Spinocerebellar ataxia type 3 (Machado–Joseph disease): severe destruction of the lateral reticular nucleus


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Summary
The lateral reticular nucleus (LRT) of the medulla oblongata is a precerebellar nucleus involved in proprioception and somatomotor automatisms. We investigated this nucleus in five individuals with clinically diagnosed and genetically confirmed spinocerebellar ataxia type 3 (SCA3, Machado–Joseph disease). Polyethylene glycol-embedded 100 μm thick sections stained for lipofuscin granules and Nissl material as well as Nissl-stained paraffin-embedded sections revealed severe destruction of the LRT in all SCA3 brains examined. Some of the few surviving neurones contained ataxin-3-immunopositive intranuclear inclusion bodies, as noted in other affected brain regions in SCA3. Along with the severe neuronal depletion, obvious astrogliosis was seen in the LRT of all SCA3 patients. The findings suggest that the LRT is a consistent target of the pathological process underlying SCA3. In view of its afferent and efferent connections, destruction of the LRT probably contributes to gait ataxia in individuals suffering from SCA3.

Keywords: ataxin; Nissl stain; precerebellar nuclei; reticular formation; SCA3

Abbreviations: LRT = lateral reticular nucleus; SCA = spinocerebellar ataxia

Introduction
Spinocerebellar ataxia type 3 (SCA3), also known as Machado–Joseph disease, is an autosomal dominantly inherited neurodegenerative disease that, together with Huntington’s disease and at least seven other diseases, comprises the so-called CAG repeat or polyglutamine diseases. These inherited diseases are caused by unstable expansions of (CAG)n trinucleotide repeat sequences at disease-specific gene loci that are translated into elongated polyglutamine tracts. In SCA3, the expanded trinucleotide repeat resides in the codon region of the MJJD1 (Machado–Joseph disease 1) gene on chromosome 14q32.1, which normally has a repeat of 12–40 CAG triplets that is expanded to ~56–84 CAG triplets in affected individuals (Kawaguchi et al., 1994; Schöls et al., 1995; Stevanin et al., 1995; Twist et al., 1995; Dürr et al., 1996; Nishiyama et al., 1996; Koshy and Zoghbi, 1997; Nance, 1997; Paulson et al., 1997; Klockgether and Evert, 1998; Koeppen, 1998; Schmidt et al., 1998; Tait et al., 1998; Trottier et al., 1998; Lieberman et al., 1999; Fujigasaki et al., 2000).

The disease gene product in SCA3 is the protein ataxin-3, whose physiological role is unknown. Ataxin-3 is widely expressed in CNS neurones. In SCA3, ataxin-3 is found in dot-like intranuclear aggregates or inclusions within select populations of neurones (Koshy and Zoghbi, 1997; Nance, 1997; Paulson et al., 1997; Klockgether and Evert, 1998; Koeppen, 1998; Schmidt et al., 1998; Tait et al., 1998; Trottier et al., 1998; Lieberman et al., 1999; Fujigasaki et al., 2000).

Clinically, SCA3 presents with progressive gait and limb ataxia, dysarthria and a variable combination of other symptoms including pyramidal signs, dystonia, oculomotor disorders, faciallingual weakness, neuropathy, progressive sensory loss and parkinsonian features (Bürk et al., 1996, 1999; Dürr et al., 1996; Abele et al., 1997; Nance, 1997; Klockgether et al., 1999; Arpa et al., 2000; Tang et al., 2000; Jardim et al., 2001).

As with the clinical features, the underlying degenerative changes in SCA3 vary to some degree. Among the CNS

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regions that may undergo neuronal loss and reactive astrogliosis are select telencephalic, cerebellar and brainstem nuclei, the anterior horn of the spinal cord, Clarke’s column and the dorsal root ganglia (Dürr et al., 1996; Robitaille et al., 1997; Koeppen, 1998; Schmidt et al., 1998; Iwabuchi et al., 1999; Kumada et al., 2000).

Up to now, only one patient clinically diagnosed as SCA3 is described with neuronal loss in the lateral reticular nucleus (LRT) of the medulla oblongata (Kanda et al., 1989). We investigated this nucleus in five brains from individuals who died with clinically and genetically confirmed SCA3 in order to see whether destruction of this nucleus is a consistent neuropathological feature in the clinical end-stage of the disease.

**Material and methods**

The brains of five patients with clinically diagnosed and neuropathologically confirmed SCA3 (one female, four males; mean age at death 54.2 ± 12.4 years; mean age at disease onset 30.2 ± 4.6 years; mean duration of disease 24.0 ± 8.4 years) autopsied in an institute for pathology (Laboratorium Pathologie Oost Nederland, Enschede, The Netherlands) were used for this study (Table 1). The SCA3 patients studied came from five different SCA3 families residing in the northern part of The Netherlands. For comparison, five brains from age- and gender-matched individuals without medical histories of neurological or psychiatric diseases (one female, four males; mean age 55.8 ± 11.3 years) were used (Table 1). Neuropathological examination showed severe neuronal loss together with astrogliosis in the substantia nigra, red nucleus, pontine nuclei (Fig. 1), subthalamic nucleus and in the internal segment of the pallidum in all the SCA3 brains. The cerebellar cortex of all SCA3 cases showed a slight reduction of the number of its Purkinje cells, and the dentate nucleus consistently exhibited severe loss of nerve cells with so-called grumose degeneration in surviving neurones. In addition, severe neuronal loss was seen consistently in the cranial nerve nuclei VI, VIII and XII, as well as in the anterior horn of the spinal cord and in Clarke’s column. Finally, in all of the SCA3 patients, a severe degeneration was found in the ventral and dorsal spinocerebellar tracts.

The onset of disease was determined as the time when the patient (or a close relative) noticed with certainty the first signs of stance and gait imbalance. All of the patients included in this study had adult onset of the disease (Table 1) and suffered from late-stage SCA3. They were wheelchair-bound, and suffered from severe dysarthria, lingual paresis, horizontal gaze and abducens paresis with hypometric and

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CAG = number of expanded CAG repeats in the SCA3 allele; n.d. = not determined; βA = stages A–C in the development of Alzheimer-related β-amyloid deposits.
slow saccades, upper limb ataxia, bradykinesia and dystonia, peripheral weakness and loss of proprioception.

The clinical diagnosis in the SCA3 patients was confirmed genetically (Table 1). To this end, genomic DNA was prepared by extraction from peripheral lymphocytes as previously described (Verschuuren-Bermelma et al., 1995) and genotyped with polymorphic dinucleotide repeat sequences that flank the SCA gene locus at chromosome 14q24.3–q32. The number of expanded CAG repeats in the disease allele ranged from 72 to 75 (Table 1).

In all SCA3 and control cases, representative tissue sections from the neocortex and allocortex underwent staining with the modified silver pyridine Campbell–Switzer method to identify β-amyloid deposits (Braak and Braak, 1991b). Neuropathological classification of cortical β-amyloidosis was performed on these sections according to the Braak and Braak staging system that distinguishes three stages (A–C) of cortical β-amyloidosis (Table 1) (Braak and Braak 1991a).

A collection consisting of the first, twentieth, twenty-first, etc. of the 100 μm thick sections was stained for lipofuscin pigment (aldehyde fuchsin) and Nissl material (Darrow red) (Braak, 1980), while the 6 μm thick sections through the pons, the spinal cord and the rostral midbrain were embedded in paraffin, cut into 6 μm frontal sections and stained with haematoxylin–eosin. In three SCA3 cases (cases 2, 4 and 5; Table 1) and three control cases (cases 7, 9 and 10; Table 1), the brainstem and spinal cord were cut perpendicular to the brainstem axis into uninterrupted series of 100 μm thick horizontal sections, while the paraffin-embedded blocks of the pons, the medulla oblongata and spinal cord were embedded in paraffin. The PEG-embedded brainstems and spinal cords were cut perpendicular to Meynert’s brainstem axis into uninterrupted series of 100 μm thick horizontal sections, while the paraffin-embedded blocks of the pons, medulla oblongata and spinal cord were cut into 6 μm uninterrupted serial sections.

In all SCA3 and control cases, representative tissue sections from the neocortex and allocortex underwent staining with the modified silver pyridine Campbell–Switzer method (Braak and Braak, 1991b), while the presence of astrocytes was investigated in adjacent sections through the LRT treated with a rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP; 1 : 500, Dako, Glostrup, Denmark) and counterstained with haematoxylin. In the same manner, the antibody CR3/43 (1 : 1.500, Dako, Glostrup, Denmark) was employed in order to visualize activated microglial cells.

In all of the SCA3 patients and control cases, immunocytochemistry for the normal and the mutant form of ataxin-3 was performed. A polyclonal anti-ataxin-3 antibody (1 : 2000) was employed for the immunolocalization of normal and mutant ataxin-3 in tissue sections through the LRT of SCA3 patients and controls (Paulson et al., 1997). These sections were counterstained with haematoxylin. In addition, to characterize ataxin-3-positive structures further, the mouse monoclonal antibody 1C2 (1 : 1000, Chemicon, Temecula, CA, USA) directed against expanded polyglutamine stretches was used (Trottier et al., 1995; Lieberman et al., 1999). The specificity of the immunostaining was analysed by omission of the primary antibodies. To demask hidden epitopes, sections were autoclaved in 10 mM citrate buffer (pH 6.0) for 20 min. For single immunostaining, incubation with the primary antibodies was performed for 40–44 h at room temperature followed by incubation with biotinylated anti-mouse immunoglobulins for 2 h at room temperature. Bound antigens were visualized with the ABC complex (Vectastain, Vector Laboratories, Burlingame, CA, USA) and 3,3-diaminobenzidine-tetra-HCl/H2O2 (DAB; D5637 Sigma, Taufkirchen, Germany).

Results

Anatomical remarks

The LRT (nucleus medullae oblongatae lateralis according to Olszewski and Baxter, 1982) is located in the ventrolateral region of the medullary reticular formation filling the space between the inferior olive on the one hand and the nucleus of the trigeminal spinal tract on the other (Fig. 2B–D). It extends from the caudal pole of the medial accessory nucleus of the inferior olive caudally to the level of the pontomedullary sulcus rostrally, where it breaks up into a number of distinct subunits (Fig. 2B–D) (Walberg, 1952; Braak, 1971; Olszewski and Baxter, 1982; Huang and Paxinos, 1995; Paxinos et al., 1990; Voogd et al., 1990, 1998; Braak and Braak, 1998).

The human LRT is composed of densely packed medium to large sized multipolar neurones harbouring numerous lipofuscin granules (Braak, 1971; Olszewski and Baxter, 1982; Paxinos et al., 1990; Braak and Braak, 1998). These morphological features permit a reliable delineation of the LRT from the neighbouring components of the medullary reticular formation, all of which are composed of less densely arranged and less intensely pigmented neurones (Fig. 3A, C and E).
Neuronal loss in the lateral reticular nucleus in SCA3

Macroscopic examination of tissue blocks of the medulla oblongata showed an obvious pallor in the region where the LRT normally resides, indicating that there is substantial loss of nerve cells in this nucleus. This macroscopic finding was confirmed by light microscopic investigation. In contrast to the control cases, in which the LRT appeared as a nucleus rich in nerve cells (Fig. 3A, C and E), the area of the LRT of all SCA3 cases was nearly devoid of nerve cells, with the result that the outlines of the LRT were not reliably discernible (Fig. 3B, D and F). In all SCA3 cases, only a few surviving LRT neurones were encountered. This was demonstrable in thick sections processed for lipofuscin and Nissl material as

Fig. 2 (A) Median view of a mediosagittal section through the human brain. The lines drawn through the medulla oblongata indicate the sectional plane of the sections presented in B–D (line B, sectional plane of the section in B; line C, sectional plane of the section in C; line D, sectional plane of the section in D). (B–D) Schematized frontal sections cut perpendicular to the brainstem axis of Meynert showing three levels of the medulla oblongata with the LRT outlined by bold lines (for orientation see A). (B) Rostral pole of the LRT at the level of the area postrema (AP). (C) Middle portion of the LRT at the caudal pole of the principal subnucleus of the inferior olive (IOP). (D) Caudal pole of the LRT at the transition between the medulla oblongata and the spinal cord. A = ambiguus nucleus; AP = area postrema; AR = arcuate nucleus; CC = central canal; COM = commissural solitary nucleus; CU = cuneate nucleus; DRT = dorsal reticular nucleus; ECU = external cuneate nucleus; G = gelatinous solitary nucleus; GR = gracile nucleus; IOD = inferior olive, dorsal accessory subnucleus; IOM = inferior olive, medial accessory subnucleus; IOP = inferior olive, principal subnucleus; IRZ = intermediate reticular zone; LRT = lateral reticular nucleus; M = medial solitary nucleus; MRT = medial reticular nucleus; PCOM = paracomissural solitary nucleus; PCRT = parvocellular reticular nucleus; pyr = pyramidal tract; sol = solitary tract; SPV = spinal trigeminal nucleus; X = dorsal motor nucleus of vagus; XII = hypoglossal nucleus.
Fig. 3 (A, C and E) Three levels of the LRT of a representative control case (case 7, Table 1) in comparison with (B, D and F) the corresponding levels of a typical SCA3 patient (case 2, Table 1) (aldehyde fuchsin–Darrow red staining, 100 μm PEG sections). The LRT of the SCA3 patient at all three levels is more or less devoid of nerve cells so that the anatomical borders of the LRT were not reliably discernible. Note that the principal and dorsal accessory subnuclei of the inferior olive of the SCA3 patient are relatively well preserved (B and D), whereas the medial accessory subnucleus of the inferior olive shows mild (F) and the medial reticular nucleus severe loss of nerve cells, and the ambiguous nucleus is absent (B, D and F). (A and B) Rostral pole of the LRT at the level of the area postrema. (C and D) Middle portion of the LRT at the caudal pole of the principal subnucleus of the inferior olive. (E and F) Caudal pole of the LRT at the transition between the medulla oblongata and the spinal cord. A = ambiguus nucleus; IOD = inferior olive, dorsal accessory subnucleus; IOM = inferior olive, medial accessory subnucleus; IOP = inferior olive, principal subnucleus; LRT = lateral reticular nucleus; MRT = medial reticular nucleus; SPV = spinal trigeminal nucleus.
well as in Nissl-stained thin sections (Fig. 4A and B). The extent of the degeneration of the LRT was virtually the same in all SCA3 cases studied irrespective of gender, age at onset and duration of the disease.

Accessory tissue changes associated with neurodegeneration in the LRT in SCA3

Due to the severe neuronal loss in the LRT of SCA3, tissue shrinkage occurs, resulting in an aggregation of cells predominantly displaying morphological features of oligodendrocytes. Accordingly, in the region corresponding to the LRT in all SCA3 cases, a dense lawn consisting of an abundance of small oligodendroglia-like cells with sparse cytoplasm and a round nucleus with dense peripheral accumulation of heterochromatin was seen (Fig. 4B).

As opposed to the control cases, in all SCA3 patients, the LRT showed several GFAP-positive thorn-shaped astrocytes (Fig. 5A and B), while immunostaining for microglial cells revealed a slight upregulation. In all control and SCA3 cases studied, the LRT was devoid of β-amyloid deposits.

Anti-ataxin-3 immunostaining showed ataxin-3 in nearly all nerve cells of the LRT of control cases, with ataxin-3 displaying a granular, mainly cytoplasmic distribution. In addition, there was considerable neuropil staining, and oligodendrocytes occasionally exhibited ataxin-3 immunoreactivity in their cytoplasm (Fig. 5C). In SCA3 brains, the surviving nerve cells of the LRT expressed ataxin-3 cytoplasmically and most displayed ataxin-3-immunoreactive dot-like intranuclear inclusions with a relative depletion of neuropil immunostaining compared with that of control brains (Fig. 5D). No specific immunostaining in the LRT of controls was observed using the 1C2 antibody.

Discussion

Our observation that the LRT is severely destroyed in five individuals suffering from clinical end-stage and genetically proven SCA3 confirms the finding of an early case report (Kanda et al., 1989) and suggests that damage to this nucleus is a constant feature of clinical end-stage SCA3. Since in the present study only brains of individuals with a moderate and comparable CAG expansion were studied, it was not possible to clarify whether the extent of the CAG expansion is correlated with the degenerative process in the LRT.

Due to the severe neuronal loss, unequivocal and reliable definition of the anatomical borders of the LRT in the SCA3 brains was impossible. Thus, the basic prerequisites for the application of the current methods of choice for obtaining quantitative information on brain structures, i.e. stereological methods aimed at estimating total neuronal numbers by using the point counting method in combination with the optical disector or the fractionator (Heinsen et al., 1994, 2000; Hof and Schmitz, 2000; Schmitz and Hof, 2000), were not fulfilled. Because the extent of the destruction of the LRT in SCA3, however, was quite similar to that seen commonly in the neostriatum in patients suffering from terminal Huntington’s disease (Heinsen et al., 1994), nerve cell loss in the LRT was demonstrable even without employing advanced stereological approaches. Due to the optical superposition of biological structures, thick sections are superior to paraffin sections when it comes to identifying discrete anatomical components of the human brain or less prominent pathological alterations (Heinsen and Heinsen, 1991; Rüb et al., 2001). Damage to the LRT was so extensive, however, that it was detectable even in thin paraffin sections. Thus, routine neuropathological methods are sufficient to test our hypothesis concerning a consistent destruction of the LRT in individuals suffering from late-stage SCA3.
To our knowledge, there are no clinicopathological correlations between selective destruction of the human LRT and clinical symptoms. Accordingly, little is known with respect to the functional role of this nucleus, making the interpretation of possible functional consequences of its destruction in SCA3 not an easy task. The LRT receives input from the motor and premotor cortices, the spinal cord and vestibular nuclei (Fig. 6) (Walberg, 1952; Olzewski and Baxter, 1982; Carleton and Carpenter, 1987; Marin and Wiesendanger, 1987; Wiesendanger and Wiesendanger, 1987; Voogd et al., 1998). It sends efferents to the vermal and hemispherical cortex of the cerebellum and is connected bidirectionally with the cerebellar fastigial nucleus (Walberg, 1952; Carleton and Carpenter, 1984; Gerrits, 1990; Voogd et al., 1990, 1998; Gilman, 1992; Braak and Braak, 1998). Due to these connections, the LRT is regarded as a precerebellar nucleus involved in proprioceptive mechanisms as well as somatomotor automatisms (Olszewski and Baxter, 1982; Marin and Wiesendanger, 1987; Paxinos et al., 1990; Voogd et al., 1990, 1998; Gilman, 1992; Braak and Braak, 1998).

Gait ataxia may result from damage to structures of the midline zone (vermal cortex, fastigial nucleus) or lateral zone of the cerebellum (cortex of the cerebellar hemisphere, dentate nucleus) and/or associated precerebellar components (Gilman, 2000). In part because of the intimate connections of the LRT with the midline zone of the cerebellum and associated components, the destruction of this nucleus probably contributes to gait ataxia in SCA3. All of our SCA3 patients suffered from severe gait ataxia and, along with severe destruction of the LRT, showed additional damage to the dentate nucleus and associated precerebellar components of the midline (i.e. spinal cord, vestibular nuclei, Clarke’s column, ventral and dorsal spinocerebellar tracts) and lateral zones (i.e. pontine nuclei). This favours the view that gait ataxia in SCA3 results from nerve cell loss at multiple sites (Schmidt et al., 1998). Unfortunately, due to the involvement of numerous ataxia-related sites, an estimation...
of the extent of the LRT’s contribution to gait ataxia is impossible, and further conclusions regarding the normal function of this nucleus cannot be drawn.

Despite recent progress in deciphering the molecular cause of SCA3, the pathogenetic mechanisms underlying neuronal death are still unclear. Some researchers assume that neurodegeneration in SCA3 and other polyglutamine diseases involves a dominant, toxic property of the disease protein (Klockgether and Evert, 1998; Schmidt et al., 1998). In addition, according to recent studies, inflammatory processes are involved in the pathogenesis of SCA3 (Evert et al., 2001). Regardless of the precise mechanism of toxicity, it is generally agreed that polyglutamine disease proteins must be expressed in neurones for degeneration to occur. The LRT in SCA3 fulfills this criterion; the disease protein ataxin-3 normally is expressed in LRT neurones. In the LRT of healthy individuals, ataxin-3 expression is primarily cytoplasmic, whereas in the LRT of SCA3 diseased brains, ataxin-3 forms intranuclear aggregates in the surviving nerve cells. The basis for the apparent selective vulnerability of LRT neurones is unknown. Clearly, expression of ataxin-3 is not sufficient to cause degeneration since neurones in several other brain regions are spared despite expressing ataxin-3 (Paulson et al., 1997; Schmidt et al., 1998; Trottier et al., 1998). The precise role with respect to disease of intranuclear aggregates of mutant ataxin-3 is also uncertain (Schmidt et al., 1998; Trottier et al., 1998). Intranuclear aggregates of ataxin-3 originally were observed in SCA3 brain regions that show significant neuronal loss (Paulson et al., 1997; Schmidt et al., 1998; Trottier et al., 1998), but recently were also demonstrated in brain regions that have been categorized as spared in SCA3 (Yamada et al., 2001). In view of their formation in both affected and presumably spared regions, it remains an open question whether intranuclear inclusions are pathogenic structures directly linked to the disease process underlying SCA3. Accordingly, further investigations aimed at defining both the physiological role of ataxin-3 and its dysfunction in the disease state, including the significance of intranuclear inclusions, are needed. Answers to these questions may lead to rational therapies that slow or prevent neuronal loss in SCA3 patients.

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References


Destruction of the lateral reticular nucleus in SCA3


