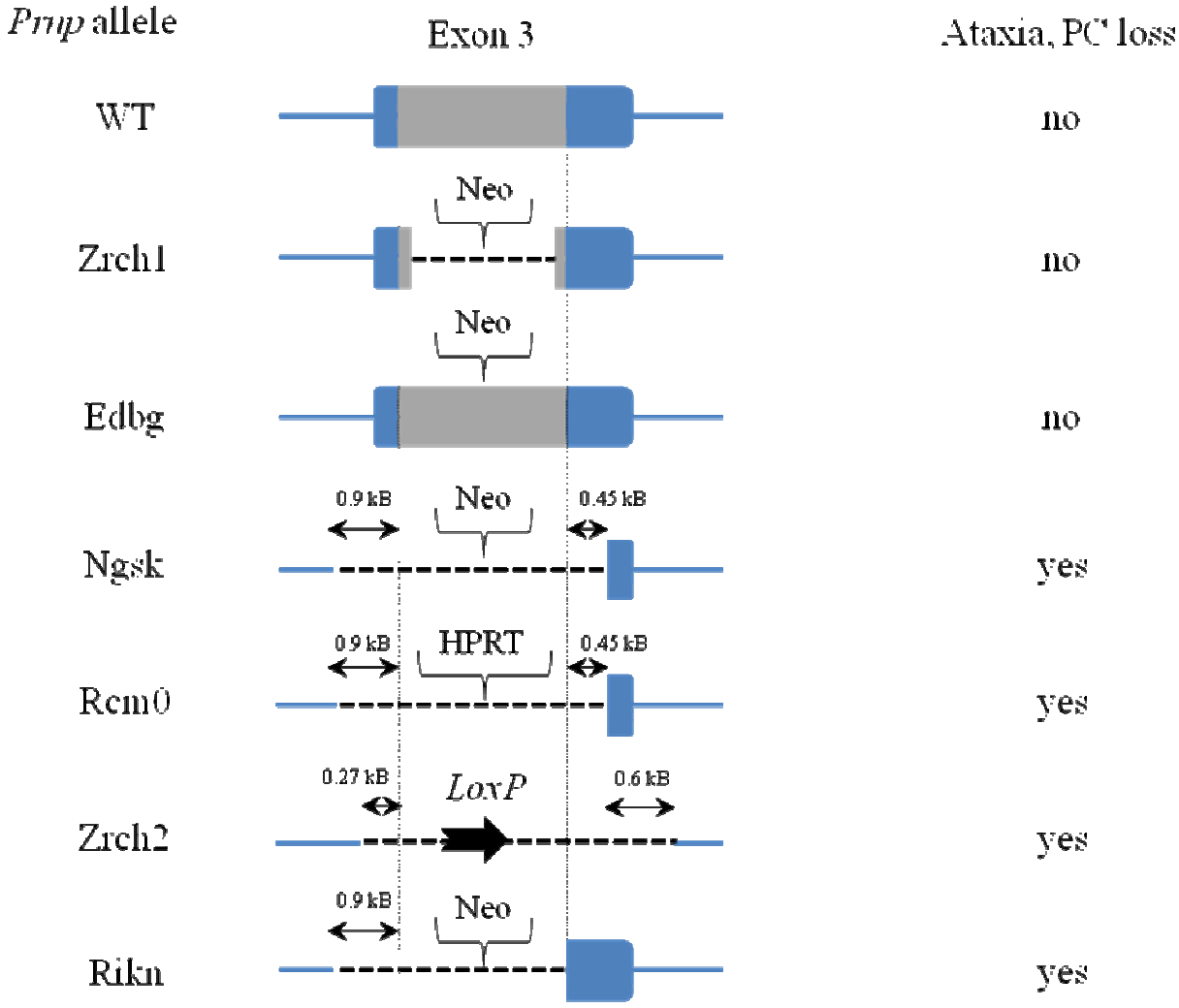

Figure 16: Various strategies used to disrupt the locus of the *Prnp* gene to generate different strains of *Prnp* knockout mice. (Modified from Rossi et al., 2001)

Prnp ORF is in grey and non-coding sequences are in blue. The dotted line indicates the deleted *Prnp* sequence and the brace indicates an inserted neomycine phosphotransferase (Neo) or a hypoxanthine phosphoribosyltransferase (HPRT). *LoxP* is a 34 Kb recombination site from phage 1.

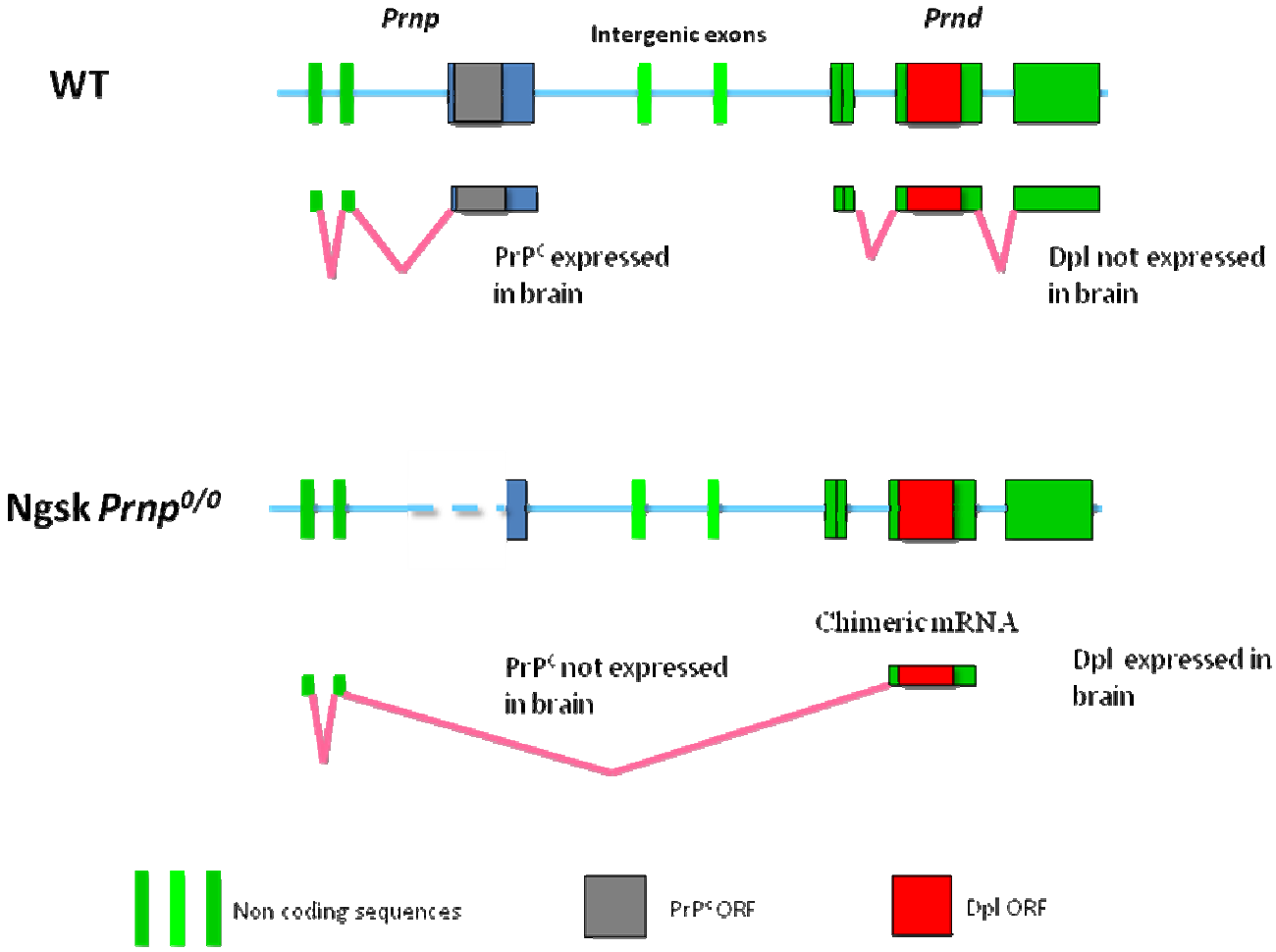

Figure 17: *Prnd* expression depends on *Prnp* promoter in Nagasaki $Prnp^{0/0}$ ($NP^{0/0}$) mouse. (Modified from Weissmann and Aguzzi, 1999)

When deletion of *Prnp* coding region and flanking regions includes the splice acceptor of intron 2, the resulting chimerical mRNAs comprising *Prnp* exons 1 and 2 are spliced to the Dpl-coding *Prnd* exon.

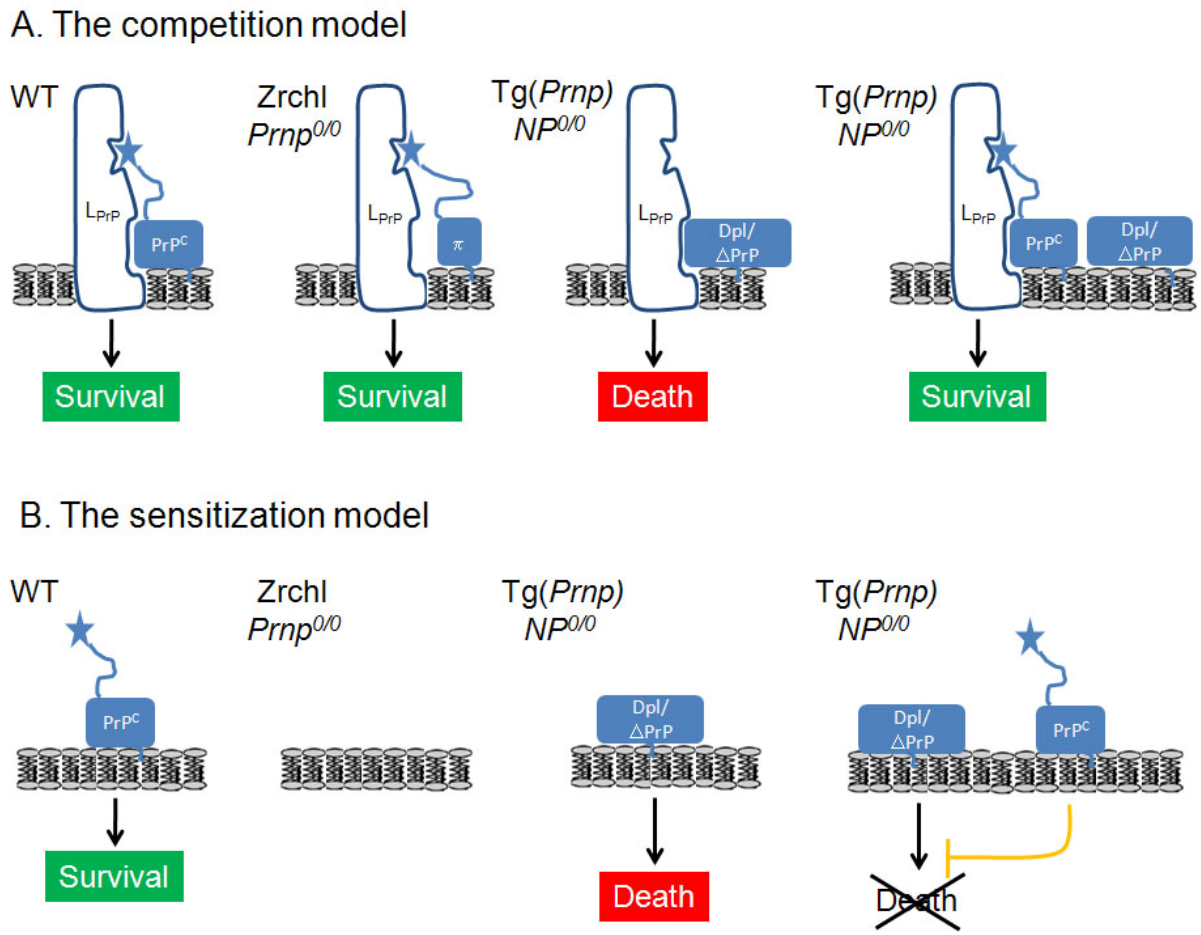

Figure 18: Dpl-PrP^{C} interaction models. (Modified from Watts and Westaway, 2007)

A. The competition model. In wild-type mouse, PrP^{C} binds to a hypothetical ligand L_{PrP} and elicits a survival signal. In *Prnp*$^{0/0}$ mouse such as ZrchI, a hypothetical PrP^{C}-like protein π binds to L_{PrP} with a lower affinity and induces the same survival signal. When Dpl or ΔPrP bind to L_{PrP}, an improper signal leading to cell death is initiated. It is counteracted by reintroduction of PrP^{C}.

B. The sensitization model. PrP^{C} favors cell survival in wild-type mouse and its absence in ZrchI mouse has no specific effect. In *NP*$^{0/0}$ mice, Dpl like ΔPrP initiates a cell death signal which is directly abrogated by PrP^{C}.

A

Group	Treatment schedule	Mean number of NASP treated PCs	Mean number of untreated PCs
WT 1	12 days treatment	429.4 ± 90.8	423 ± 60.7
GluRδ2$^{Lc/+}$ 2	12 days treatment	446.7 ± 36.7	258.24 ± 22.9
WT 3	6 days treatment	512 ± 62.2	453 ± 43.1
GluRδ2$^{Lc/+}$ 4	6 days treatment	530.7 ± 163	494 ± 58
GluRδ2$^{Lc/+}$ 5	10 days feeding and 2 days treatment	324.3 ± 35	258.24 ± 29.9
GluRδ2$^{Lc/+}$ 6	8 days feeding and 4 days treatment	388.6 ± 49.14	
GluRδ2$^{Lc/+}$ 7	6 days feeding and 6 days treatment	401 ± 41	
GluRδ2$^{Lc/+}$ 8	6 days treatment and 6 days feeding	252 ± 24	

B

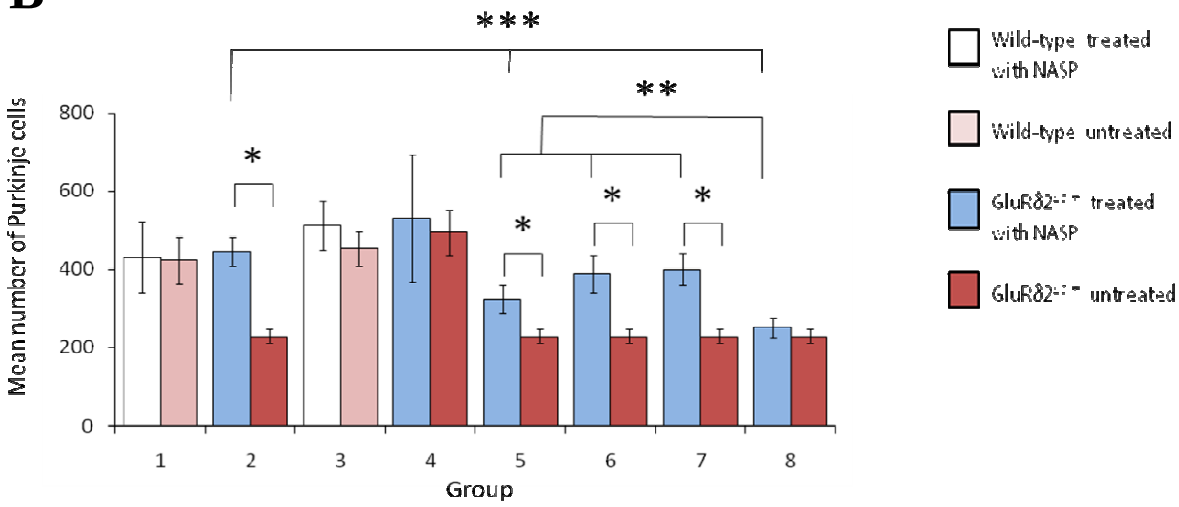

Figure 19.

A. Mean numbers of GluRδ2$^{Lc/+}$ and wild-type Purkinje cells submitted to different NASP treatment schedules ± SD.

B. Blockade of GluRδ2$^{Lc/+}$-induced excitotoxicity by NASP treatment is efficient to significantly rescue GluRδ2$^{Lc/+}$ Purkinje cells after at least 6 DIV in organotypic cerebellar cultures from P0 cerebellum. 1 to 8 are groups of wild-type and GluRδ2$^{Lc/+}$ mice indicated in A.

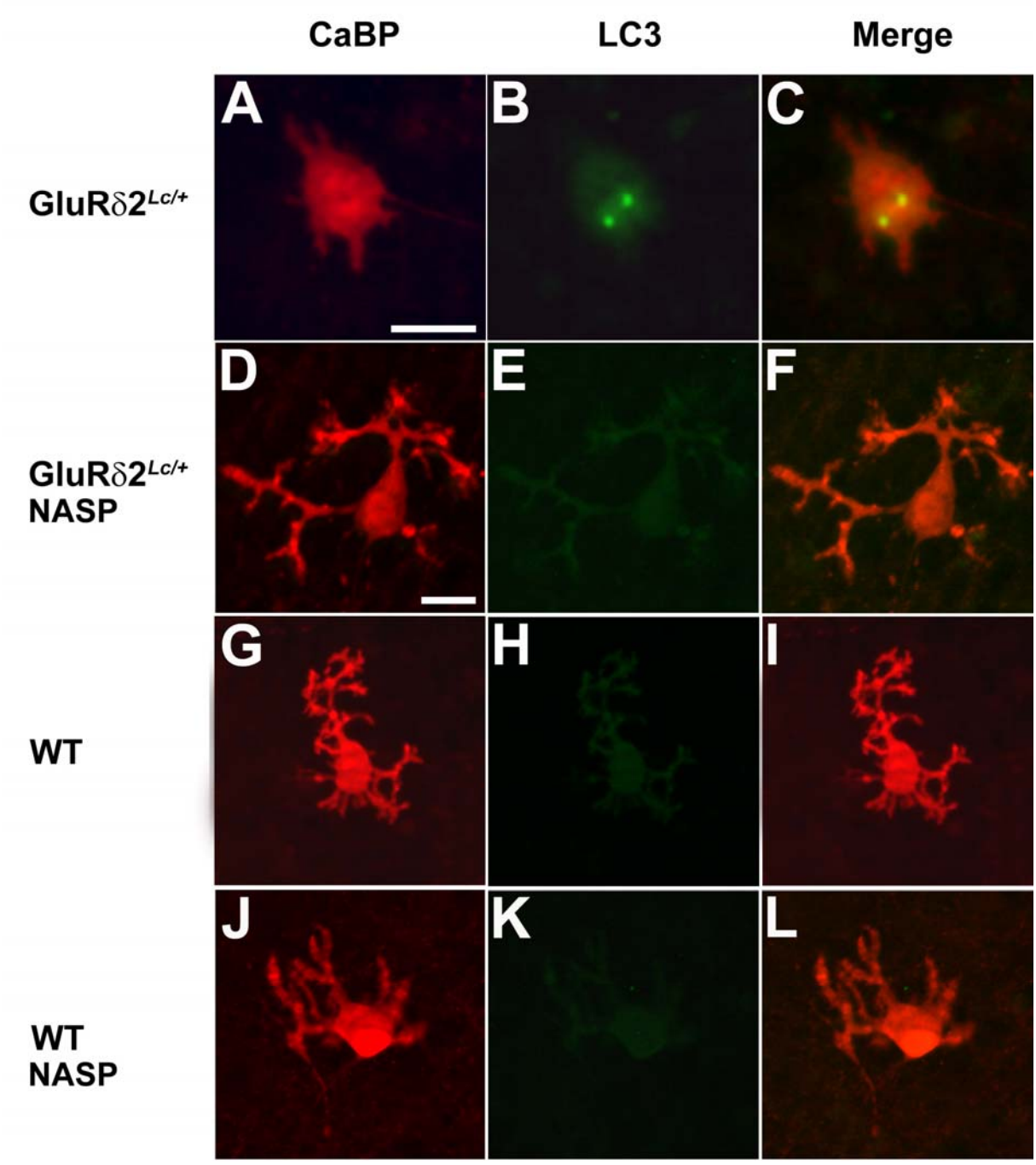

Figure 20. Somatic dots of LC3-immunofluorescent labeling (B, C) reveal autophagy in the CaBP-immunofluorescent GluRδ2$^{Lc/+}$ Purkinje cells (A, C) displaying dendritic atrophy in 12 DIV organotypic cerebellar cultures. Autophagy could not be detected (E, F) in GluRδ2$^{Lc/+}$ Purkinje cells treated with NASP for 12 DIV (D, F). This treatment also restored the dendritic tree of GluRδ2$^{Lc/+}$ Purkinje cells to its wild-type dimension but had no effect on wild-type Purkinje cells (G-L). Bar = 20µm for A-L.

A

Group	Treatment schedule	Mean dendritic area of treated PCs	Mean dendritic area of untreated PCs	Mean maximum dendritic length of treated PCs	Mean maximum dendritic length of untreated PCs
WT 1	12 days treatment	1584.6 ± 142.8	1516.5 ± 191.5	23.9 ± 1.3	24.1 ± 1.2
GluRδ2$^{Lc/+}$ 2	12 days treatment	1483.4 ± 117	933.32 ± 127.05	23.5 ± 1.27	18.23 ± 1.18
GluRδ2$^{Lc/+}$ 5	10 days feeding and 2 days treatment	1068.18 ± 116		20.77 ± 2.3	
GluRδ2$^{Lc/+}$ 6	8 days feeding and 4 days treatment	1085.4 ± 120.85		22.55 ± 2.1	
GluRδ2$^{Lc/+}$ 7	6 days feeding and 6 days treatment	1451.7 ± 116.9		23.54 ± 1.09	
GluRδ2$^{Lc/+}$ 8	6 days treatment and 6 days feeding	878.13 ± 36.32		15.69 ± 0.85	

B

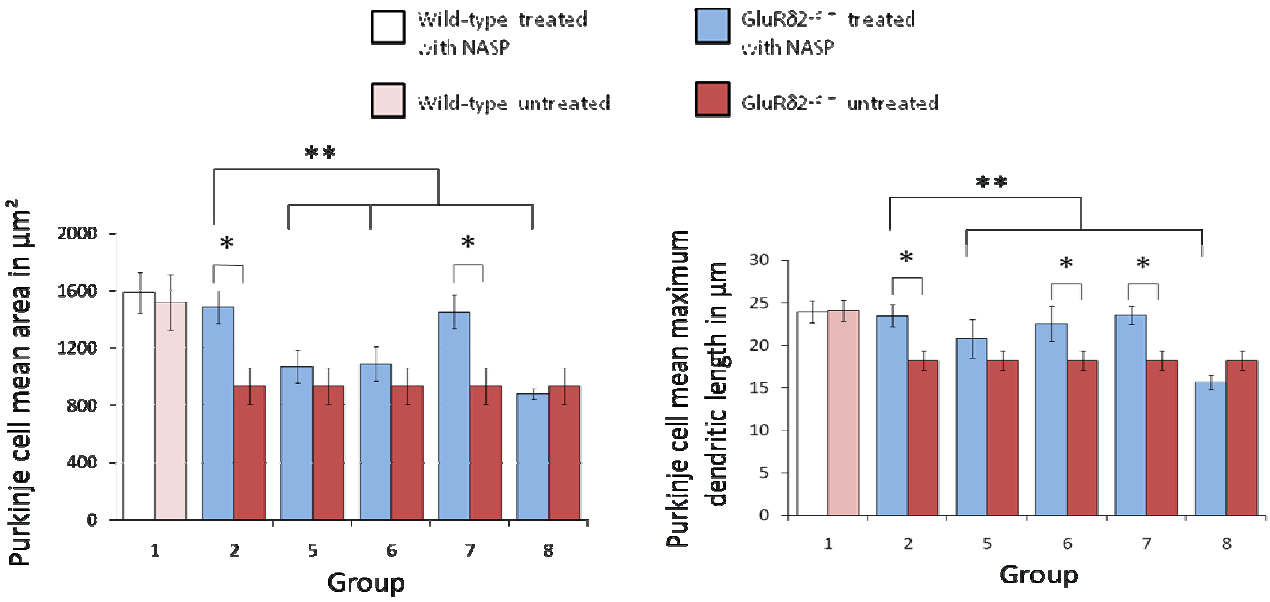

Figure 21. A. Mean area (µm²) and maximum dendritic length (µm) ± SD of GluRδ2$^{Lc/+}$ and wild-type Purkinje cells submitted to different NASP treatment schedules in 12 DIV organotypic cerebellar cultures.

B. Blockade of GluRδ2$^{Lc/+}$-induced excitotoxicity by NASP treatment is efficient to significantly restore GluRδ2$^{Lc/+}$ Purkinje cell dendritic growth after at least 6 DIV in organotypic cerebellar cultures from P0 cerebellum. 1 to 8 are groups of wild-type and GluRδ2$^{Lc/+}$ mice indicated in A.

Genotype	Mean number of Purkinje cells	Mean dendritic area	Mean maximum dendritic length	Mean numbers of primary dendrites	Mean numbers of secondary dendrites	Mean numbers of tertiary dendrites
WT	998 ± 116	3138 ± 272	98 ± 1	1.58 ± 0.7	4.87 ± 1.59	53.2 ± 11
GluRδ2$^{ho/ho}$	681 ± 173	3037 ± 819	83 ± 5	1.61 ± 0.8	3.95 ± 1.4	18.13 ± 4.7

Table 1. Mean number of Purkinje cells, mean area and maximum length of Purkinje cell dendritic tree and mean numbers of primary, secondary and tertiary dendrites of GluRδ2$^{ho/ho}$ and wild-type Purkinje cells ± SD.

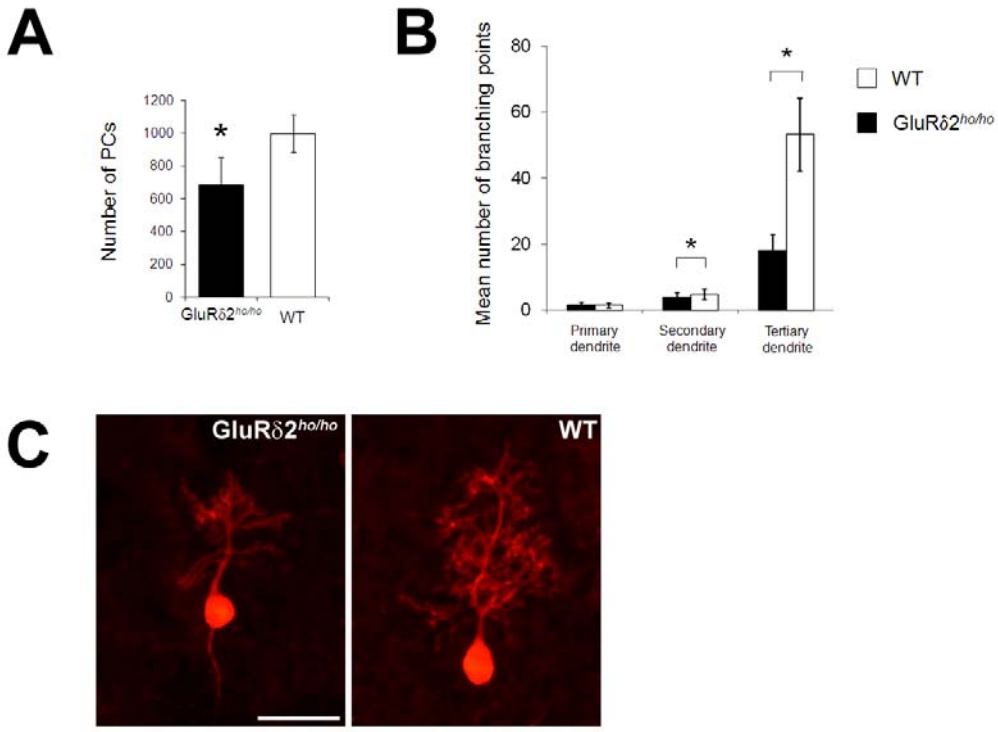

Figure 22. A and B. Significant Purkinje cell loss and atrophy of secondary and tertiary Purkinje cell dendrites in GluRδ2$^{ho/ho}$ cerebellar cultures. C. Representative CaBP-immunofluorescent Purkinje cells in GluRδ2$^{ho/ho}$ and wild-type organotypic cerebellar cultures. Bar = 50 µm.

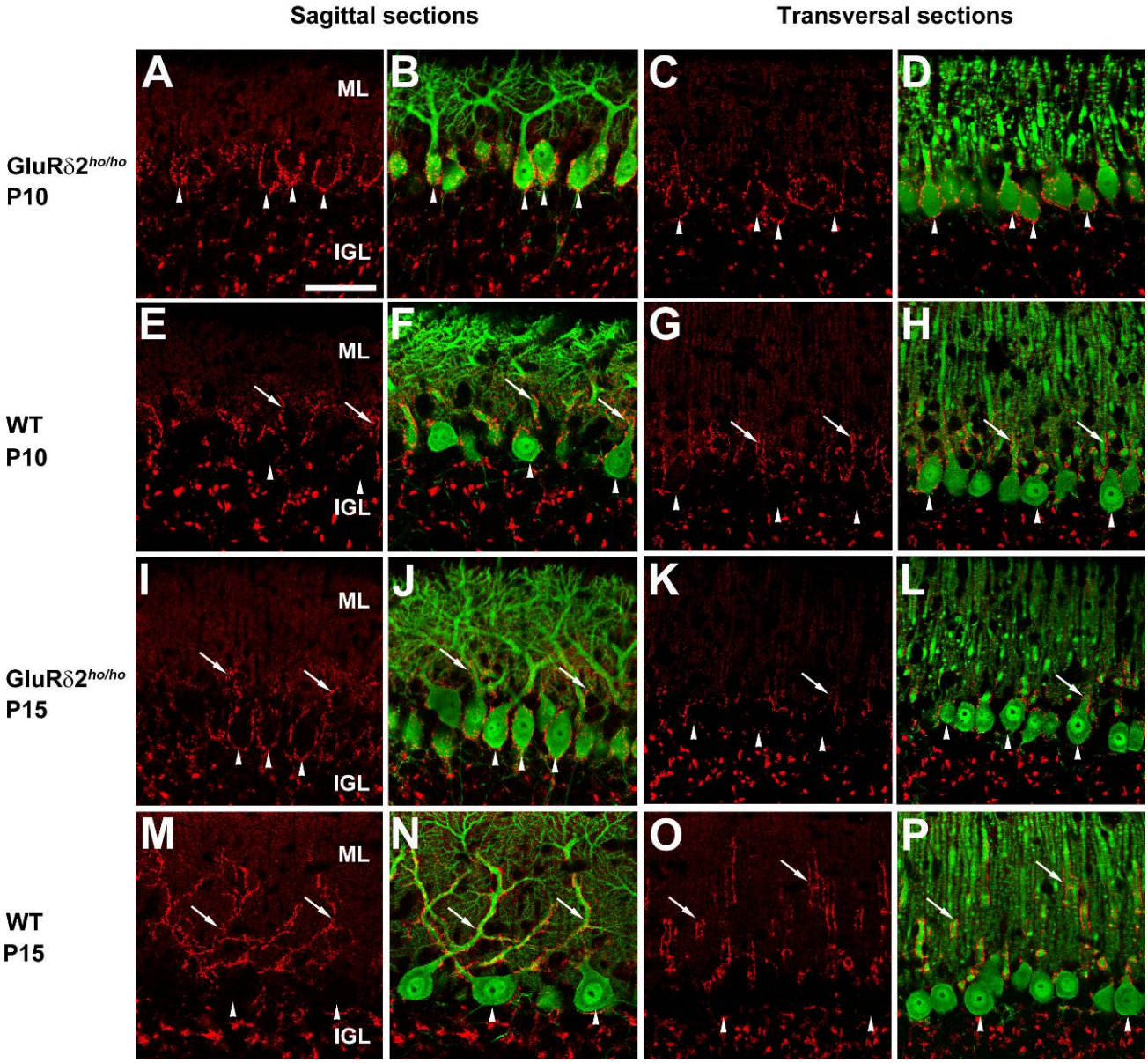

Figure 23. Climbing fiber (arrows) translocation along the soma and dendrites of the Purkinje cells (arrowheads) is impaired in GluRδ2$^{ho/ho}$ mice.

A-H. At P10, VGlut 2-immunofluorescent (red) climbing fibers (A-D) innervate the soma but not the dendrites of the CaBP-immunofluorescent (green) GluRδ2$^{ho/ho}$ Purkinje cells (B, D) whereas climbing fiber somato-dendritic translocation (E-H) has already occurred on the proximal dendrite of the wild-type Purkinje cells (F, H). Fifteen day-old GluRδ2$^{ho/ho}$ Purkinje cells (J, L) exhibit somatic and limited dendritic climbing fiber innervation (I-L) while climbing fiber translocation to the primary and secondary dendrites is completed on the wild-type Purkinje cells (M-P). Bar = 50µm for A-P.

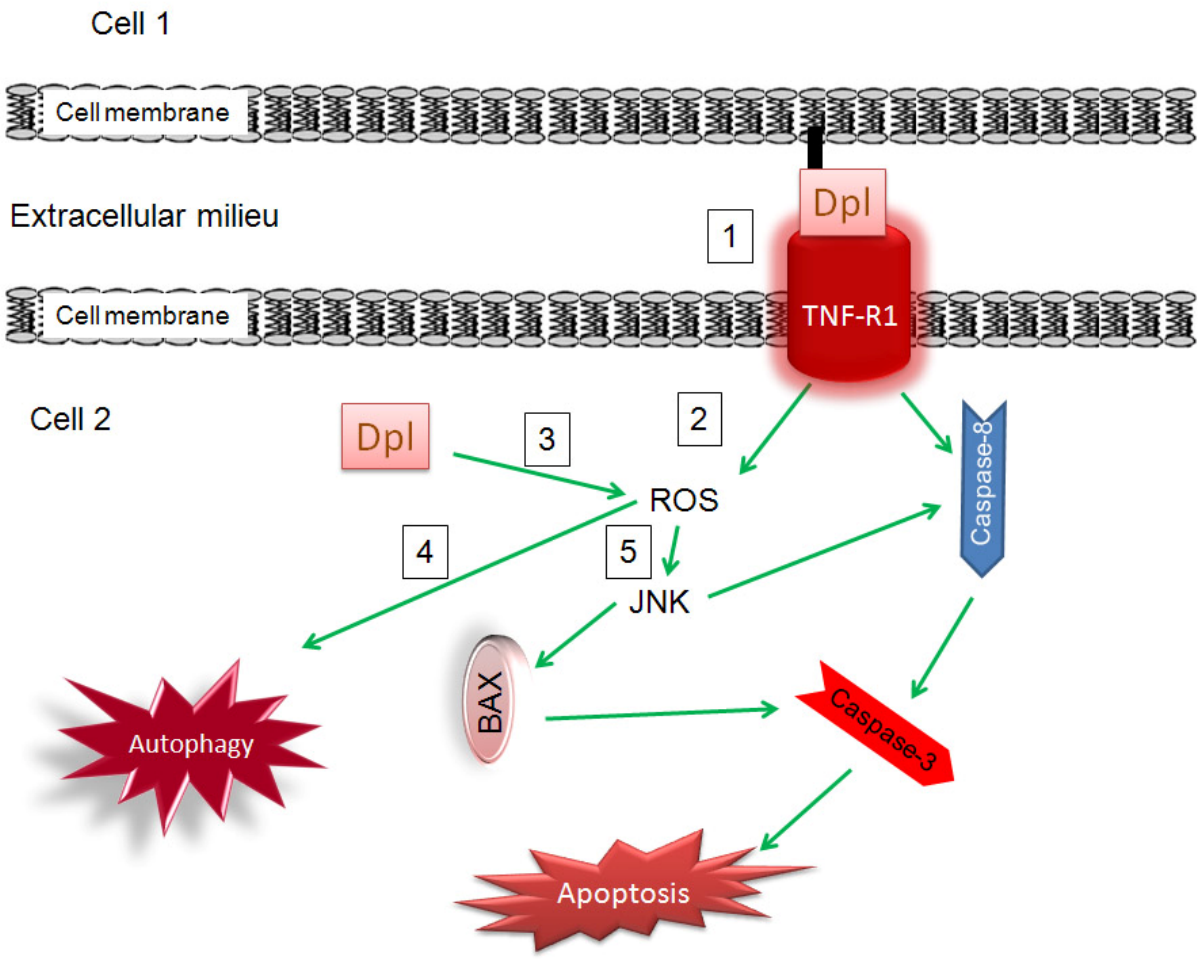

Figure 24. Hypothesis for a Dpl-induced neurotoxic mechanism.

(1) Extracellular Dpl, bound to cell membrane, can activate TNF-R1, leading to the production of ROS (2) by an unidentified mechanism (Papa et al., 2006). (3) In parallel, cytosolic Dpl can induce production of ROS by a possible calcium-dependent pathway (Brini et al., 2005). The produced ROS will either induce autophagy (4) (Djavaheri-Mergny et al., 2007) or activate JNK (5) (Papa et al., 2006) leading to the activation of caspase-8-dependent apoptosis and BAX-dependent apoptosis.

Primary antibody	**Company**	**Dilution**	**Secondary antibody**	**Company**	**Dilution**	**Experiment**
Mouse anti-CaBP	Sigma Aldrich	1/1000	Alexa 546 goat anti-mouse	Molecular Probes	1/1000	Immuno-fluorescence
Mouse anti-CaBP	Sigma Aldrich	1/1000	Alexa 488 goat anti-mouse	Molecular Probes	1/1000	Immuno-fluorescence
Rabbit anti-CaBP	From Mrs Thomasset	1/1000	Alexa 546 goat anti-rabbit	Molecular Probes	1/1000	Immuno-fluorescence
Guinea pig anti-VGlut2	Chemicon	1/3000	Alexa 546 goat anti-Guinea pig	Molecular Probes	1/1000	Immuno-fluorescence
Mouse anti-GFAP	Sigma Aldrich	1/500	Byotinilated horse anti-mouse	Vector Labs	1/200	Immuno-peroxidase
Mouse anti-GFAP	Sigma Aldrich	1/500	Alexa 546 goat anti-mouse	Molecular Probes	1/1000	Immuno-fluorescence
Rabbit anti-GFAP	Dako	1/500	Alexa 488 goat anti-rabbit	Molecular Probes	1/1000	Immuno-fluorescence
Rabbit anti-aldolase C	From Dr Sugihara	160ng/mL	Byotinilated horse anti-rabbit	Vector Labs	1/200	Immuno-peroxidase
Rabbit anti-aldolase C	From Dr Sugihara	160ng/mL	Alexa 488 goat anti-rabbit	Molecular Probes	1/1000	Immuno-fluorescence
Mouse anti-*Hu-bcl-2*	BD Biosciences	1/50	Byotinilated horse anti-mouse	Vector Labs	1/200	Immuno-peroxidase
Rabbit anti-Scrg1	From Dr Dron	1/60	Alexa 488 goat anti-rabbit	Molecular Probes	1/1000	Immuno-fluorescence
Mouse anti-LC3B	Nanotools	1/10	Alexa 488 goat anti-mouse	Molecular Probes	1/1000	Immuno-fluorescence
Mouse anti-p62	BD Transduction Labs	1/100	Alexa 488 goat anti-mouse	Molecular Probes	1/1000	Immuno-fluorescence

Table 3. Antibodies used for immunohistochemical staining experiments.

Method	Fixative
Transmission electron microscopy for ultrastructural analysis	4% paraformaldehyde – 2% glutaraldehyde in PB
Scrapie responsive gene 1 (Scrg1) pre-embedding immunogold	1% paraformaldehyde in PB
γ amino butyric acid (GABA) post-embedding immunogold	2% paraformaldehyde - 2% glutaraldehyde in PB

Table 4. Fixative used for transmission electron microscopy and immunocytochemistry.

	GluRδ2$^{Lc/+}$ mouse		$NP^{0/0}$ mouse	
		Reference		Reference
Apoptosis	Increased Bax IR in GluRδ2$^{Lc/+}$ Purkinje cells	523	DNA fragmentation in Dpl-overexpressing hippocampal neurons	237
	TUNEL labeling of GluRδ2$^{Lc/+}$ Purkinje cells	522, 338	TUNEL labelling of Dpl overexpressing granule cells	324
	Caspase-3 IF in GluRδ2$^{Lc/+}$ Purkinje cells	427	ROS producing enzymes upregulation in Dpl-overexpressing neurons	94, 521
	Delayed death of *Bax*$^{-/-}$:GluRδ2$^{Lc/+}$ Purkinje cell	428	Activation of caspases 3 and 10 by Dpl in N2A and astrocytes	376
	Inactivation of extrinsic pathway delays *tPA*$^{-/-}$:GluRδ2$^{Lc/+}$ Purkinje cell death	279	*Bcl-2* overexpression delays caspase-3 activation in the cerebellum of ΔPrP transgenic mouse	333
	ROS are induced in GluRδ2$^{Lc/+}$ Purkinje cells	307	*Bcl-2* overexpression and *Bax*$^{-/-}$ partly rescue $NP^{0/0}$ Purkinje cells	171, 172
Autophagy	GluRδ2 is linked to Beclin1 via nPIST in cerebellar extracts and HEK cells	541	Scrg1 IR of $NP^{0/0}$ Purkinje cells	111, 173
	LC3 IR of GluRδ2$^{Lc/+}$ Purkinje cells *in vivo* and *in vitro*	499		
	GluRδ2^{Lc} induces autophagy *in vitro* in HEK cells and *in vivo* in *Lurcher* Purkinje cells	541	Early autophagy in $NP^{0/0}$ Purkinje cells	173
	Autophagy is induced by GluRδ2^{Lc} in GluRδ2$^{Lc/ho}$ Purkinje cells	429		
	Induction of axonal autophagy in GluRδ2$^{Lc/+}$ Purkinje cell	499	Upregulation of p62 in $NP^{0/0}$ Purkinje cells	173
	Absence of p62 upregulation in GluRδ2$^{Lc/+}$ Purkinje cells	499		

Table 2. Experimental evidences for apoptosis and autophagy in *Grid2* mutant mice and in Nagasaki *Prnp*-deficient mice overexpressing Dpl.