Annals of Anatomy xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.com/locate/aanat

Cell death and neurodegeneration in the postnatal development of cerebellar vermis in normal and Reeler mice

Claudia Castagna*, Adalberto Merighi, Laura Lossi

University of Turin, Department of Veterinary Sciences, Italy

ARTICLE INFO

Article history:
Received 21 December 2015
Accepted 25 January 2016
Available online xxx

Keywords:
Cerebellum
Apoptosis
Autophagy
Ultrastructure
Granule cells
Mouse
Development

SUMMARY

Programmed cell death (PCD) was demonstrated in neurons and glia in normal brain development, plasticity, and aging, but also in neurodegeneration. (Macro)autophagy, characterized by cytoplasmic vacuolization and activation of lysosomal hydrolases, and apoptosis, typically entailing cell shrinkage, chromatin and nuclear condensation, are the two more common forms of PCD. Their underlying intracellular pathways are partly shared and neurons can die following both modalities, according to the type of death-triggering stimulus.

Reelin is an extracellular protein necessary for proper neuronal migration and brain lamination. In the mutant *Reeler* mouse, its absence causes neuronal mispositioning, with a notable degree of cerebellar hypoplasia that was tentatively related to an increase in PCD. We have carried out an ultrastructural analysis on the occurrence and type of postnatal PCD affecting the cerebellar neurons in normal and *Reeler* mice. In the forming cerebellar cortex, PCD took the form of apoptosis or autophagy and mainly affected the cerebellar granule cells (CGCs). Densities of apoptotic CGCs were comparable in both mouse strains at P0-P10, while, in mutants, they increased to become significantly higher at P15. In WT mice the density of autophagic neurons did not display statistically significant differences in the time interval examined in this study, whereas it was reduced in *Reeler* in the P0-P10 interval, but increased at P15. Besides CGCs, the Purkinje neurons also displayed autophagic features in both WT and *Reeler* mice. Therefore, cerebellar neurons undergo different types of PCD and a Reelin deficiency affects the type and degree of neuronal death during postnatal development of the cerebellum.

1. Introduction

During development, the embryonic nervous system undergoes a complex and highly regulated sequence of events resulting in the generation of all neurons and glial cells of the adult brain, spinal cord and peripheral nervous system. In laminar brain regions, such as the cerebral and cerebellar cortices, neurons perform long distance migration and extend their axons to make highly precise synaptic connections with targets. Genetic mutations, as well as insults of heterogeneous nature, that concern the ability of neural cells to perform these sequenced steps, result in developmental arrest and may lead to death of the affected cells (Yamaguchi and Miura, 2015).

Reeler is the first described mouse cerebellar mutation (Falconer, 1951) but the mutated protein, named Reelin, was only discovered many years later (D'Arcangelo et al., 1995). The *Reeler* phenotype is characterized by typical alterations in gait ("reeling"), associated with the total absence of Reelin in the recessive homozygous mice (*reln*$^{-/-}$). Reelin is an extracellular matrix glycoprotein secreted during neurogenesis and represents the first molecule of a complex intracellular cascade regulating neuronal migration (D'Arcangelo and Curran, 1998; Lambert de Rouvroit and Goffinet, 1998). The absence of Reelin induces anatomical anomalies in a large number of different areas of the central nervous system (CNS), including the olfactory bulb, neocortex, hippocampus, anterior colliculus, substantia nigra, the pontine nuclei, inferior olivary nucleus, some nuclei of the cerebral trunk associated with the encephalic nerves, cerebellum, and spinal cord (Katsuyama and Terashima, 2009; Cendelin, 2014).

The *Reeler* mouse cerebellum is profoundly hypoplastic and, at its surface, only shows very shallow notches, probably the remnants of a tentative foliation (Mikoshiba et al., 1980).

A series of severe histological alterations corresponds to these gross anatomy anomalies: the molecular layer (ML) of the cerebellar cortex is irregular in thickness; the Purkinje cell layer is discontinuous with only few scattered Purkinje neurons (PNs)

* Correspondence to: Largo Braccini, 2, I-10095 GRUGLIASCO (TO) Italy, EU.
E-mail address: claudia.castagna@unito.it (C. Castagna).

http://dx.doi.org/10.1016/j.aanat.2016.01.010
0940-9602/

aligned in their normal position between the more superficial ML and the deeper granular layer (GL), which displays a lower cellular density and a variable thickness, while the remaining PNs are ectopically localized mainly in a central cellular mass intermingled with the white matter (Mariani et al., 1977; Heckroth et al., 1989; Yuasa et al., 1993). Of these alterations, particularly the reduction in GL largely contributes to the atrophy that is macroscopically observed in the *Reeler* mice (D'Arcangelo and Curran, 1998).

The notion that a form of programmed cell death (PCD) affects the neurons and glia during normal nervous system development, plasticity, and aging, but also in neurodegenerative diseases, is now fully accepted (for a very recent review see Lossi et al., 2015). From the initial observations, several forms of PCD have been described after ultrastructural examination. Schweichel and Merker (1973) were the first to propose a classification of PCD into three types on the basis of role of lysosomes in cell disruption. The first type was originally referred to as heterophagocytosis and mainly corresponds to apoptosis in subsequent classifications (Clarke, 1990). In this case cell death results from phagocytosis and activation of tissue macrophages, without activation of endogenous cell lysosomes. In the second type, sharing several features with necrosis (Clarke, 1990), cell death is induced by an external insult without an obvious lysosome intervention. In the third type, autophagocytosis, cells are eliminated through the activation of their own lysosomal enzymes. This third mode of cell death according to Schweichel and Merker (1973) corresponds to the later described (macro)autophagy (Maiuri et al., 2007).

Among the three forms of PCD, apoptosis has been widely described to massively affect the cerebellar granule cells (CGCs) in the course of their postnatal maturation and migration in several altricial species, including humans (Marzban et al., 2015). Nonetheless, dying neurons displaying the characteristic ultrastructural features of autophagy were also previously observed, albeit occasionally (Lossi and Merighi, 2003).

Although evidence supports the hypothesis that an absence of Reelin causes a loss of the CGCs and the PNs in the cerebellar cortex (Cendelin, 2014), very limited information is available on the occurrence and type of postnatal cell death in mutants. Our group has demonstrated that the CGCs of the *Reeler* mouse undergo apoptosis after TUNEL labeling of fragmented DNA, and that apoptosis in mutants is significantly higher than in normal wild-type (WT) littermates (Cocito et al., 2016). However, the existence of other death modalities was not investigated, and ultrastructural confirmation is required to assess the type of cell death beyond any reasonable doubt, as DNA fragmentation may also occur in death types other than apoptosis (Lossi et al., 2015).

Therefore, we have expanded our light microscopic investigations on cell death and neurodegeneration during the course of the postnatal cerebellar development of the *Reeler* mouse, and provided here the first comprehensive ultrastructural description of the modes of death affecting the cerebellar neurons in this mutant. For simplicity, in comparing data between mutants and their WT littermates, we will refer to cell death/neurodegeneration as to different forms of PCD. One should however have well in mind that PCD in WT mice is *bona fide* naturally occurring neuronal cell death (NOND), whereas in *Reeler* it is complicated by the effects of the mutation.

2. Materials and methods

2.1. Animals

Studies were performed on 16 mice, ranging in age from birth (P0) to postnatal day 15 (P15). *Reeler* and littermate WT mice at P0, P5, P10 and P15 were used for qualitative and quantitative analysis ($n = 4$/each postnatal age). The number of animals was kept to a minimum and all efforts were made to minimize their suffering. All experiments were authorized by the Italian Ministry of Health and the Bioethics Committee of the University of Turin. Animal procedures were carried out according to the guidelines and recommendations of the European Union (Directive 2010/63/UE) as implemented by current Italian regulations on animal welfare (DL no. 26-04/03/2014). Before being used in this study, all mice were genotyped to ascertain their appropriate genetic background (D'Arcangelo et al., 1996).

2.2. Histology and ultrastructural analysis

Animals were euthanized intraperitoneally with an overdose of sodium pentobarbital and then perfused with 2% glutaraldehyde + 1% paraformaldehyde in Sörensen buffer 0.1 M pH 7.4. Dissected cerebella were then cut in 200 μm thick parasagittal vibratome slices and sections of the vermis were processed according to standard TEM procedures.

For quantitative analysis, a single ultrathin parasagittal section of the entire vermis was collected onto a 75-mesh grid. Due to the small cerebellar size, the area of the grid was sufficient to host the entire section obtained from both WT and *Reeler* mice at all ages examined. Two different ultrathin sections from the same animal, collected at a distance of at least 200 μm along the transverse plane, were subject to analysis. Cells were individually scanned at a magnification ranging between 4000 and 10,000× and classified on the basis of their ultrastructure as described in Section 3.

Quantitative results were expressed as the numerical density of cells undergoing PCD per area unit (number of cells undergoing PCD/mm^2). Numerical density was calculated as the ratio of the total number of cells undergoing PCD versus the total number of meshes occupied by tissue. The values so obtained were normalized to area unit by dividing them for the total area of one mesh (0.812 mm^2).

2.3. Statistics

Cell counts obtained from each section pair from the same mouse were subjected to statistical analysis. There were no statistically significant differences in measurements taken from sections belonging to the same animal, thus values were averaged and their means used for analysis. A Student's *t*-test was used to compare the means obtained for each animal of the same group and between groups. Data were expressed as the mean of the number of cells undergoing to apoptosis or autophagy/mm^2 ± SEM. Results were considered statistically significant at $p \leq 0.05$.

3. Results

Table 1 summarizes the key ultrastructural features that were used in this study to differentiate between cells undergoing apoptosis or autophagy. A third category of "non-canonical" or dark degenerating cells was also defined. Cells falling into this category: (i) did not display the typical characteristics of apoptosis or autophagy, but rather showed a mixture of the features of the two. In some instances some traits typical of necrotic degeneration were also observed; or (ii). presented an ultrastructural pattern similar to the so called dark degeneration that was previously described to affect the neurons and glia in the weaver mutant mouse (Migheli et al., 1997), and, more recently, in a murine model of neurodegeneration (Yang et al., 2008). Notably we never found cells with the typical ultrastructural features of pure necrosis.

Please cite this article in press as: Castagna, C., et al., Cell death and neurodegeneration in the postnatal development of cerebellar vermis in normal and Reeler mice. Ann. Anatomy (2016), http://dx.doi.org/10.1016/j.aanat.2016.01.010