Friedreich Ataxia: Failure of GABA-ergic and Glycinergic Synaptic Transmission in the Dentate Nucleus

Arnulf H. Koeppen, MD, R. Liane Ramirez, MS, Alyssa B. Becker, BA, Paul J. Feustel, PhD, and Joseph E. Mazurkiewicz, PhD

INTRODUCTION

Friedreich ataxia (FA) results from the deficiency of a small mitochondrial protein, frataxin. The most common underlying mutation is a recessively inherited pathogenic homozygous expansion of guanine-adenine-adenine (GAA) trinucleotide repeat expansion in intron 1 of the frataxin gene (chromosome 9q21). Compound heterozygotes, such as those with a GAA trinucleotide repeat expansion on 1 allele and a point mutation or deletion on the other, are much rarer; however, such patients often have a similar neurologic phenotype. Total deletion of the frataxin gene in a mouse model of FA and consequent complete absence of frataxin are lethal to the embryo (1). Human patients with FA have abnormally low levels of tissue frataxin but survive an average of 26 years after FA becomes clinically evident (2). Onset of the disease has been difficult to define because the neurologic deficit remains hidden for years. After a child or young adult becomes overtly ataxic, parents or other family members may recall that the patient did not develop motor skills at the same pace as his or her siblings who remained well. Several hypotheses have been presented to explain delayed manifestations and progression to a state of profound disability. Declining frataxin levels caused by somatic GAA trinucleotide repeat expansions are an attractive explanation (3–5), and mismatch repair enzymes may be involved (4, 5). Beyond impairment of transcription by the formation of triplex deoxyribonucleic acid (6), frataxin gene regulation is also subject to epigenetic factors (7). The dentate nucleus (DN) is highly vulnerable to FA (8), and the profound ataxia in FA should also be attributed to atrophy of large glutamatergic DN neurons. Clinical disease progression can be measured by several available rating scales (9), and the paramagnetic effect of DN iron on magnetic resonance images is emerging as a biomarker of the disease (10, 11).

In contrast to clinical and imaging measures, however, the literature contains no information on the severity of the DN lesion as a correlate of age at onset, disease duration, length of GAA trinucleotide repeats, or frataxin levels. The DN receives the bulk of its afferent fibers from Purkinje cells, the principal neurohumoral transmitter is γ-aminobutyric acid (GABA). Although the DN loses its large glutamatergic neurons, small GABA-ergic neurons survive (8). The DN also contains small glycineric neurons, but the role of glycine as an inhibitory transmitter in the human cerebellar circuitry is not well characterized.

The work presented here advances our hypothesis that large DN neurons fail to express GABA-ergic and glycineric receptors, or the receptor-anchoring protein gephyrin, and thereby lose trophic support from Purkinje cells in the cerebellar cortex and intrinsic glycineric neurons in the DN. Selective...
atrophy of large DN neurons has major clinical implications because they are the main source of efferent cerebellar fibers. It is widely recognized that the cerebellar cortex in FA only rarely displays abnormalities (12). Survival of Purkinje cells, however, is functionally irrelevant because corticonuclear connections are interrupted by the loss of large DN neurons. In mice, Purkinje cells show little if any morphologic evidence of retrograde degeneration after axonal interruption (13). The putative reason is recovery of trophic support by axonal sprouting from the divided main axon. Survival of Purkinje cells in FA may be analogous, but no prior information exists on how these neurons recover trophic support after most of the large DN neurons have disappeared.

MATERIALS AND METHODS

Specimens

Autopsy specimens were obtained through a formal tissue donation program supported by Friedreich’s Ataxia Research Alliance and National Ataxia Foundation or by the courtesy of other physicians. The program includes the collection of personal health information of the deceased subject and family history. The institutional review board of the Veterans Affairs Medical Center has approved this research. The Table lists basic clinical information on 29 patients with FA from whom paraffin-embedded DN samples were available for immunohistochemistry and immunofluorescence. In 13 of these cases, frozen DN tissue was available for the assay of frataxin. Confirmation of the mutation was available in 28 patients either by information gained from the clinical record or by amplification of the GAA trinucleotide repeat expansion in deoxyribonucleic acid extracted postmortem from the cerebellar cortex. One patient, FA 26 (Table), declined testing during life, and all tissue samples had been fixed at the time of autopsy and amplification of DNA extracted from formalin-fixed tissues was inconclusive. The neuropathologic and cardiac phenotypes, however, were diagnostic of FA.

Fourteen normal autopsy samples containing DN (9 males, 5 females) were secured from the academic autopsy service of Albany Medical College and Veterans Affairs Medical Center, Albany, NY. In 8 of these control samples, the amount

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Mean ± SD 12.8 ± 11.2 37.7 ± 18.3 24.9 ± 11.8 696 ± 225 880 ± 213
Range 2–54 10–83 5–54 106–1,114 235–1,200

Patients were sorted according to age at onset. Patients FA2 and FA4 were siblings.
F, female; GAA1, GAA2, guanine-adenine-adenine trinucleotide repeat expansion at allele 1 or allele 2; M, male; NA, not available.
of frozen tissue was sufficient for biochemical assay of frataxin. The age of death of the control subjects was 64.4 ± 8.4 years (mean ± SD). The FA tissue repository also included the DN of 2 subjects who were FA carriers. Both were men, aged 79 and 86 years, and their GAA trinucleotide repeats were 863/33 and 560/7, respectively. Autopsy delay for the FA cases was 20 ± 19 hours (mean ± SD) with a range of 3 to 96 hours. Autopsy delay for the control cases was 15 ± 8 hours (mean ± SD) with a range of 3 to 26 hours.

ELISA of Frataxin

Portions of the gray matter ribbon of the DN were dissected with a No. 11 blade. Weighed samples of wet tissue (48–137 mg) were dispersed by 3 to 5 5-second-long bursts of ultrasonication in a lysis buffer containing 100 mmol/L Tris-HCl, pH 7.5, 150 mmol/mL NaCl, 1% each of the nonionic detergents Triton X-100 and Nonidet P-40, 5 mmol/L ethylenediamine tetraacetic acid, 5 mmol/L ethylene glycol tetraacetic acid, and 1% protease inhibitor cocktail (vol/vol: Sigma, St. Louis, MO) (14). The mixtures were chilled on ice between bursts. The homogenates were centrifuged for 2 hours at 14,000 × g and 4°C. The supernatant was collected, and aliquots were diluted 1:10 in PBS to reduce the detergent concentrations to 0.1%. The diluted extracts were then filtered through a centrifugal filter device with a molecular weight cutoff of 30 kDa (EMD Millipore, Billerica, MA) at 14,000 × g for 45 minutes. The filtrate was collected, and ELISA of frataxin proceeded as follows: polystyrene ELISA plates (Santa Cruz Biotechnology, Santa Cruz, CA) were coated with monoclonal anti-frataxin (0.33 μg/mL; Abcam, Cambridge, MA) in 0.05 mol/L carbonate buffer (pH 9.6) by an overnight incubation at 4°C. The plates were washed 3 times with a 1% solution of nonfat dry milk in PBS, containing 0.1% Tween 80 (NFDM-PBS-Tween 80). Well surfaces were then covered for 4 hours at room temperature with NFDM-PBS-Tween 80 to block nonspecific absorption of antibodies. The next step was the application of diluted tissue lysate or recombinant human frataxin in NFDM-PBS-Tween 80. After an overnight incubation, the wells were drained and washed with NFDM-PBS-Tween 80. The detecting antibody was rabbit polyclonal anti-frataxin (whole serum) that was diluted 1:2000 in NFDM-PBS-Tween 80. A second overnight incubation at 4°C followed. After washing the plates 3 times with NFDM-PBS-Tween 80, the wells were filled with biotinylated anti-rabbit IgG (0.75 μg/mL) in NFDM-PBS-Tween 80 and maintained at room temperature for 2 hours. Unbound biotinylated antibody was removed by washing once with NFDM-PBS-Tween 80 and 3 times with plain PBS. The next step was a 1-hour incubation at room temperature in a solution of horseradish peroxidase–labeled streptavidin (0.25 μg/mL) in PBS. After 3 washes with PBS, a chromogenic solution of ortho-phenylenediamine (2 mmol/L) and H₂O₂ (0.01%) in 0.1 mol/L citric acid–sodium phosphate buffer (pH 5.0) was added to each well. A distinct color gradient developed within 2 to 3 minutes, and the addition of 2.5 mol/L sulfuric acid (50 μL) stopped the reaction. Absorbance at 492 nm was determined using an ELISA plate reader (SpectraMax Plus; Molecular Devices, Sunnyvale, CA). The amount of frataxin in tissue lysates was determined by reference to a calibration standard curve, and results were expressed as nanograms per gram original wet weight.

Antibodies for Immunohistochemistry and Immunofluorescence

The following antibodies to the listed proteins were obtained from commercial sources (abbreviation, host and type, and supplier in parentheses): glutamic acid decarboxylase (GAD, mouse monoclonal; MBL International, Woburn, MA); class III β-tubulin (mouse monoclonal; R & D Systems, Minneapolis, MN); GABA-A-receptor γ2-subunit (GABA-A-Rγ2, goat polyclonal; Santa Cruz Biotechnology); glycine receptor α1/2-subunits (GlyRα1/2, rabbit polyclonal; Thermo Fisher Scientific, Waltham, MA); gephyrin (mouse monoclonal; Santa Cruz Biotechnology); glycine transporter 2 (GlyT2, rabbit polyclonal; Santa Cruz Biotechnology); synaptophysin (rabbit polyclonal; Millipore, Temecula, CA); and nonphosphorylated neurofilament protein (mouse monoclonal; Covance, Emeryville, CA).

Antigen Retrieval and Immunohistochemistry

Antibody concentrations were optimized by trial and error; protein concentrations ranged from 0.15 μg/mL (for polyclonal anti-synaptophysin) to 2 μg/mL (for monoclonal anti-nonphosphorylated neurofilament protein and polyclonal anti-GlyRα1/2). Three antigen retrieval methods were used on 6-μm-thick rehydrated paraffin sections (antigens in parentheses): Incubation at 95°C for 30 minutes in a 1× solution (vol/vol) of DIVA, a proprietary antigen retrieval mixture (Biocare Medical, Concord, CA) (GAD and GlyRα1/2); incubation at 98.5°C for 20 minutes in 0.1 mol/L Tris hydrochloride buffer (pH 9.5) containing 5% urea, followed by a 10-minute cooldown period (GABA-A-Rγ2, GlyT2, and nonphosphorylated neurofilament protein); incubation in 0.01 mol/L citric acid–sodium citrate buffer (pH 6) at 95°C for 10 minutes, followed by a 10-minute cooldown period (gephyrin, class III β-tubulin, and synaptophysin).

The sequence of incubations for the visualization of immunohistochemical reaction products was as previously described (15) and included amplification by biotinylated anti-rabbit, mouse, or goat IgG (0.6 μg protein/mL for DIVA-treated sections, 3 μg protein/mL for all others), horseradish peroxidase–labeled streptavidin (0.4 μg protein/mL for DIVA-treated sections, 2 μg protein/mL for all others), and dianisobenzidine/urea/hydrogen peroxide as the chromogen (Sigma).

Immunofluorescence

Double label immunofluorescence was used to detect localization and colocalization of synaptophysin and class III β-tubulin and of GABA-A-Rγ2 and GlyRα1/2. The initial steps included section rehydration and suppression of nonspecific signals. Antigen retrieval and antibody dilution were identical to those of immunohistochemistry, but oxidation in H₂O₂-containing methanol was omitted. For double label detection of class III β-tubulin and synaptophysin, the sequence of incubations was as follows (washing steps omitted): overnight incubation at 4°C in the first primary antibody (mouse monoclonal anti–class III β-tubulin), application
of Alexa Fluor 488–labeled donkey anti-mouse IgG (2 μg protein/mL) for 4 hours at room temperature, resuspension in 10% normal donkey serum, an additional overnight cold incubation in the second primary antibody (rabbit polyclonal anti-synaptophysin), followed by incubation in Cy3–labeled donkey anti-rabbit IgG (2 μg protein/mL) for 4 hours at room temperature. After washing, the sections were covered by a solution of 50% glycerol in PBS. The protocol for double label immunofluorescence of GABA-A-R and GlyR was similar. Incubation with primary antibodies (goat IgG and rabbit IgG, respectively) was followed by Alexa Fluor 488– and Cy3–labeled secondary antibodies (to goat and rabbit IgG, respectively). The fluorescent secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Measurement of DN Thickness

Three nonoverlapping linear or curvilinear portions of each GAD-stained DN were photographed at a magnification of 100× with a Zeiss Axiophot microscope. The stored images were displayed and analyzed with the aid of a Zeiss AxioVision computer program (version 4.7.1; Carl Zeiss, Gottingen, Germany). Measurements were made by drawing 10 straight lines at intervals across the thickness of each DN, as shown in Figure 1. Measuring 3 DN portions in this manner provided a total of 30 values for each patient or normal control. The computer program also generated data tables that were used to calculate mean DN thicknesses and SDs.

Data Analysis

The thickness of the DN was correlated with age at onset, disease duration, GAA trinucleotide repeat expansion in allele 1 (GAA1) and allele 2 (GAA2), and frataxin levels by standard regression analysis using MiniTab version 17.1 (MiniTab Inc, State College, PA) and OriginPro v.9.1 (OriginLab Corp, Northampton, MA).

RESULTS

Clinical Data

All patients listed in the Table for whom genetic testing was possible (n = 28) had homozygous GAA trinucleotide repeat expansions. Onset (aged 2–54 years) and duration (5–54 years) ranged over a wide spectrum. As expected, disease onset correlated significantly with the length of the shorter GAA trinucleotide repeat (R² = 0.43 in linear regression y = 13.12x + 860.52, wherein y = GAA1 and x = age at onset in years; p < 0.001). The shortest GAA1 trinucleotide expansion was 106, conveying onset at age 54 years (FA29) (Table). During the life of this patient, examiners diagnosed dominant ataxia with incomplete penetrance rather than recessive ataxia and therefore did not request genetic testing for FA. The diagnosis was established on DNA extracted from frozen postmortem tissue. In 24 patients, heart disease contributed to or was the main cause of death (82.8%) (Table).

Frataxin Levels and Correlation With DN Atrophy

Frataxin levels in the DN of 8 control cases were 195.0 ± 55.3 ng/g wet weight (mean ± SD). In 13 FA cases with sufficient frozen DN tissue, the frataxin level was 6.8 ± 3.1 ng/g.
In the 2 carrier cases, the levels were 181.8 and 102.2 ng/g, respectively. Frataxin levels in FA correlated significantly with the thickness of the DN ($R^2 = 0.56$ in linear regression: $y = 117 + 6.3x$, where $y$ = DN thickness and $x$ = frataxin in nanograms per gram; $p = 0.003$) but not with age at onset, disease duration, GAA1, or GAA2. The highest frataxin levels were present in the DN of Patients FA14, FA19, and FA20, measuring, in nanograms per gram wet weight, 12.3, 10.0, and 12.5, respectively. The corresponding DN thicknesses were 186, 184, and 186 μm. Friedreich ataxia Patients FA2 and FA3 showed frataxin levels at or below the lower detection limit of 4 ng/g wet weight, corresponding to DN thicknesses of 143 and 111 μm.

### Thickness of the DN and Correlation With Clinical Data

Dentate nucleus thicknesses also negatively correlated with longer disease duration (Fig. 2). Dentate nucleus thickness in 14 normal controls was $238 \pm 21 \mu m$ (mean ± SD) with a range of 207 to 272 μm. In 2 FA patients with short disease durations (7 and 5 years in FA10 and FA22, respectively), DN thickness fell into the normal range. In an exceptional case of FA (Patient FA16), DN thickness was normal at 213 μm despite a disease duration of 28 years. Dentate nucleus thickness did not correlate significantly with age at onset ($R^2 = 0.3; p = 0.8$), GAA expansions on either the long ($R^2 = 0; p = 0.99$) or the short allele ($R^2 = 0.03; p = 0.48$). Dentate nucleus thicknesses were 142 and 202 μm in the 79- and 86-year-old FA carriers, respectively. Both values were below the range of the normal controls (207–272 μm) (Fig. 2).

### Neurons and Axons in the DN of FA

Reactivities of the neuropil of the DN in 1 normal control and 3 cases of FA with antibodies to nonphosphorylated neurofilament protein are shown in Figure 3A to D and to class III β-tubulin in Figure 3E to H. The main observations in FA are loss of large neurons and retention of small nerve cells.

**FIGURE 2.** Correlation of dentate nucleus (DN) thickness and disease duration in Friedreich ataxia (FA). Normal controls are shown by open red triangles, FA carriers by blue circles, and FA cases by open black squares. Progressive thinning of the DN occurs with longer disease durations. Normal DN thickness is $238 \pm 21 \mu m$ (mean ± SD). The values for both carriers are below the normal range. The linear regression line shown includes only the 29 FA patients and yields a slope of $1.29$ at $R^2 = 0.24$, $p < 0.01$.

**FIGURE 3.** Immunohistochemistry of nonphosphorylated neurofilament protein and class III β-tubulin in the cerebellum of a normal control case and 3 cases of Friedreich ataxia (FA). (A, E) In the normal dentate nucleus (DN), neuron sizes are heterogeneous. Small neurons are generally located at the junction of the DN gray matter and adjacent white matter (arrows). (B, D, F–H) In the FA cases, both immunostains show loss of large neurons in the DN, whereas small neurons remain intact (arrows). Insets show the contrast between morphologically intact Purkinje cells and DN neurons in FA on the same tissue block. (A–D) Nonphosphorylated neurofilament protein. (E–H) Class III β-tubulin. (B, F) Patient FA20. (C, G) Patient FA9. (D, H) Patient FA29. Scale bar = 50 μm.
FIGURE 4. (A–D) Double label immunofluorescence of class III β-tubulin and synaptophysin in Friedreich ataxia (FA) (Patient FA27) (A, B) and a normal control (C, D). Class III β-tubulin is shown in Alexa Fluor 488 (green); synaptophysin in Cy3 (red). The images were acquired by laser scanning confocal microscopy and resolved as maximum intensity projections. (A) A focus of synaptophysin-positive grumose reaction is surrounded and penetrated by a dense network of delicate axons. A neuronal cell body is not distinct in the center of the grumose cluster. (B) Two small neurons (“N”) display an abnormally thick layer of synaptic terminals about their cell bodies and proximal dendrites (arrows). This appearance is thought to represent grumose “regeneration” rather than “degeneration.” Many thin axons traverse the neuropil. Some are in the immediate vicinity of the synaptic cluster and may be the source of the crowded terminals. (C) Two large normal dentate nucleus (DN) neurons and many nearby dendrites are lined by thin layers of synaptic terminals. Axons in the neuropil are generally thicker than in FA (A, B) and less abundant. (D) A small neuron located in the white matter adjacent to the main DN shows a thin layer of synapses. Axons around the neuron are thicker than in a comparable location of the DN in FA (B). Scale bar = 20 μm.

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Small DN nerve cells are probably not completely exempt from destruction in FA. An example is shown in Figure 3C (Patient FA9), in which only a single neuron stains for anti-class III β-tubulin. Despite extensive neuronal loss, thin axons persist to a large degree in the depleted DN (Fig. 3F–H). The better preservation of the DN in the FA patient with late onset (FA29) is apparent on both stains (Fig. 3D, H). The insets in Figure 3B to D and 3F to H of the FA cases display the integrity of Purkinje cells in the cerebellar cortex that lies immediately adjacent to the DN.

**Grumose Reaction in the DN of FA**

Figure 4 displays the status of synaptic terminals in the DN of 1 case of FA (Patient FA27) and a normal control. Double label fluorescence staining for synaptophysin and class III β-tubulin shows grumose reaction in FA (Fig. 4A) and numerous thin axons within and around the clusters of synaptic terminals. Neuronal cell bodies are no longer distinct (Fig. 4A). In Figure 4B, a surviving small neuron is also surrounded by numerous thin axons. Short segments of proximal dendrites show a thick layer of compact synaptic terminals, representing grumose reaction (Fig. 4B). The normal large DN neurons in Figure 4C show a delicate layer of synaptic terminals about their somata and dendrites. The normal DN lacks the thin hairlike axons that are characteristic of FA (Fig. 4A). A small neuron of the normal DN (Fig. 4D) displays intense fluorescence of axosomatic and axodendritic synapses but does not show the grumose clusters surrounding cells of comparable size in FA (Fig. 4B).

**GABA-ergic and Glycinergic Terminals in the DN of FA**

Immunohistochemical results with antibodies to GAD and GlyT2 as indicators of GABA-ergic and glycinergic transmission, respectively, are shown in Figure 5. In 2 cases of FA (Fig. 5B, C; Patients F20 and F9, respectively), the GAD stain shows disorganization and grumose reaction of the DN. The bulk of the GAD reaction product occurs in synaptic terminals (Fig. 5A), and prompt fixation also allows visualization of this protein in the cytoplasm of small nerve cells (Fig. 5A, D, insets). Figure 5D shows the DN of the FA case with late onset and disease duration of 29 years (FA29). The GAD-reactive neuropil is more abundant, although negative images of large neurons are absent. The stain of GlyT2 generates punctate reaction product and delicate varicose axons in the normal DN (Fig. 5E). Small neurons with GlyT2-reactive somata are infrequent (Fig. 5E, arrow, inset). In FA, the GlyT2 stain suggests reduced density of reactive axons in 2 of 3 cases (Fig. 5G, H). Glycinergic terminals do not appear to contribute to grumose reaction in FA (Fig. 5F–H).

**GABA-A-Rγ2, GlyRα1/2, and Gephyrin in the DN of FA**

Immunohistochemical reaction products of the GABA and glycine receptors and of the inhibitory receptor-anchoring proteins GABA-A-Rγ2, GlyRα1/2, and Gephyrin in the DN of 1 normal control case (A, E) and 3 cases of Friedreich ataxia (FA) (B–D, F–H). GAD reaction product in different terminals in the normal DN (A) is very dense and generates negative images of large neurons (“N”). Small GAD-positive neurons are also present. The inset shows a small neuron with GAD-reactive cytoplasm and a layer of GAD-positive synaptic terminals. The cytoplasm of an adjacent large nerve cell (“N”) is GAD negative but displays a thin layer of axosomatic terminals. In FA patients, GAD immunohistochemistry shows a disorganized DN and several regions of grumose reaction (B, C, arrows). The DN in Patient FA29 (D) seems less disorganized, although it is thinner than normal and devoid of negative images of neurons and grumose reaction. The inset in (D) shows 2 surviving small γ-aminobutyric acid (GABA)-ergic neurons. The GlyT2 reaction shows punctate product and scattered varicose axons in the normal DN (E). When present, cytoplasmic reaction product occurs only in small nerve cells (F, arrow, and inset). In (F) (Patient FA20), punctate GlyT2 reaction product does not appear reduced although the DN is thinner; GlyT2-positive neurons are absent. Reduced GlyT2 reaction product is more convincing in (G) and (H), corresponding to Patients FA9 and FA29, respectively. (A–D) GAD. (E–H) GlyT2. (A, E) Normal control. (B, F) Patient FA20. (C, G) Patient FA9. (D, H) Patient FA 29. Scale bars = 50 μm; insets, 20 μm.

FIGURE 5. (A–H) Immunohistochemistry of glutamic acid decarboxylase (GAD) (A–D) and glycine transporter 2 (GlyT2) (E–H) in the dentate nucleus (DN) in 1 normal control case (A, E) and 3 cases of Friedreich ataxia (FA) (B–D, F–H). GAD reaction product in different terminals in the normal DN (A) is very dense and generates negative images of large neurons (“N”). Small GAD-positive neurons are also present. The inset shows a small neuron with GAD-reactive cytoplasm and a layer of GAD-positive synaptic terminals. The cytoplasm of an adjacent large nerve cell (“N”) is GAD negative but displays a thin layer of axosomatic terminals. In FA patients, GAD immunohistochemistry shows a disorganized DN and several regions of grumose reaction (B, C, arrows). The DN in Patient FA29 (D) seems less disorganized, although it is thinner than normal and devoid of negative images of neurons and grumose reaction. The inset in (D) shows 2 surviving small γ-aminobutyric acid (GABA)-ergic neurons. The GlyT2 reaction shows punctate product and scattered varicose axons in the normal DN (E). When present, cytoplasmic reaction product occurs only in small nerve cells (F, arrow, and inset). In (F) (Patient FA20), punctate GlyT2 reaction product does not appear reduced although the DN is thinner; GlyT2-positive neurons are absent. Reduced GlyT2 reaction product is more convincing in (G) and (H), corresponding to Patients FA9 and FA29, respectively. (A–D) GAD. (E–H) GlyT2. (A, E) Normal control. (B, F) Patient FA20. (C, G) Patient FA9. (D, H) Patient FA 29. Scale bars = 50 μm; insets, 20 μm.
protein gephyrin are shown in Figure 6. Large and small neurons of the normal DN show GABA-A-R\(_{\text{F}2}\) and GlyR\(_{>1/2}\) immunoreactivity close to or within the plasma membrane of somata and dendrites. This localization is best seen on images obtained under higher magnification and oil immersion (Fig. 6A–H, insets). In contrast to the receptor proteins, gephyrin reaction product is present throughout the cytoplasm of soma and dendrites (Fig. 6I–L). In FA, the receptor stains show a decline in number and size of neurons and a general disorganization of the DN neuropil. The loss of large DN neurons is very evident on the anti-gephyrin stained sections (Fig. 6J–L). Only a few small neurons remain in the FA patients. Some nerve cells have lost their receptor or gephyrin immunoreactivity (Fig. 6B, F, J–L, insets, arrows). Panels D, H, and L of Figure 6 confirm the relative preservation of the receptor proteins and gephyrin in the FA case with late onset (FA29), matching the GAD (Fig. 5D) and nerve cell stains (Fig. 3D, H).

Laser confocal double label immunofluorescence images of GABA-A-R\(_{\text{F}2}\) and GlyR\(_{>1/2}\) in the DN of 1 normal control and a case of FA (FA20) are shown in Figure 7. The GABA and glycine receptors occur in close apposition along the plasma membranes of neuronal somata and dendrites.

**DISCUSSION**

**Frataxin Deficit and Progression of the DN Lesion in FA**

Tissue frataxin concentrations reported here for the DN in FA show a greater deficit than reported for lymphoblasts (16), peripheral blood cells, whole blood, buccal cells (17, 18), or skeletal muscle (19), obtained from living FA patients. The mean level of 6.8 ng/g wet weight in DN represents 3.5% of normal, in contrast to the reported 20% to 30% in blood cells (16, 17). The average frataxin level of 2 carriers (142 ng/g wet weight) represents 72.8% of the normal mean, which is similar to the previously reported percentage in leukocytes (16, 17). Two reasons probably account for the very low frataxin concentrations in the DN in FA patients: 1) the DN is fully differentiated at the time of disease onset; 2) neurons, which

![FIGURE 6. (A–L) Immunohistochemistry of γ-amino-butyric acid receptor (GABA)-A-R\(_{\text{F}2}\), glycine receptor α1/2-subunits (GlyR\(_{\alpha1/2}\)), and gephyrin in the dentate nucleus (DN) of 1 normal control case and 3 cases of Friedreich ataxia (FA). In the normal case, GABA-A-R\(_{\text{F}2}\) (A) and GlyR\(_{>1/2}\) (E) reaction products are most abundant near the plasma membrane of neuronal somata and proximal dendrites, which is more distinctly visible at higher power under oil immersion (insets). In FA cases (B–D, F–H), immunohistochemistry of the receptor proteins generates a peculiar plaquelike distribution of reaction product. Some intact nerve cells also display receptor reaction products (insets). Reaction product is absent from some small neurons (B, F, insets, arrows). Receptor reaction products are more abundant in (D) and (H) from Patient FA29 whose disease began late and continued for 29 years. (I–L) Gephyrin reaction product is present in the cytoplasm of nerve cell bodies and dendrites of the control (I). Immunohistochemistry with anti-gephyrin shows severe loss of large neurons and retention of small nerve cells in FA patients (J–L). In (L) (Patient FA29), the neuropil is better preserved than in the cases of early onset (J, Patient FA20; K, Patient FA9). Some neurons of small or intermediate size are gephyrin negative (J–L, insets, arrows). (A–D) GABA-A-R\(_{\text{F}2}\). (E–H) GlyR\(_{\alpha1/2}\). (I–L) Gephyrin. (A, E, I) Normal control. (B, F, J) Patient FA 20. (C, G, K) Patient FA9. (D, H, L) Patient FA29. Scale bars = 50 μm; insets, 20 μm.

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account for the bulk of mitochondrial frataxin in the DN (2), do not regenerate. It cannot be assumed, however, that frataxin levels in the DN represent neurologic “end stage” of FA because the most common cause of death is cardiomyopathy (Table). The aforementioned regression analysis of DN thickness as a function of frataxin level suggests that relatively high residual levels of the protein may convey some protection against neuronal atrophy. It is unknown, however, whether other gray matter regions of the CNS that are not affected in FA contain more frataxin than reported here for the DN.

**Measuring FA Progression by GAD Immunohistochemistry of the DN**

The GAD is a strongly antigenic protein, and the compact organization of GABA-ergic terminals defines the measurable thickness of DN (Fig. 2). Loss of large neurons accounts for thinning of the DN, but regression analysis cannot determine the start of the disease progress (Fig. 2). Rapidly progressive fatal cardiomyopathy such as in Patients FA1, FA10, and FA22 (Table) provided the best insight into the correlation between DN thickness and short duration of the neurologic disease (FA1, 8 years; FA10, 7 years; FA22, 5 years).

**Normal Purkinje Cells in FA and Grumose Reaction**

The apparent morphologic integrity of Purkinje cells in FA must be reexamined. Why do Purkinje cells survive loss of their synaptic target (Fig. 3), which is contrary to other neurons of the CNS? Panels F to H of Figure 3 show a peculiar abundance of axons that traverse the DN, although depletion of afferent fibers would be expected. It is likely that grumose reaction in the DN in FA (Figs. 4A; 5B, C) is analogous to sprouting from transected Purkinje cell axons in experimental animals (13, 20, 21). The intense immunoreactivities with antibodies to synaptophysin (Fig. 4A) and GAD (Fig. 5B, C) establish derivation of these clusters from GABA-ergic Purkinje cell axons. The term “grumose degeneration” should, therefore, be revised to “grumose reaction” because proliferation of terminals may be beneficial. From Figure 4B, it appears likely that axonal sprouts establish contact with small surviving neurons (Fig. 5B), and this new

**FIGURE 7. (A–F)** Double-label immunofluorescence of γ-amino-butyric acid receptor (GABA-A-Rγ2) and glycine receptor α1/2-subunits (GlyRα1/2) in the dentate nucleus (DN) of a control patient (A–C) and Patient FA20 with Friedreich ataxia (FA) (D–F). Images were generated by laser scanning confocal microscopy; fluorescence was obtained by Alexa Fluor 488– and Cy3-labeled secondary antibodies. The 2 normal DN neurons (A) (shown as “N”) and the single DN neuron in FA 20 (D) (“N”) display densely packed GABA and glycine receptors in close apposition to each other. Abundance and density of the receptors in the FA neuron do not appreciably differ from the normal state. (A, D) GABA-A-Rγ2. (B, E) GlyRα1/2. (C, F) Merged images. Scale bar = 10 µm.
connection may provide the necessary retrograde trophic support to Purkinje cells. Purkinje cells also have collateral connections in the cerebellar cortex that may contribute to their survival when large DN neurons disappear. The ex- emption from FA, however, may only be temporary. In some FA patients, small and intermediate-size DN neurons are sparse (Fig. 3C, G), and some lack GABA-A-Ry2 (Fig. 6B, inset), GlyRα1/2 (Fig. 6F, inset), or gephyrin immunoreactivity (Fig. 6J, K, insets). They may ultimately disappear, at which time Purkinje cells may become atrophic.

Glutamatergic, GABA-ergic, and Glycinergic Neurons of the DN in FA

Based on the absence of established markers of inhibitory transmission such as GAD or GlyT2, large neurons in the DN are thought to be glutamatergic and excitatory. Considering the role of large DN neurons as the main source of efferent cerebellar output, the morphologic integrity of neurons, axons, and terminals in the molecular and granular layers of the cerebel- lum becomes functionally irrelevant for motor control FA(8).

In contrast to the relatively abundant small GAD- positive neurons in the DN, GlyT2-reactive nerve cells are sparse (Fig. 5). The paucity of glycineergic somata and axons may be misleading because normal large and small neurons of the DN express abundant GlyRα1/2 (Figs. 6, 7). Inhibitory glycineergic transmission in the DN may be more important than previously considered (22). It is also possible that cotransmission of GABA and glycine from the same terminal occurs in the human DN, in analogy to the cerebellar nuclei of rats (23). GlyT2 immunoreactivity also occurs elsewhere in the cerebellum (24) but, from the immunohistochemical re- action product shown in Figure 5, synaptic connections in the human DN may be largely intrinsic.

The deficit of GABA-ergic and glycineergic synaptic transmission in the DN of FA patients is highlighted by loss of GAD, GABA-A-Ry2, and GlyRα1/2 immunoreactivities (Figs. 5, 6). Selection of an antibody to the γ2 subunit of the GABA receptor was based on the wide distribution and abundance of this subunit in the CNS, its essential function in benzodiazepine responsiveness (25), and its prominent inter- action with gephyrin (26). The presence of GABA-A-Ry2 on small neurons correlates with the potential of these cells to generate a beneficial grumose reaction in response to axonal sprouts arising from Purkinje cell axons (Fig. 4B).

Gephyrin, named after the Greek word for “bridge” (27), is of interest to the study of the DN in FA because it anchors both GABA and glycine receptors to the cytoskeleton of neurons just beneath the plasma membrane (28). Any dis- turbance in this mechanism in FA would have serious conse- quences on inhibitory and likely, trophic, input to the neurons of the DN. The localization of gephyrin reaction product in the cytoplasm (Fig. 6I–L) is not necessarily contrary to its function at the plasma membrane. The observation of gephyrin staining in neuronal cytoplasm is not new (29) and may indicate rapid biosynthesis and degradation of the protein (28). We hypothesize that intact biosynthesis of gephyrin in small DN neurons (Fig. 6J–L) maintains the correct positioning of GABA-A-Ry2 and GlyRα1/2 and, consequently, proper GABA-ergic and glycine transmission. Because these small nerve cells do not send efferent fibers to the thalamus, their survival seems of limited importance to the ataxic dis- turbance of FA patients. Gephyrin reaction product is generally robust (Fig. 6I–L), and the absence of an immunoreaction in nerve cells of small and intermediate size (Fig. 6J–L, insets, arrows) may imply failed protein biosynthesis.

Interrupted GABA-ergic and Glycinergic Synapses and Frataxin Deficiency

Frataxin deficiency causes a lack of iron-sulfur clusters that affects complexes I, II, and III of the mitochondrial electron transport chain and the citric acid cycle enzyme aconitase. The severe lack of frataxin in the DN in FA reported here, however, does not reduce mitochondrial biogenesis, as determined by immunohistochemistry with an antibody to mitochondrial complex V (30, 31). Frataxin deficiency should affect all mitochondria-rich cells of the DN, including large and small neurons, with comparable severity. The remarkable survival of small GABA-ergic neurons and their dentato-olivary fibers is not consistent with a shared lack of a single protein, and it may be necessary to interpret failed GABA-ergic and glycine transmission as a primary event rather than secondary to neu- ronal damage. Under this hypothesis, trophic loss because of impaired alignment of GABA-A-Ry2, GlyRα1/2, and associ- ated proteins precedes atrophy of large glutamatergic neurons. Iron-mediated oxidative injury may not properly apply to the lesion of the DN, although the high levels of iron and copper in this gray matter ribbon and the admixture of these metals in the collapsing DN may be a source of toxic oxygen species (15). Several investigators have sought to connect frataxin deficien- cy with cytoskeletal abnormalities in FA (32–34). Recently, Bayot et al (34) suggested that destabilization of the actin network in cultured FA fibroblasts occurred because FA also silenced a gene located upstream from the frataxin gene, namely, phosphatidylinositol-4-phosphate-5-kinase 1B. Poor growth of cultured FA fibroblasts has been known for many years (35), but it is unlikely that observations on mesenchymal cells are completely relevant to DN neurons. Immunohisto- chemistry alone will not provide sufficient insight into the role of receptor-associated proteins in FA. In addition to gephyrin, many other proteins of interest have established or putative roles in the linkage of receptors at inhibitory synapses to actin filaments and microtubules (36, 37), among which neuroligin 2 and collybistin have already received detailed attention (37). The methods of proteomics appear well suited to the study of all proteins that contribute to the function of inhibitory synap- ses in the normal DN and the DN in FA. The hypothesis that intact GABA-ergic input to the large glutamatergic neurons of the DN provides a critical trophic function gains support from analogous observations on the inferior olivary nuclei in FA (8). Intact GABA-ergic dentato-olivary fibers arising from small DN neurons in FA support the neurons of the inferior olivary nuclei and their efferent glutamatergic climbing fibers. Any neoplastic or non-neoplastic lesion that interrupts the dentato- olivary tract and thereby deprives the inferior olivary nucleus of GABA-ergic input leads to neuronal hypertrophy, vacuola- tion, and transsynaptic death of olivary nerve cells.
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