Axonal torpedoes in cerebellar Purkinje cells of two normal mouse strains during aging

Abstract The present study systematically investigated the proportional evolution of Purkinje cell (PC) axonal swellings, also termed torpedoes, during aging of the two unrelated mouse strains B6CBA and C57BL/6J. Torpedoes were identified using monoclonal antibodies against the calcium-binding protein calbindin D-28k in mice ranging in age from 8 days postnatally up to 32 months. The relative density of PCs bearing torpedoes in animals up to 6 months of age was less than 0.1%. The number increases between 6-8 months and rises further in older mice almost linearly up to 13.7% affected PCs in the oldest animal (32 months) studied. In contrast, PC loss, as indicated by parvalbumin-immunoreactive empty baskets, is only at a very moderate level (less than 0.5%) in these strains. While the proximal axonic segments often show two and occasionally up to five swellings and frequently appear to be hypertrophied as a whole, the dendritic trees and neuronal somata of the affected PCs exhibit normal morphology. On rare occasions adaptive reactions indicated by "arciform axons" and enlarged varicosities of recurrent collaterals were observed. The results demonstrate that in addition to age-related PC loss of whatever degree, axonal disturbances of PCs, indicated by torpedoes, are present, leading most probably to a graded loss of cerebellar cortico-fugal projections.

Key words Torpedoes • Purkinje cell • Aging Calbindin D-28k • Cerebellum

Introduction Round or spindle-shaped axonal swellings, also termed spheroids or torpedoes, are reported to be a relatively common characteristic of a wide range of neuropathies. Torpedoes are found in various neurodegenerative diseases with cerebellar involvement like Creutzfeldt-Jakob's disease [23], Kuru [29], Tay-Sachs-idiocy, syphilis, dementia paralytica, Arnold Chiari's malformation, olivo-ponto-cerebellar atrophy and Friedreich's ataxia [3]. Focal cerebellar lesions due to neoplasms that disrupt the cortico-nuclear projections of Purkinje cells (PCs) [48] and culturing of PCs [16, 18] are also described as causing torpedoes. In myelin deficiencies like multiple sclerosis in man [47] or in genetically unrelated mouse-mutants with a paucity in myelination (quaking, shiverer and jimpjy) [16, 38, 46] these bulbous swellings are found in addition. Disruptions in the neurofilament network or microtubules also induce these spheroids [2, 8], and the antiepileptic drug phenytoin [27] as well as several neurotoxins [2, 18] are known to lead to swellings in PC axons. Ultrastructural investigation of torpedoes revealed a massive accumulation of misaligned neurofilaments, tubular profiles of the smooth endoplasmic reticulum and mitochondria [31, 40, 46]. From this short survey it seems obvious that torpedoes are a relatively unspecific sign most probably secondary to axonal damage, disturbance of the neuronal metabolism, defective myelination or cytoskeletal dysfunctions. However, axonal degeneration indicated by the presence of torpedoes must not necessarily imply cell damage or neuron loss, as can be concluded from several cerebellarly affected mouse mutants with various extents of PC degeneration. PCs of Leaner (tg la) [39], Lurcher (lc) [14], Nervous (nr) [42, 51], Nodding (nd) [41], the hyperspiny Purkinje cell mutant (hpc) [19, 40] and the Niemann-Pick type C mouse (npc) [22] are affected by torpedoes but in these mutants the fate of degeneration does not reach each single PC bearing a torpedo. In nr more than 90% of the PCs die, but most of the surviving exhibit a torpedo [42, 51]. In hpc practically all PCs are characterized by these swellings and the terminal domains of the cerebellum are largely denervated, but only 27% of the PCs undergo degeneration [19, 40]. In tg la, lc, nd and npc surviving PCs also bear torpedoes. In another mutant, the Purkinje cell degeneration mutant (pcd), in which less than 1%
of the PCs survive [32, 49], virtually all PC terminals positive for the calcium-binding protein calbindin D-28k (CaBP) are lost [7], however, to our knowledge hitherto no torpedoes are described in the literature. We show in the present report that even some of the very few surviving PCs in this mutant also bear a torpedo. Therefore, although degeneration of the distal axonic segment is indicated by torpedoes, one has to be cautious in equating the occurrence of torpedoes with the degeneration of the whole cell. Moreover, axonal swellings are also reported during normal postnatal cerebellar development of the healthy rat without indications of PC death [18].

In a much earlier study in man [28] cerebellar torpedoes were observed in all individuals over 60 years of age investigated, regardless of underlying disease. From several other studies in different brain areas and in different species, it was concluded that the number of spheroids increases with advancing age [17, 26, 34, 45, 46], but in regard to the cerebellum this has not been investigated systematically. In contrast, age-related loss of PCs has been studied widely during the last century but its extent is still a subject for controversy (for review see [4]). Almost as many studies as those that found a remarkable PC loss during aging [25, 33, 36, 37, 44, 50], revealed only a very moderate PC loss, if at all [4, 11-13]. The present study does not reinvestigate the question of neuron loss in the cerebellum, although some observations supporting the idea of a relatively mild loss of PCs are presented, but systematically studies the occurrence of torpedoes in PC axons during aging in two different mouse strains, using monoclonal antibodies (mAbs), which in the cerebellum are Purkinje cell specific, directed against CaBP [5, 7]. Regarding the integrative function of the PC, in view of the fact that PCs provide the only output of the cerebellar cortex and in consideration of the motor disturbances in advanced age, the incidence of axonal torpedoes is of particular interest, as axonal degeneration without neuron loss can lead to similar or even more distinct functional disturbances than PC degeneration. Parts of this study have been published in abstract form [6].

**Material and methods**

**Animals**

For CaBP and parvalbumin (Parv) immunocytochemistry a total of 18 mice were used, the parents of which originated from the Jackson Laboratories (Bar Harbor, Me). The offspring were maintained at the Department of Physiology, Freie Universität Berlin, on a natural dark/light rhythm and food and water ad libitum. For CaBP immunocytochemistry 8 C57BL/6J wild types and 10 B6CBA wild types were used. In some cases several sections of the same brains were incubated with an antibody directed against Parv to gain an estimate of the PC loss in these aging animals.

**Tissue preparation**

Initially the animals received a lethal dose of chloral-hydrate (Merck; Darmstadt, Germany, cat. no. 2425) (1.75 g/kg body weight). Perfusion started with normal saline (0.9 %) in 0.067 M phosphate buffer at pH 7.4 and was followed by the fixative containing 1 % paraformaldehyde (Merek; cat. no. 4005) and 1 % glutaraldehyde (Merek; cat. no. 4239) in 0.1 M phosphate buffer at pH 7.4 for 15 min. After 2 h of postfixation in the same fixative, brains were cut in the corona plane at 30 µm on a vibratome (Series 1000, Technical Products International Inc., St. Louis, Mo.).

**Antibodies**

The CaBP antibody used was a monoclonal mouse-IgG (code no. 300) produced by hybridization of mouse myeloma cells with mouse spleen cells, immunized with CaBP from chicken gut. Specification and potency of this clone have been fully characterized by various tests [10] and numerous studies (for review see [1]).

The Parv antibody (clone 235) is a mAb raised in hybrids of mouse spleen cells fused with mouse myeloma cells and is directed against Parv from carp muscle conjugated to tetanus toxoid using carbodiimide. Specification and characterization of this clone are fully described in Celio et al. [9].

Both mAbs used were initially a gift from Dr M. R. Celio and later commercially obtained from SWant (Swiss antibodies, Bellinzona, Switzerland).

**Immunocytochemistry**

Immunocytochemistry was performed according to the avidin-biotin-peroxidase complex (ABC) method [24]. Sections were treated first with 10 % methanol and 3 % H2O2 in 0.01 M PBS (phosphate-buffered saline) at pH 7.5 to block endogenous peroxidase activity. This was followed by washing twice in distilled water for 5 min, and subsequent washing in TRIS-buffered saline (TBS) consisting of 0.05 M TRIS-HCl, pH 7.6 and 0.9 % NaCl. To prevent nonspecific binding of the first antibody, preincubation with a solution containing 10 % normal rabbit serum (NRS; Gibco BRL, Paisley, Scotland), 0.1 % bovine serum albumin (BSA; Sigma, St. Louis, Mo.) and 1 % Triton X-100 (Sigma) in TBS was performed. In preliminary test various dilutions of the first antibody were screened to obtain optimal immunostaining: intense, Golgi-like staining of cerebellar PCs was achieved with a dilution of 1:6000 of the CaBP antibody in TBS containing 5 % NRS and 0.3 % Triton X-100. Best results with the Parv antibody were obtained using a dilution of 1:1000 in the same solution as above. The first antibody incubation was performed at room temperature for 1 h under gentle agitation. Control sections were incubated in the same solution with added NRS but without the first antibody, and did not show any immunostaining. Three washes in TBS followed prior to incubation with the second antibody, biotinylated rabbit anti-mouse (Dako, Hamburg, Germany) 1:200, diluted in the same solution as the primary antibody. Sections were then washed again three times for 5 min in TBS and were incubated in ABC (Dako) for 30 min. After washing three times for 5 min in TBS, the presence of the peroxidase enzyme was revealed using 0.05 % diaminobenzidine (DAB, Sigma) in TBS containing 1 % H2O2 for 15 min.

**Quantification**

The numerical density of PCs bearing torpedoes was quantified as the percentage of CaBP-positive axons with spheroids in the granular layer in relation to the total number of immunopositive PCs in the corresponding PC row. PCs with more than one torpedo were counted as one; therefore, the given percentage is a term of affected PCs and not of the number of torpedoes.