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The cerebellar component of Friedreich's ataxia

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Abstract

Lack of frataxin in Friedreich's ataxia (FRDA) causes a complex neurological and pathological phenotype. Progressive atrophy of the dentate nucleus (DN) is a major intrinsic central nervous system lesion. Antibodies to neuron-specific enolase (NSE), calbindin, glutamic acid decarboxylase (GAD), and vesicular glutamate transporters 1 and 2 (VGluT1, VGluT2) allowed insight into the disturbed synaptic circuitry of the DN. The available case material included autopsy specimens of 24 patients with genetically defined FRDA and 14 normal controls. In FRDA, the cerebellar cortex revealed intact Purkinje cell somata and dendrites as assessed by calbindin immunoreactivity. The DN, however, displayed severe loss of large NSE-reactive neurons. Small neurons remained intact. Labeling of Purkinje cells, basket fibers, Golgi neurons, and Golgi axonal plexuses with antibodies to GAD indicated normal intrinsic circuitry of the cerebellar cortex involving γ -aminobutyric acid (GABA). In contrast, the DN displayed severe loss of GABA-ergic terminals and formation of GAD- and calbindin-reactive glumose degeneration. The surviving small GAD-positive DN neurons provided normal GABA-ergic terminals to intact inferior olivary nuclei. The olives also received normal glutamatergic terminals as shown by VGluT2-reactivity. VGluT1-immunocytochemistry of the cerebellar cortex confirmed normal glutamatergic input to the molecular layer by parallel fibers and the granular layer by mossy fibers. VGluT2-immunoreactivity visualized normal climbing fibers and mossy fiber terminals. The DN, however, showed depletion of VGluT1- and VGluT2-reactive terminals arising from climbing and mossy fiber collaterals. The main functional deficit underlying cerebellar ataxia in FRDA is defective processing of inhibitory and excitatory impulses that converge on the large neurons of the DN. The reason for the selective vulnerability of these nerve cells remains elusive.

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Keywords

Cerebellar nuclei; Friedreich's ataxia; γ -aminobutyric acid; Glutamic acid; Olivary nucleus; Synapses

Introduction

Degeneration of the dentate nucleus (DN) is a characteristic central nervous system (CNS) lesion in Friedreich's ataxia (FRDA). In 1907, Mott [18] contributed the first detailed description of the DN in FRDA, but the abnormality received little attention until many years later [21, 29]. Loss of proprioceptive and spinocerebellar fibers does not fully explain the ataxia of FRDA patients. Atrophy of the DN is the more likely cause of disabling dysmetria, dysarthria, and dysphagia.

The consequence of the mutation in FRDA is deficiency of frataxin, a small mitochondrial protein that participates in iron homeostasis of the entire cell [1]. Frataxin levels in the DN of FRDA patients are low [14] but the pathogenesis of selective DN atrophy remains elusive. Evidence reported in the present study implicates interrupted transmission between Purkinje cells and large neurons of the DN. Small γ -aminobutyric acid (GABA)-containing neurons in the DN remain intact and maintain GABA-ergic dentato-olivary connections. Therefore, olivary neurons do not undergo transsynaptic atrophy and issue normal climbing fibers to Purkinje cells. Glutamatergic collaterals to the DN that arise from climbing and mossy fibers disappear as atrophy of large neurons progresses.

Materials and methods

Autopsy tissues

Paraffin-embedded tissues of cerebellar cortex, DN, and medulla oblongata were available from 24 patients (6 male; 18 female) with genetically confirmed FRDA. Mean age of onset in years ($\pm$ standard deviation) was 13.2 ± 10.7 (range 2–50 years); age of death was 39.2 ± 21.3 (range 10–83). All patients had homozygous guanine–adenine–adenine (GAA) trinucleotide repeat expansions. The mean expansion on the longer allele was 784 ± 207 (range 236–1,200); the mean expansion on the shorter or equally long allele was 593 ± 246 (range 106–1,200). Matching specimens of 14 patients (9 male, 5 female) without neurological disease served as controls (mean age of death $\pm$ standard deviation: 44.4 ± 20 years [range 12–77 years]).

Antisera and antibodies

Mouse monoclonal antibodies to neuron-specific enolase (NSE; Chemicon, Temecula, CA, USA) and glutamic acid decarboxylase (GAD; MBL International, Woburn, MA, USA) were purchased from commercial sources. A mouse monoclonal antibody against vesicular glutamate transporter 1 (VGluT1) was obtained from the University of California Davis/ National Institutes of Health Neuromab Facility (Davis, CA, USA). Rabbit polyclonal anti-VGluT2 was raised and immuno-purified under contract with Anaspec (San José, CA,

USA). The antigenic peptide, CHEDELDEETGDITQNYINY [⁹], was linked to keyhole limpet hemocyanin. Polyclonal rabbit anti-calbindin D-28K was from Chemicon.

Immunocytochemistry

Paraffin sections of 6 µm thickness were rehydrated and oxidized by standard methods. Details of the immunocytochemical protocol were published previously [¹⁴]. Optimal antigen retrieval for the visualization of NSE and GAD was incubation for 30 min at 95°C in a diluted de-cloaking solution termed “DIVA” by the supplier (Biocare Medical, Concord, CA, USA). Immunoreactivity of VGluT1 and VGluT2 was improved by incubation for 20 min at 95°C in 0.1 M tris buffer (pH 9.5). The antigen retrieval method for calbindin was exposure to 0.1 M citric acid/sodium citrate buffer (pH 6) for 3 min at 95°C, followed by cooling to room temperature over 20 min. The sequence of incubations after full rehydration, oxidation, and antigen retrieval was background “suppression” with normal horse serum (10% by vol) in phosphate-buffered saline (PBS), containing 4% bovine serum albumin (BSA; weight/vol) → primary antibody → secondary biotinylated anti-mouse IgG (anti-rabbit IgG for polyclonal antibodies) (Vector, Burlingame, CA, USA) → horseradish peroxidase (HRP)-tagged streptavidin (Sigma, St. Louis, MO, USA) → diaminobenzidine/hydrogen peroxide generated from tablets containing this chromogen and urea hydrogen peroxide (Sigma). Sections were covered with a xylene-soluble mounting medium (Surgipath, Richmond, IL, USA). When DIVA was the antigen retrieval method, the BSA concentration in the suppressor solution was reduced to 0.5%.

Optimal dilutions of antibodies and antisera were determined empirically, and protein concentrations ranged from 0.1 to 1.0 µg/ml. Biotinylated secondary antibody concentrations were 3 µg protein/ml (0.6 µg/ml if antigen retrieval involved DIVA). HRP-labeled streptavidin was applied at a concentration of 2 µg protein/ml (0.4 µg protein/ml after DIVA).

Results

Immunocytochemistry disclosed similar abnormalities in FRDA irrespective of age of onset, age of death, disease duration, or the lengths of the GAA trinucleotide repeats.

Calbindin immunocytochemistry (Fig. 1a, c) confirmed the preservation of Purkinje cells in FRDA. In contrast, the DN showed severe loss of large neurons (Fig. 1b). Small neurons were normal, displaying strong NSE reaction product (Fig. 1b).

Figure 2 illustrates immunoreactivity in the cerebellar cortex and DN after application of an antibody to GAD. Pale reaction product is present in the cytoplasm of Purkinje cell bodies, but more robust staining reveals axonal baskets around Purkinje cells, GABA-ergic fibers in the molecular layer arising from basket and stellate neurons, and the axonal plexuses of Golgi neurons in the granular layer (Fig. 2a, c). In some sections, the cell bodies of Golgi neurons show dense reaction product (Fig. 2d). Reaction product in the cerebellar cortex of FRDA (Fig. 2a) is very similar to a control (Fig. 2c). GAD-reactive torpedoes are relatively common in FRDA (Fig. 2a). Immunocytochemistry of the normal DN with anti-GAD shows a great abundance of terminals about large GAD-negative neurons (Fig. 2e). Small neurons

reveal cytoplasmic GAD and GAD-reactive afferent terminals (Fig. 2e, arrow). In the DN of FRDA, GAD reaction product is disorganized and generally less abundant (Fig. 2b). Grumose degeneration is GAD-reactive (Fig. 2b, inset). Small neurons with cytoplasmic GAD reaction product stand out more clearly in comparison with the normal DN as many axons and terminals in the adjacent neuropil have disappeared (Fig. 2b, arrow).

Figure 3 illustrates reaction product in the DN after application of anti-calbindin. The stain shows loss and disorganization of terminals and grumose degeneration (Fig. 3a and inset). The blebs of grumose degeneration are coarser than the finely granular aggregates revealed by GAD immunocytochemistry (Fig. 2b), presumably because the subcellular distribution of the proteins in the terminals is not identical. Calbindin reaction product in the normal DN is similar to GAD, generating negative images of larger neurons. Smaller neurons also show pale cytoplasmic reaction product (Fig. 3b).

Figure 4 shows a comparison of cerebellar cortex and DN after staining for VGluT1. There are no differences in the distribution of reaction product in the molecular and granular layers of FRDA (Fig. 4a, b) and control (Fig. 4d, e). Dense, finely granular reaction product surrounds the dendrites of Purkinje cells, yielding negative images of these neurons (Fig. 4b, e). In the granular layer, mossy fiber terminals are strongly VGluT1 reactive. In the DN of FRDA, only a few VGluT1-reactive terminals remain (Fig. 4c). Grumose degeneration is non-reactive (not illustrated). Figure 4f shows VGluT1 reaction product abutting cell bodies and proximal dendrites of large neurons ("N") in the normal DN.

In contrast to anti-VGluT1, anti-VGluT2 generates reaction product only in the climbing fibers of the cerebellar molecular layer (Fig. 5). Abundance and distribution are similar in FRDA (Fig. 5a, b) and normal control (Fig. 5d, e). The normal DN shows sparse VGluT2-positive granules (Fig. 5f). Very few are present in FRDA (Fig. 5c). Grumose degeneration is VGluT2-negative (Fig. 5c, inset).

The inferior olivary nucleus in FRDA displays normal abundance and distribution of GAD reaction product in FRDA (Fig. 6a) compared to the normal control (Fig. 6c). VGluT1 immunoreactivity is absent from the inferior olives of FRDA patients and normal controls, though terminals in the nearby arcuate and lateral reticular nuclei are strongly reactive (not illustrated). In contrast to VGluT1, VGluT2 shows dense investment of inferior olivary neurons by reactive terminals without obvious difference between FRDA and normal control (Fig. 6b, d).

Discussion

GABA-ergic afferent and efferent connections of the DN

Immunocytochemical reaction product after the application of anti-GAD is now generally accepted as evidence of the GABA-ergic properties of nerve cells, axons, and synaptic terminals. Purkinje cells provide most GAD-positive axosomatic and axodendritic terminals in the cerebellar nuclei of experimental animals and the DN of humans (Fig. 2e), and there is little doubt that these corticonuclear connections are inhibitory. The discovery of small GAD-reactive nerve cells in cerebellar nuclei [19] and the GABA-ergic cerebello-olivary

pathway [3, 6, 20] led to a clearer understanding of olivary function in motor control. Small GABA-ergic neurons may also have a role in the intrinsic circuitry of the DN [2]. In humans, evidence of a dentato-olivary pathway is indirect and based on the structural lesions visible at the time of autopsy. Destruction of the DN or the central tegmental tracts causes identical transneuronal changes of the olivary nuclei [5, 26]. The lesion, now universally termed “olivary hypertrophy”, is contralateral to the affected DN but ipsilateral to the destroyed central tegmental tract. Lapresle and Ben Hamida [17] systematically investigated olivary hypertrophy due to the DN lesions in serial sections and established that a topistic relationship exists between olivary and DN neurons. The authors cited earlier work by Guillain and Mollaret [8] who postulated a triangular reciprocal relationship between DN and contralateral inferior olivary nucleus. Neurologists have long accepted a “triangle of Guillain and Mollaret” in their interpretation of palatal myoclonus, which is the characteristic clinical correlate of olivary hypertrophy. Ruigrok et al. [25] and De Zeeuw et al. [4] recognized the importance of an afferent GABA-ergic deficit in experimental olivary hypertrophy in cats. A systematic study of the human equivalent has not yet been published though GAD-immunocytochemistry is suitable for archival paraffin-embedded tissues of the medulla oblongata. A key question is: Why does such a severe DN lesion in FRDA as illustrated in this report not cause transneuronal changes in the inferior olives? The likely answer is the exemption of small GABA-ergic neurons from the disease. This interpretation implies that GABA has trophic properties on the neurons of the inferior olivary nuclei in the adult CNS. Non-synaptic properties of GABA are well established but appear restricted to the developing nervous system [22]. It is speculative to assume that the adult inferior olivary nucleus of humans is an exception and that GABA is the actual trophic factor allowing these neurons to survive.

It is not clear why glutamatergic afferents to the human inferior olivary nucleus are VGluT1-negative. In a systematic study of the rat CNS, Kaneko et al. [12] found immunoreactivity of the inferior olivary nuclei with antibodies to both VGluT1 and DNPI (a prior term for VGluT2). The inferior olives receive connections from many sites [2], and transmitters other than GABA and glutamate are involved [4]. Many neurophysiological experiments have confirmed the role of GABA and glutamate in the modulation of electrotonic coupling of nerve cells in the inferior olivary nuclei. Lang et al. [15] reported that injection of picrotoxin, a GABA inhibitor, into the inferior olivary nucleus and lesioning of the nucleo-olivary pathway in rats had a similar effect on complex Purkinje cell spikes, namely, increased firing rate and synchrony. It is evident that the small neurons of the DN in the human cerebellum provide a comparable control of olivary function. The direct injection of glutamate receptor antagonists into the inferior olives of rats reduced the firing rate of evoked complex Purkinje cells spikes but did not hinder spontaneous olivary activity [16]. Normal VGluT2-positive terminals in the human inferior olivary nuclei (Fig. 6b) suggest that FRDA does not affect excitatory afferents. Koeppen et al. [13] showed that the inferior olivary nuclei in FRDA retain all synapses as determined by immunofluorescence of synaptosome-associated protein 25 (SNAP-25).

Glutamatergic afferents in the DN

Immunocytochemistry with antibodies to VGluT1 (Fig. 4) and VGluT2 (Fig. 5) suggests that FRDA does not destroy glutamatergic input to Purkinje cells by parallel (VGluT1; Fig. 4a, b) or climbing fibers (VGluT2; Fig. 5a, b). VGluT2 is absent from parallel-fiber synapses in the molecular layer, and all VGluT2-positive terminals may be attributed to climbing fibers [7, 10]. The robust visualization of these olivocerebellar connections is further confirmation of the integrity of the inferior olivary nuclei in FRDA. A similar differential distribution does not exist for the glutamatergic afferents of the DN because the terminals of mossy fiber collaterals are positive for both VGluT1 and VGluT2 [7, 10]. Therefore, the deficit of glutamatergic terminals in the DN of FRDA (Figs. 3, 4) cannot be readily assigned to climbing or mossy fibers. All of these glutamatergic terminals appear to arise from delicate collateral branches rather than main fibers [2, 27, 28]. The afferent fibers provide input from the inferior olivary nuclei, spinal cord, basis pontis, and other extracerebellar regions. It is evident from Figs. 4c and 5c that all glutamatergic terminals are less numerous in the DN of FRDA than normal. It is also noteworthy though not unexpected that grumose degeneration is VGluT1- and VGluT2-negative. Only GABA-ergic terminals appear to contribute to the formation of these unusual clusters of abnormal synaptic boutons (Fig. 2b). Their reactivity with anti-calbindin also supports their origin from axon terminals of Purkinje cells (Fig. 3a and inset). Neither GAD- nor calbindin-immunoreactivity provides insight into the mechanism by which selective atrophy of large neurons causes grumose degeneration.

Localizing the problem of FRDA to large neurons of the DN

The evidence presented here supports the conclusion that FRDA causes selective atrophy of the large neurons of the DN, sparing small GABA-ergic neurons. Grumose degeneration may be a reparative response. It involves corticonuclear connections but Purkinje cells appear to survive loss of their main efferent target. Two observations may be cited to explain this lack of retrograde atrophy: (1) Persistent synaptic contacts with small neurons in the DN may be sufficient to prevent a retrograde response; and (2) Purkinje cells display unique plasticity following axonal interruption [24]. Grumose degeneration, however, is not unique to FRDA. It is present in progressive supranuclear palsy and spinocerebellar ataxia type 3 (Machado-Joseph disease; SCA-3/MJD). SCA-3/MJD spares the inferior olivary nucleus presumably due to the similar survival of small neurons in the DN. Olivary degeneration is more variable in dentatorubropallidoluysian atrophy (review in ref. [11]). In some cases of this disorder, the olives show frank neuronal hypertrophy, vacuolation, and large astrocytes [23]. This astroglial reaction is well known from cases of palatal myoclonus and differs greatly from small-cell gliosis of retrograde olivary degeneration.

Clinicoanatomic correlations

Clinicians must consider impairment of efferent output from large neurons of the DN as they seek to explain the clinical phenotype of FRDA. Both GABA-ergic and glutamatergic motor controls are lost at this anatomical site. The survival of inferior olivary neurons differs greatly from those spinocerebellar ataxias that show prominent cortical cerebellar atrophy. Nevertheless, intact Purkinje cells and olivary neurons in FRDA are irrelevant because neuronal loss in the DN blocks GABA-ergic and glutamatergic transmission anyway. The

disappearance of VGluT1- and VGluT2-positive terminals in the DN of FRDA is most likely due to a retrograde effect. Nerve cells providing these glutamatergic collaterals appear to tolerate the partial loss of their targets without atrophy, presumably because their main connections in the molecular and granular layers remain intact. The reason for the selective vulnerability of large DN nerve cells is unknown. FRDA also causes loss of spinocerebellar input to the cerebellar hemispheres due to transneuronal atrophy of the dorsal nuclei of Clarke. The mossy fiber terminals of the spinocerebellar tracts mingle with those from other sources, and a numerical loss of synapses may not be detectable. Additional research is needed to explain the susceptibility of DN neurons to FRDA and attempt therapeutic restoration of cerebellar output.

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Fig. 1. Cerebellar cortex and DN in FRDA and a normal control. **a, b** FRDA; **c, d** control. Purkinje cells in FRDA (**a**) are comparable to the normal control (**c**). In contrast, the DN in FRDA (**b**) differs greatly from the control due to severe loss of large neurons. Only small neurons with dense immunocytochemical NSE reaction product remain (**b**, *arrow*). Immunocytochemistry with antibodies to calbindin (**a, c**) and NSE (**b, d**). Bars 50 µm

Fig. 2. GAD immunocytochemistry of cerebellar cortex and DN in FRDA and a normal control. **a**, **b** FRDA; **c–e** control. In the cerebellar cortex, the GAD stain reveals pale reaction product in the cytoplasm of Purkinje cells (**a**, **c**). More intense reaction product labels Purkinje cell baskets (**a**, **c**), GABA-ergic parallel fibers in the molecular layer (**a**, **c**), Golgi neurons (*Gn*) and a long apical dendrite (**d**, arrows), and the axonal plexuses of Golgi neurons in the granular layer (**a**, **c**). The cerebellar cortex in FRDA (**a**) does not differ substantially from the normal control (**c**) though a GAD-reactive axonal expansion is present in the granular layer of the FRDA case (**a**, arrow). In the DN (**b**, **e**), small neurons show pale reaction product (**b**, **e**, arrows). GAD immunoreactivity reveals an overall depletion of GABA-ergic terminals in the DN of FRDA (**b**) and stains grumose degeneration with great intensity (**b**, and inset). Large neurons, labeled as “*N*” in **e**, are GAD-negative. Bars 50 μm in **a–e**, 20 μm in (**b**, inset)

Fig. 3.

Calbindin immunocytochemistry of the DN in FRDA and a normal control. **a** FRDA; **b** control. The DN in FRDA (**a**) shows loss and disorganization of calbindin-reactive terminals and grumose degeneration. Reaction product in grumose degeneration is coarser than that generated by anti-GAD (Fig. 2b and inset for comparison). In the normal DN (**b**), calbindin reaction product shows larger neurons as voids (*N*) though smaller nerve cells display pale cytoplasmic staining (*arrows*). Bars 50 μm in **a** and **b**, 20 μm in (**a**, *inset*)

Fig. 4. VGlut1 immunocytochemistry of cerebellar cortex and DN in FRDA and a normal control. **a–c** FRDA; **d–f** control. The stain shows intense, finely granular, reaction product in the molecular layer of FRDA (**a**) and control (**d**), revealing negative images of Purkinje cells (**b**, **e**). In the granular layer, mossy fiber terminals are VGlut1-positive (**a**, **d**). In the DN of FRDA (**c**), VGlut1-reactive terminals are small and infrequent when compared with the control (**f**). *N* negative images of large nerve cells in the control DN (**f**). Bars 100 μ m in **a** and **d**; 20 μ m in **b**, **c**, **e**, and **f**

Fig. 5. VGlut2 immunocytochemistry of cerebellar cortex and DN in FRDA and a control. **a–c** FRDA; **d–f** normal. VGlut2-positive climbing fiber terminals are present in the molecular layer of both FRDA (**a, b**) and control (**d, e**). They are restricted to the major dendrites of Purkinje cells. The somata of Purkinje cells are outlined by interrupted lines in **b** and **e**. VGlut2 reaction product is also present in mossy fiber terminals of the granular layer in FRDA (**a**) and control (**d**). The dentate nucleus shows depletion of VGlut2-positive terminals in FRDA (**c**) in comparison with the normal control (**f**). Grumose degeneration in FRDA is VGlut2-negative (*inset* in **c**, *arrow*). *N* negative images of neurons (**f**). Bars 100 μm in **a** and **d**; 20 μm in **b, e, c**, *inset* of **c**, and **f**

Fig. 6. GAD and VGluT2 immunocytochemistry of the inferior olivary nucleus in FRDA and a normal control. **a, b** FRDA; **c, d** control; **a** and **c** GAD; **b** and **d** VGluT2. The inferior olivary nuclei show no differences in their reactivity with anti-GAD and anti-VGluT2. Negative images of neurons are present throughout the course of the olivary gray matter. Bars 100 μ m