the following procedure. First, univariate logistic regression was performed to identify SNP genotypes associated ($P \leq 0.05$) with a particular disease. Pairs of associated SNPs and their interactions were then included in logistic regression models. SNPs involved in a significant interaction or that remained significant in two-locus models were then assessed in higher order models, and were included in the final model for the disease if they retained their significance. Age, gender and *APOE* genotype were assessed as potential confounders using a 10% change in the ORs for any genetic effect as the criterion for retention in the final model.

ALS-G, PDC-G and GD in Guam may represent pleiotropic effects or variable expressivity of a single altered gene as originally hypothesized for ALS-G and PDC-G by Plato *et al.* (18), or a single set of altered genes. The power of our analyses to detect associations with ALS-G alone was limited because DNA was available from only 54 ALS-G cases. Therefore, the best genetic model as determined by analyses of PDC-G and GD was also fit using only ALS-G cases, as well as all three disease groups combined, compared to the control group.

Haplotype frequencies were estimated with an EM algorithm by the program HAPFREQS (73) in individuals homozygous for the H1 inversion polymorphism. Frequencies were estimated separately in the controls, in each case sample, and in the combined sample, as well as, for comparison, in the HapMap Japanese (JPT) sample as the closest match to Guam in ethnic origin. Haplotype frequencies in the Guam samples were estimated for SNPs 6 and 9, and also for SNPs 4, 6 and 9. The latter 3-SNP haplotypes were used as a surrogate for the three SNPs previously reported to tag the H1c haplotype associated with PSP and CBD (36): rs1467967 (7.2 kb proximal to SNP4), rs242557 (SNP6) and rs2471738 (8.7 kb distal to SNP9). To compare haplotype status of the high-risk haplotype found in the current study with the H1c haplotype, haplotype frequencies were estimated in the JPT sample for all five SNPs. From these frequencies, we computed the conditional probability of haplotype H1c (AxAxT) as P(AxAxT | xTACx) and P(AxAxT | xxACx), where x indicates any SNP allele in a particular haplotype position, A, T,G and C are the particular SNP alleles, and the SNPs are indicated in their chromosomal order: rs1467967–SNP4–SNP6–SNP9–rs2471738. Similar analysis using the Caucasian (CEU) HapMap sample lead to the same conclusion regarding the conditional probability of the H1c haplotype (data not shown).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Original Article

Magnesium deficiency over generations in rats with special references to the pathogenesis of the parkinsonism–dementia complex and amyotrophic lateral sclerosis of Guam

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Parkinsonism–dementia complex (PDC) and amyotrophic lateral sclerosis (ALS) are fatal neurological diseases. The incidence on Guam was very high between 1950 and 1965 but decreased dramatically after 1965. It is thought that drinking water containing low levels of calcium (Ca) and magnesium (Mg), and high levels of aluminum and of a plant excitatory neurotoxin are involved in the pathogenesis of these diseases. The present experiment was performed in rats that were exposed to low Ca and/or Mg intake over two generations, thus simulating the conditions human life on Guam, where several generations live continuously in the same environment. Significant loss of dopaminergic neurons was identified exclusively in the substantia nigra in 1-year-old rats that had been exposed continuously to low Mg intake (one-fifth of the normal level) over generations. The present study suggests that low Mg intake over generations may be involved in the pathogenesis of substantia nigra degeneration in humans.

Key words: Guam, magnesium, multigeneration, parkinsonism–dementia, substantia nigra.

INTRODUCTION

Some metallic elements are required for the normal functioning of the nervous system. For instance, it has been established that calcium (Ca) and magnesium (Mg) are essential for synaptic conduction, and iron oxidation plays an important role in the respiratory chain in mitochondria.[1] In addition, it has been reported that Mg is a cofactor in hundreds of enzymatic reactions. For example, it activates phosphotransferase and hydrolases such as ATPase, which are of central importance in the biochemistry of the cell, particularly in energy metabolism. Mg concentrates ribosomes and is involved in the attachment of mRNA to ribosomes. Thus, Mg is required for protein and nucleic acid synthesis, cell-cycle activity, cytoskeletal and mitochondrial integrity, and for the binding of substances to the plasma membrane. Mg frequently modulates ion transport via pumps, carriers, and channels, and thus may modulate signal transduction and cytosolic concentrations of Ca and potassium.[1]

It has been reported that dietary Mg deficiency plays an important role in humans in the pathogenesis of ischemic heart disease, congestive heart failure, cardiac arrhythmia, vascular complications of diabetes mellitus, pre-eclampsia, and hypertension.[1] It has also been proposed that Mg deficiency is involved in the pathogenesis of parkinsonism–dementia complex (PDC) and amyotrophic lateral sclerosis (ALS) in the Chamorro population on Guam, one of the Mariana islands in the western Pacific Ocean, as well as in the Kii peninsula of Japan and

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in West New Guinea.[2,3] ALS is a motor neuron disease affecting the Betz cells in the cerebral cortex, and facial and hypoglossal nuclei in the brainstem and anterior horn cells in the spinal cord. Patients with ALS usually die of respiratory failure within 5 years of disease onset. PDC is a disease entity that was established by Hirano *et al.* in 1961[4,5] that affects the neurons in the substantia nigra, brainstem, and temporal and frontal cortex. The disease is characterized by the presence of neurofibrillary tangles in the remaining neurons, disease-specific granular hazy inclusions in the astrocytes,[6,7] and tau-positive fine granules in the cerebral white matter.[8] Patients exhibit parkinsonism and dementia, and usually die within about 5 years from infectious diseases.[4,5,9]

Intake of water containing low Mg and Ca, high aluminum (Al)[2,3] and plant neurotoxins,[10] and a certain genetic predisposition[11] have all been proposed as possible causes of PDC and ALS on Guam. Recently, a gene variant of transient receptor potential melastatin 7 (TRPM7), which is a protein containing channel and kinase domains that is a Mg^{2+} concentration dependent cationic current regulator was reported in some Chamorro patients with ALS and PDC.[12] These hypotheses led researchers to experimental studies focusing on intake of low levels of Mg and Ca, and high levels of Al and plant neurotoxins. Repeated oral administration of α-amino-β-methylaminopropionic acid, a putative toxic factor that was extracted from the flower of the *Cycas circinalis* palm, to macaque monkeys produced chromatolysis of Betz cells, simple atrophy of spinal anterior horn cells, and neuritic swelling in the substantia nigra.[10] Mice fed cycad flour for 3 months showed decreased tyrosine hydroxylase labeling density in the striatum and number of spinal motor neurons.[13] In addition, a low-Ca, high-Al diet in monkeys induced neurofibrillary pathology that is characterized by the accumulation of phosphorylated neurofilaments in the anterior horn cells.[14] A low-Ca, low-Mg, high-Al diet administered to mice over a long period (11–31 months) induced loss of neurons and the development of tau-immunopositive neurons in the cerebral cortex.[15] Despite decades of research, no animal model completely replicates either PDC or ALS. However, most of the experiments carried out so far have used only a single generation of adults or infantile animals. The symptoms of PDC and ALS in humans develop at between 50 and 60 years of age, but patients with the diseases do not begin to take low-Mg, low-Ca, high-Al water or cycad flour at these ages. In the study presented here, we conducted an experiment in which rats were exposed over a long duration to a low-Ca and/or low-Mg diet over two generations, in order to reproduce the actual way of life on the island, that is, where several generations live in the same circumstances.

MATERIALS AND METHODS

Replicating the Chamorro population environment for rats

The animals used here were handled in accordance with the 'Guidelines for the Experiment of Tokyo Metropolitan Institute for Neuroscience', and adequate measures were taken to minimize pain and discomfort to the animals.

The animal feed was mixed so that it contained the test trace metals (Ca and Mg) in six different ratios, and were given to the animals as follows (Table 1): one-half or one-fifth of the normal Ca level (groups #1 and #2, respectively) and Mg level (groups # 3 and #4, respectively), and both low Ca and low Mg (one-half and one-fifth normal levels for both metals, groups #5 and #6, respectively). Density of Al in the chows was 2 ppm (examined by atomic absorption spectrophotometry). Distilled and deionized milli-Q water (DDW) was given to drink. The food and water were given *ad libitum*.

The albino Wistar rats used were 2 months old at the start of the experiments. The critical period, during which lesions can be induced, was established by assigning animals to one of five groups with different durations of exposure to food with different metal contents: group (i), starting 1 month before mating until the subsequent birth of the pups; group (ii), starting 1 month before mating until 1 month following the subsequent birth of the pups; group (iii), starting 1 month before mating until 1 years after the birth of the pups; group (iv), starting from the birth to 1 month after the birth of the pups, during which time they were breast fed; group (v), starting 2 months after birth of the pups until they were 1 year old (Fig. 1). The adult albino Wistar rats in groups (i) (ii), and (iii) were mated after 1 month of exposure to food with different metal contents (Fig. 1).

The combination of food types (i.e. with differing metal composition), exposure times, and gender with normal

Table 1 Mineral content of the animal feed

Foods	No.	Mineral content (mg/100 g)		Drinking water
		Ca	Mg	
Standard		892	78	DDW
Low Ca	#1	400	78	DDW
Low Ca	#2	190	78	DDW
Low Mg	#3	892	40	DDW
Low Mg	#4	892	14	DDW
Low Ca/Mg	#5	400	40	DDW
Low Ca/Mg	#6	190	14	DDW

Ca, calcium; Mg, magnesium; DDW, distilled and deionized milli-Q water. Standard; normal rat feed.

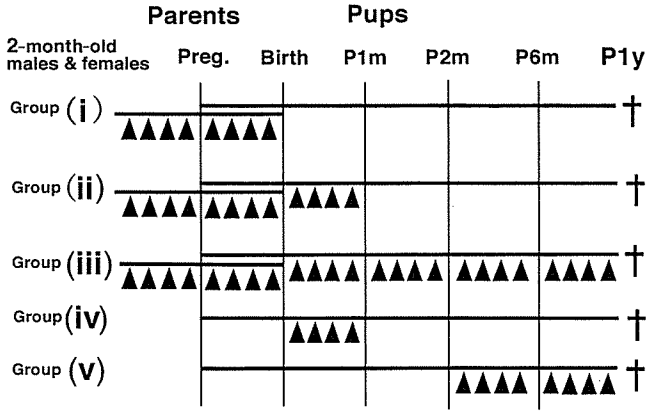


Fig. 1 Five exposure time groups (triangle: intake of the food). P1m, 1 month after birth etc; P1y, 1 year after birth. The cross indicates the time at which perfusion fixation was performed.

controls turned out that there were 62 experimental groups involving 827 rats (Appendix I).

Methods of examination

The body weight of the pups born by mating of the original 2-month-old rats was measured at embryonic day 16 (E16), on the day of birth, every 7 days thereafter until 28 days after their birth, and then every month until they were 1 year old. The pups were sacrificed at E16, on the day of birth (P1d), or 1 month (P1m), 6 months (P6m), or 1 year after birth (P1y) (Fig. 1). In addition, normal control rats were sacrificed at 2 years and 6 months after birth to examine the findings with age. They were deeply anesthetized with ethyl ether and then fixed by perfusion via the aorta with 4% paraformaldehyde (PFA) or 2.5% glutaraldehyde (GA) in a 0.1 M phosphate buffer (PB; pH 7.2). A sample of blood was taken at sacrifice, and the serum contents of Ca (colorimetric method), Mg (colorimetric method), iron (colorimetric method), potassium (electrode method), chloride (electrode method), and inorganic phosphorus (colorimetric method) were measured. As to the fetuses, blood was taken from the mother at E16 and on the day of parturition.

The pups' brains and spinal cords were removed and immersed in the 4% PFA in 0.1 M PB for 24 h. Following dehydration in a graded ethanol series, the tissues were embedded in paraffin. Five-micrometer-thick coronal sections of the cerebrum, and transverse sections of the brainstem and spinal cord were stained with HE, KB, and modified Gallyas staining procedures for light microscopic examination. Some of the sections were subjected to immunohistochemical staining using ABC with a Vectastain ABC kit (Vector, Burlingame, CA, USA). The primary antibodies used were rabbit anticow ubiquitin polyclonal antibody (dilution 1:150, Dakopatts A/S, Glostrup, Denmark), rabbit antihuman tau polyclonal antibody (dilution 1:1000), rabbit anti-β-peptide polyclonal antibody (dilution 1:500; both gifts from Prof. Y. Ihara), monoclonal antiphosphorylated tau antibody (AT-8, dilution 1:1000, Innogenetics, Ghent, Belgium), antiphosphorylated neurofilament monoclonal antibody (SMI31, dilution 1:1000, Sternberger Monoclonals, Baltimore, Maryland, USA), antiα-synuclein monoclonal antibody (dilution, 1:1000, BD Biosciences, San Diego, CA, USA), antiα-synuclein polyclonal antibody (c-20:sc7011, dilution, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), antiphosphorylated α-synuclein monoclonal antibody (#64, dilution 1:3000, gift from Prof. T. Iwatsubo), antityrosine hydroxylase (TH) monoclonal antibody (dilution 1:1000 Chemicon, Temecula, CA, USA), anti-iba1 polyclonal antibody (dilution 1:300, Wako, Osaka, Japan), and GFAP polyclonal antibody (dilution 1:500, Dakopatts A/S, Glostrup, Denmark). Antigenicity was increased for ubiquitin immunostaining by pretreating the sections with 0.025% trypsin for 15 min at room temperature, and for β-peptide immunostaining, with 99% formic acid for 3 min again at room temperature. Non-specific binding of the biotin/avidin system reagents was blocked by pretreating the sections with a blocking solution from the kit (Vector), and the sections were incubated with the required primary antibody for 2 days at 4°C. The sections were then incubated with the secondary reagent containing biotinylated antirabbit or antimouse IgG (diluted 1:200) for 2 h, and finally with the ABC solution for 1 h. The sections were subjected to the peroxidase reaction using freshly prepared 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6, for 10 min at room temperature. As antibody controls, the primary antisera were either omitted or were replaced with normal rabbit or mouse serum. Several specimens of neural and non-neural tissue from the rats served as positive or negative tissue controls, respectively.

The number of dopaminergic neurons in the substantia nigra was examined by using TH-immunostained three 6 μm-thick serial sections 24 μm apart at the level of 'Plate 25' by Paxinos and Watson.[16] TH-immunopositive neurons with nucleolus were counted in four rats in each group, and Abercrombie's correction factor[17] was applied for split cell error counting.

In situ terminal dUTP nick-end labeling (TUNEL) was carried out using an *in situ* apoptosis detection kit (ApopTag, Intergen, New York, NY, USA) on 5-μm-thick tissue sections. Deparaffinized 5-μm-thick 4%-PFA-fixed, paraffin-embedded sections were digested with proteinase K (20 μg/mL, Roche Diagnostics, Basel, Switzerland), then incubated with digoxigenin-labeled dUTP in the presence

of terminal deoxynucleotide transferase (TdT). Sections were further incubated with fluorescein-conjugated anti-digoxigenin antibody, resulting in localized green fluorescence within the nuclei of apoptotic cells. As a negative control, DNAase-digested sections were used, and distilled water or phosphate-buffered saline was substituted for the TdT solution.

An electron microscope (H-9000, Hitachi, Tokyo, Japan) was used to examine the substantia nigra. The GA-fixed tissues were postfixed with 1% osmium tetroxide, dehydrated through a graded ethanol series, and then embedded in Epon 812 resin. Semi-thin, 1-μm-thick sections were cut, stained with toluidine blue and examined with the aid of a light microscope. Ultrathin sections of appropriate areas were then cut, stained with uranyl acetate and lead citrate, and then examined with the aid of an electron microscope at 100 kV.

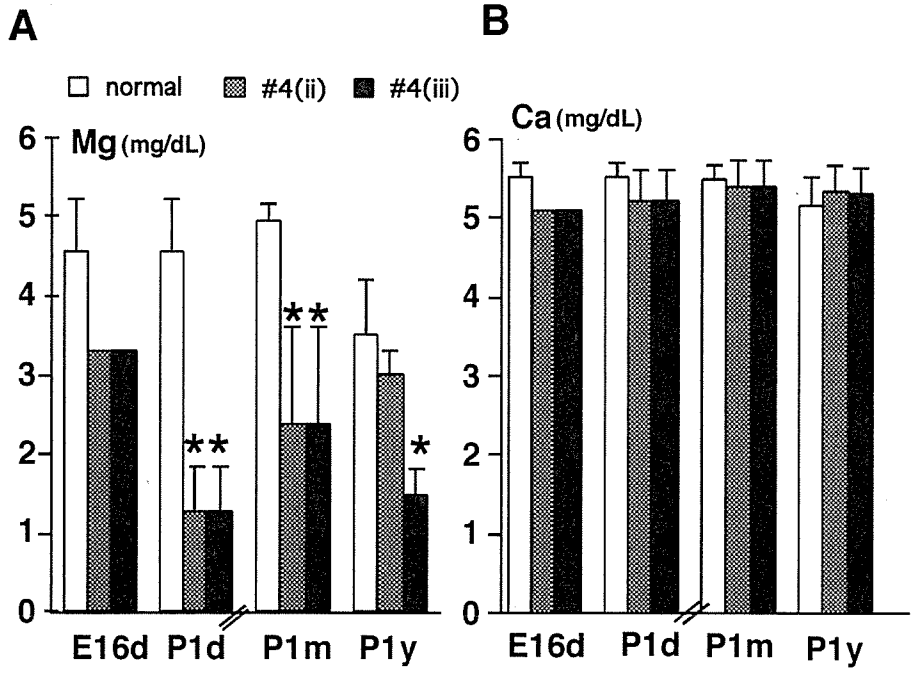
Myelinated fibers in the pars compacta of the substantia nigra were examined quantitatively with the above-mentioned Epon-embedded 1-μm-thick sections stained with toluidine blue. The substantia nigra was divided into two equal parts medially and laterally, and photographs were taken at the mid-medial and mid-lateral portions (1475 μm^2 each at 1000-fold magnification) of the pars compacta. Enlarged prints (6000-fold magnification) were made and the shortest diameter of the myelinated fibers (being perpendicular to the longest diameter of the myelinated fibers) was obtained using a digitizer (Measure 5, System Supply, Nagano, Japan). The data for the two portions in the pars compacta were summed and the frequency distribution of the myelinated fiber diameters, in 0.2 μm increments, was determined.

The data were compared statistically using the Kruskal–Wallis *H* and Mann–Whitney *U*-tests.

RESULTS

Serum mineral contents and clinical symptoms

Serum Mg was less than half of the control value at E16, on the day of birth, and 1 month, 6 months, and 1 year after birth in group (iii) animals (continuous consumption for two generations) that were fed group #4 food (containing one-fifth of the normal Mg concentration), and from E16 until 1 month after the birth of the pups in group (ii) animals (consumption for two generations but until 1 month after the birth of the pups) fed the same food. Serum Mg concentration slightly decreased only at parturition and 1 month after birth in group (ii) and (iii) animals fed group #6 food (containing one-fifth of the normal Mg and Ca con-



	E16d	P1d	P1m	P1y
Control	4	4	5	8
#4 (ii)	1	4	5	2
#4 (iii)	1	4	5	5

Number of subjects examined.

Fig. 2 Serum concentration of Mg (A) and Ca (B). Serum was taken from the dams on embryonic day 16 (E16d) and the day parturition (P1d), and from the pups at 1 month after birth (P1m) and 1 year after birth (P1y). Bars indicate S.D.

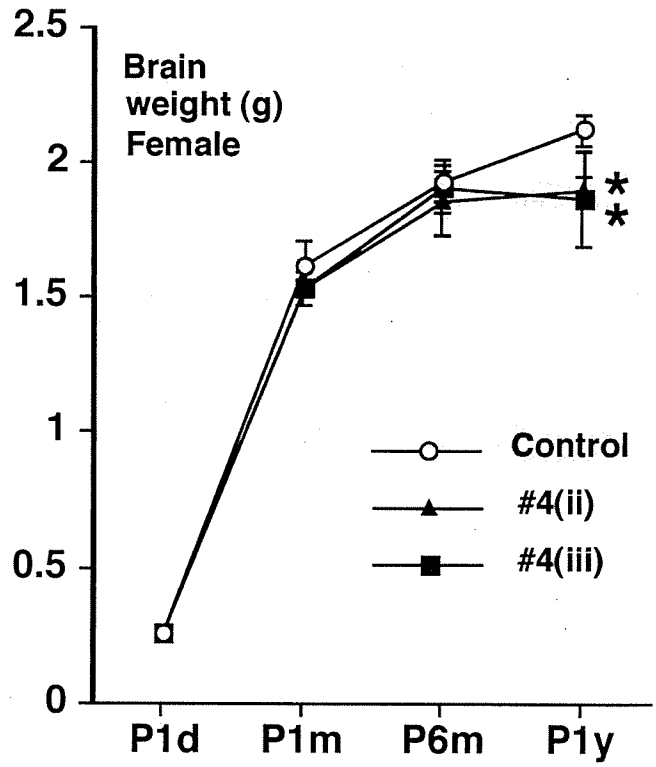
centrations). Serum Ca concentration was about half of the control value at parturition and 1 month after birth in group (ii) and (iii) animals fed group #2 food (containing one-fifth of the normal Ca concentration). Serum Ca concentrations in animals fed group #4 food did not change, and that of group (ii) and (iii) animals fed group #6 food at 1 month after birth of the pups showed only a slight decrease (Fig. 2). Serum concentrations of inorganic phosphorus was one-third to one-half of control values at the day of the pups' birth and 1 month afterwards in group (ii) and (iii) animals fed group #2, #4, or #6 food. Serum concentrations of chloride slightly increased at 1 month after birth in group (ii) and (iii) animals fed group #2, #4, or #6 food. Serum concentrations of Fe remained unchanged in all animals fed group #2 or #4 food.

There was no significant difference between the body weight of the E16 fetuses and pups at parturition in every group, and the appropriate normal controls. However, low body weight was observed, especially in the continuous Mg-deficient groups after postnatal day 7, in animals in groups (ii) and (iii) that were fed groups #4 and #6 food. Body weight was about two-thirds of the control value in those animals fed group #4 food, followed by those fed groups #1, #2, #3, #5, and #6 food. The ratio did not change until 1 year after birth. The rats in group (iii) that were fed group #4 food exhibited decreased activity at 1 year after birth. Tremor or shivering was not evident in any of the groups.

Exclusive reduction of dopaminergic neurons in the substantia nigra in Mg-deficient animals

The weight of the brain in normal control animals continued to increase for up to 1 year after birth. In the experimental groups, the weight of the pups' brains increased until 6 months after birth, but by 1 year after birth, brain weight of the group (ii) and (iii) animals fed group #4 food had decreased by approximately 10% compared with controls (Fig. 3). There were no significant differences between the weight of the brain of control animals and those in any other experimental group.

Histological examination of the brain and spinal cord revealed no evident alteration in animals at E16 or on the day of birth in any of the experimental groups. In group (iii) animals fed group #4 food, the CNS was not remarkable until 6 months; the neurons and neuropil of the cerebral cortex, including neocortex and Ammon's horn, were slightly atrophic and GFAP-immunopositive reactive gliosis was found there by 1 year after birth. However, the most significant changes were observed in the substantia nigra at 1 year, at which point the CNS exhibited marked atrophy in group (iii) rats fed group #4 food (continuous Mg deficiency over the generations until 1 year after



	P1d	P1m	P6m	P1y
Control	7	5	6	6
#4 (ii)	5	4	4	3
#4 (iii)	5	4	7	6

Number of subjects examined.

Fig. 3 Brain weight of the pups in the Mg-deficient groups. The circle, triangle and square indicate the mean, and the bars show the S.D. $P < 0.05$.

birth of the pups) (Fig. 4A,B). The neurons decreased in number, and appeared smaller in these animals. Immunohistochemistry for TH revealed that the size of TH-immunopositive dopaminergic neurons were small and the numbers low. The number of TH-immunopositive dendrites or axons of substantia nigra neurons severely decreased in 1-year-old rats in group (iii) that were fed group #4 food (Fig. 4C–F). GFAP-immunopositive reactive gliosis (Fig. 4G,H) and a mildly increased number of iba1-immunopositive microglia were observed in the substantia nigra at 1 year in group (iii) rats fed group #4 food. Conversely, the number of TH-immunopositive dopaminergic neurons and the dendrites in the ventral tegmental area and hypothalamus looked quite well preserved, even in these same rats (Fig. 4C,D). Quantitatively, the number of TH-immunopositive neurons in the substantia nigra decreased significantly to two-thirds of the normal control value in group (iii) rats fed group #4 food at 1 year

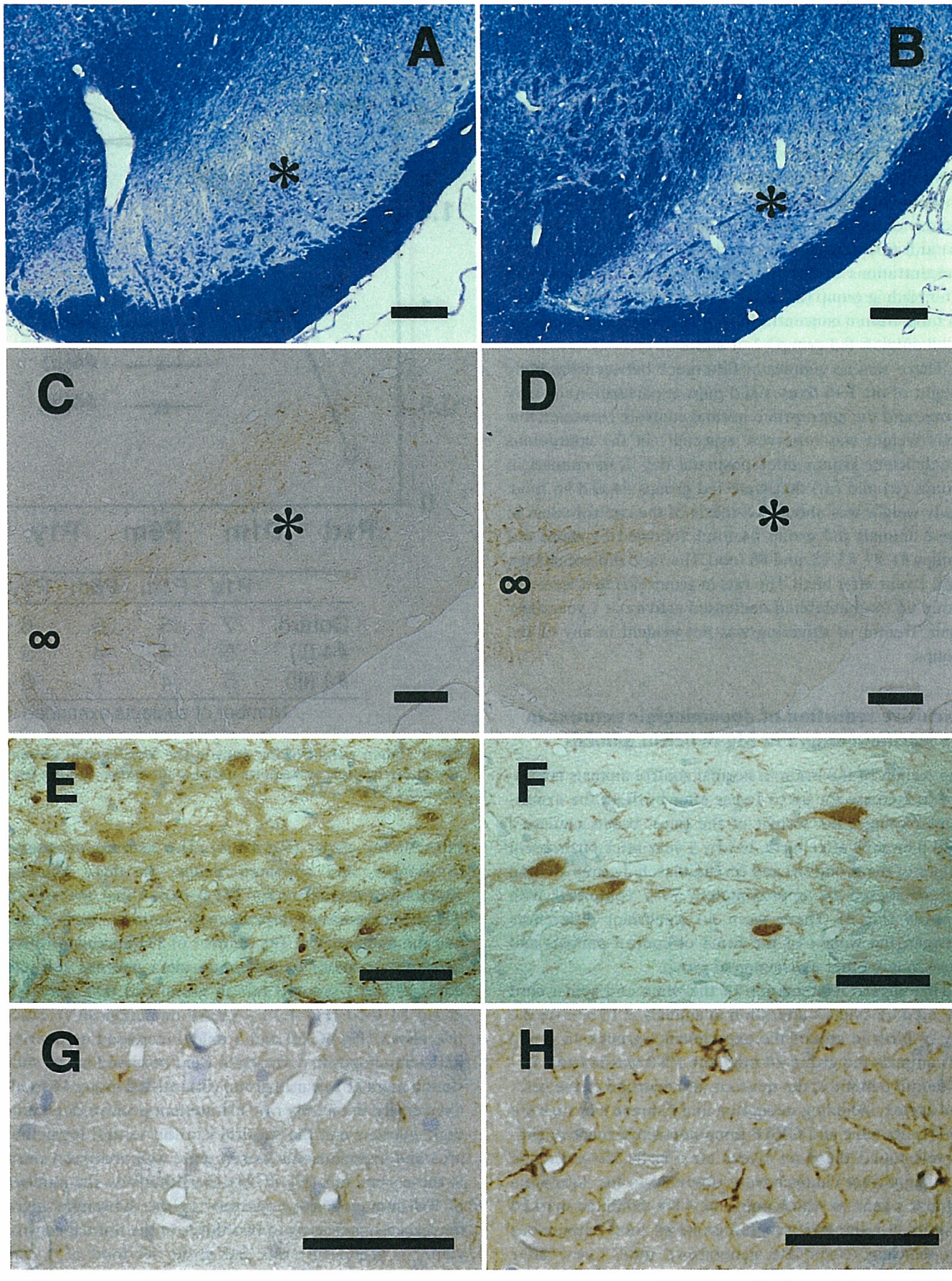


Fig. 4 Light-microscope findings from the substantia nigra of group (iii) rats fed group #4 food (group (iii) #4) compared with controls at 1 year. (Group (iii) animals were continuously Mg deficient over two generations, having one-fifth of the normal Mg level.) The substantia nigra (asterisks) showed marked atrophy at 1 year after birth (B) compared with control animals (A) (KB staining). Bars: 200 μm. The number of tyrosine hydroxylase (TH)-immunopositive dopaminergic neurons decreased exclusively in the substantia nigra (asterisks) of this group (D) compared with controls (C). However, TH-immunopositive neurons in the ventral tegmental area (∞) were quite well preserved (TH-immunohistochemistry). Bars, 200 μm. High-power view of the substantia nigra showing a marked decrease in number of TH-immunopositive dendrites or axons of the dopaminergic neurons group (iii) #4 (F) compared with a control animal (E) (TH-immunohistochemistry). Bars, 50 μm. Reactive astrocytosis was also observed in the substantia nigra (H) compared with controls (G) (GFAP-immunohistochemistry). Bars, 50 μm.

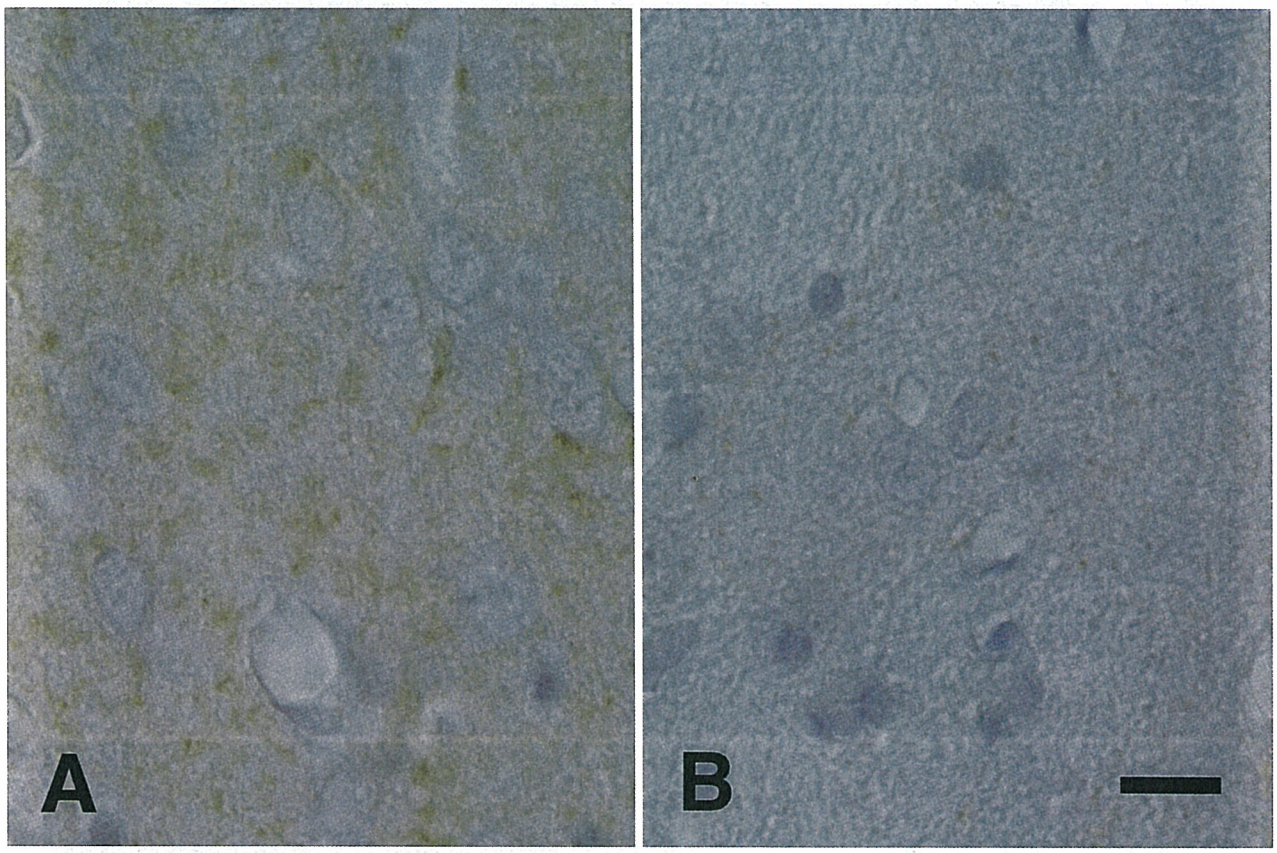


Fig. 5 TH-immunopositive neurites decreased in number in the caudate-putamen in 1-year-old Mg-deficient group (iii) rats that were fed group #4 food (B) as compared with a control rat (A). Bar; 10 μm.

(Table 2). The number of TH-immunopositive neurites in the caudate-putamen decreased in 1-year-old rats in group (iii) (Fig. 5).

At 1 year, the group (iii) rats fed group #6 food and group (ii) rats fed group #4 food showed a suspicious loss of TH-immunopositive dopaminergic neurons in the substantia nigra. However, 6-month-old group (iii) rats fed group #4 food, and the other groups at any stage until 1 year showed no evident loss of dopaminergic neurons.

Light microscopic examination of toluidine-blue-stained, 1-μm-thick epon resin sections revealed scanty cytoplasm and nuclear membrane indentation in the remaining nigral neurons of 1-year-old Mg-deficient rats from group (iii) that were fed group #4 food compared with controls (Fig. 6A,B). Electron microscope observation revealed a decrease in the number of mitochondria, rough endoplasmic reticulum and free ribosomes, the presence of

Table 2 Number of tyrosine-hydrolase-immunopositive dopaminergic neurons with nucleolus in the substantia nigra at 1 year

	No. neurons (mean ±SD)
Control ($n = 4$)	526.5 ± 74.6
Low Mg ($n = 4$)†	394 ± 63.1*

†Indicates a group – (iii)#4 – with continuous magnesium (Mg) deficiency over generations until 1 year after birth of the pups. n, number of rats examined. *$P < 0.05$.

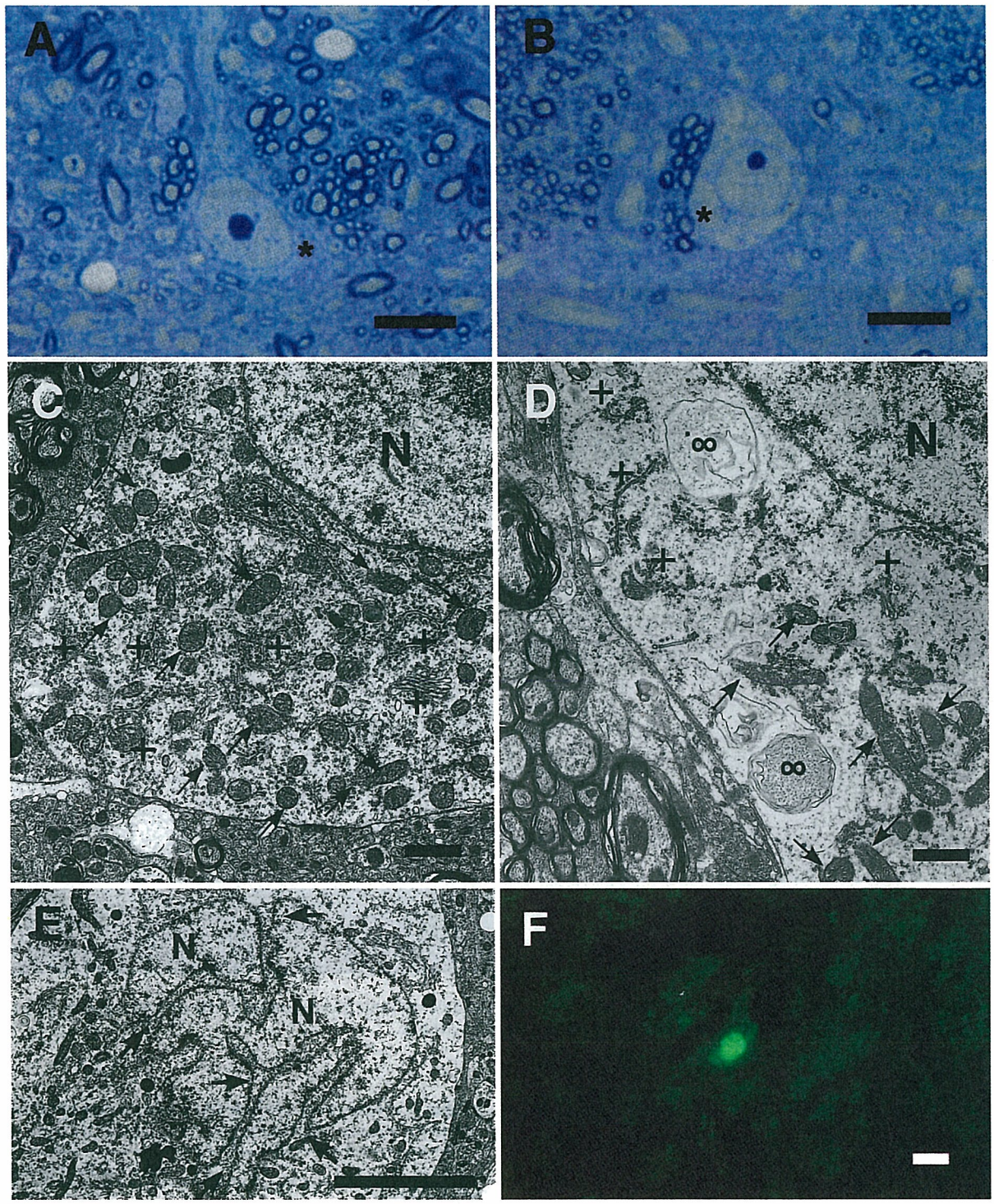


Fig. 6 Degenerating mechanism of the neurons in the substantia nigra of group (iii) rats fed group #4 food (group (iii) #4) compared with controls at 1 year. (Group (iii) animals were continuously Mg deficient over two generations, having one-fifth of the normal Mg level.). Light microscope examination of toluidine-blue-stained, 1-μm-thick epon resin sections revealed scanty cytoplasm (*) of the remaining nigral neurons (B) compared with a control rat (A). Bars, 10 μm. Electron microscope observation of group (iii) #4 substantia nigra neurons (D) revealed decreased mitochondria (arrows), rough endoplasmic reticulum (+) and free ribosomes, and the presence of degenerated materials (∞) compared with a control rat (C) (uranyl acetate and lead citrate staining). Bars, 1 μm. Nuclear membrane indentation (arrows) appeared frequently in the remaining neurons (E) of the substantia nigra of these experimental rats (N: nucleus) (uranyl acetate and lead citrate staining). Bar, 5 μm. At 1 year, *in situ* TUNEL staining conjugated with fluorescein was positive in some of the remaining substantia nigra neurons (F) of group (iii) #4. Bar, 10 μm.

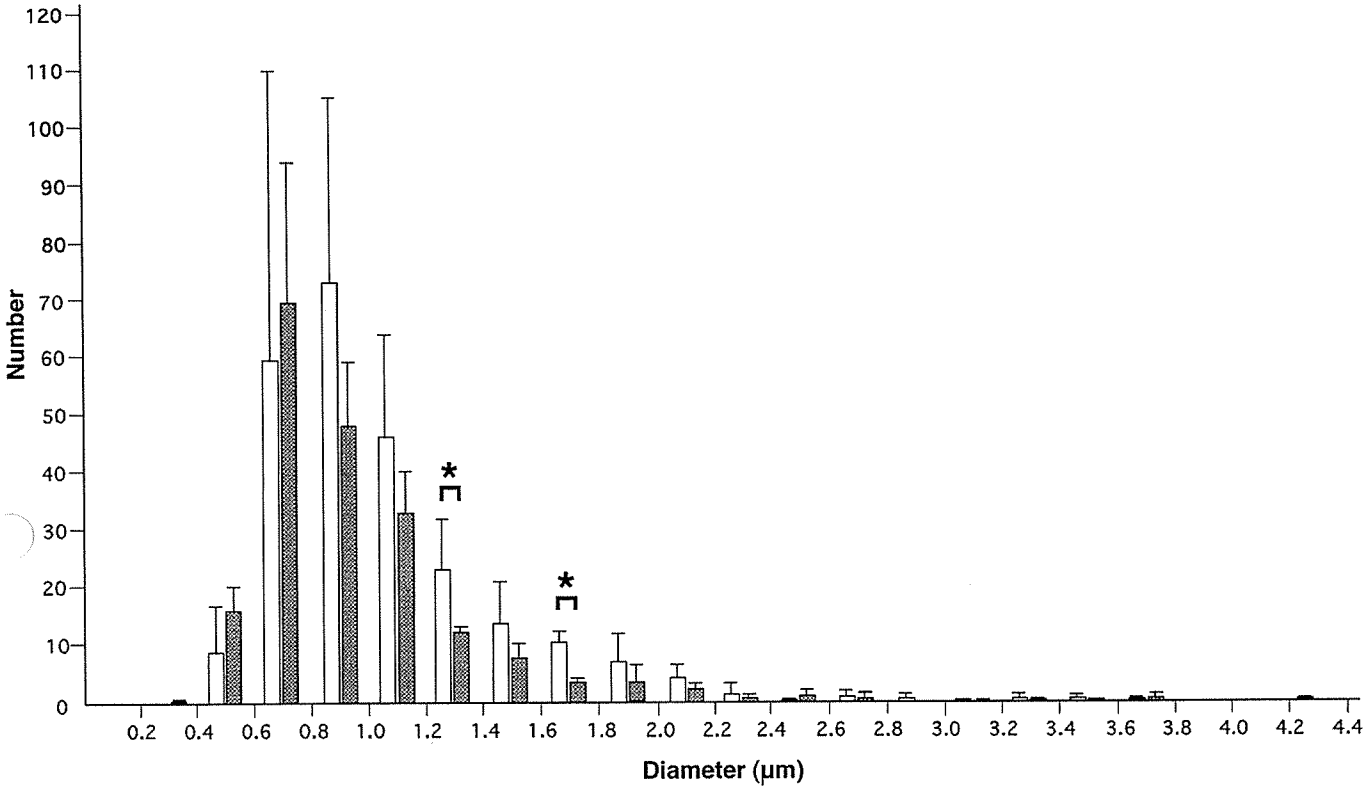


Fig. 7 Fiber-size distribution of the myelinated fibers in the pars compacta of the substantia nigra at 1 year. Normal rats showed a prominent peak at 1.0 μm, but in the rats of group (iii) fed group #4 food (continuous Mg deficiency over the generations until 1 year after birth of the pups), the peak was at 0.8 μm. The number of myelinated fibers with diameters of 1.2–1.4 μm and 1.6–1.8 μm was also significantly decreased in this group. *White bar;* control subject and *hatched bar*; Mg-deficient rats. Values given are means + SD; $*P < 0.05$.

Table 3 Number of myelinated fibers in the pars compacta of the substantia nigra at 1 year

	≤1.2 μm	1.2–2.2 μm	>2.2 μm	Total
ntrol	141.5 ±91.8	101.5 ±30.1	9.5 ±6.5	252.5 ±116.4 ($n = 4$)
ow Mg (iii)#4	134.5 ±33.5	59.8 ±3.5*	5.8 ±2.5	200.0 ±33.8 ($n = 4$)

$*P < 0.05$. Shortest diameter of the myelinated fibers at the mid-medial and mid-lateral portions (1475 μm² each) was examined. Values are given as the mean ± S.D. n, number of rats examined.

degenerated materials, and nuclear membrane indentation in the remaid with controls (Fig. 6C–E). Similar but less marked changes were observed at 1 year in group (iii) rats fed group #6 food and in group (ii) rats fed group #4 food. There were no evident changes in the substantia nigra in any other group at any other period. The findings noted above were not observed in the normal rats, even at 2 years and 6 months.

TUNEL staining was positive in some of the remaining neurons of the substantia nigra at 1 year in group (iii) rats fed group #4 food (Fig. 6F), but not in any other groups, including the controls. However, no apoptotic body was observed in the substantia nigra on electron microscopic observation. Immunohistochemistry for α-synuclein, tau, ubiquitin, and β-peptide failed to show evidence of Lewy bodies, neurofibrillary tangles, or senile plaques.

The fiber size distribution of the myelinated fibers in the pars compacta of the normal rats at 1 year showed a prominent peak at 1.0 μm, but in the rats of group (iii) fed group #4 food, the peak was at 0.8 μm. The number of myelinated fibers with diameters of 1.2–1.4 μm and 1.6–1.8 μm was decreased significantly in this group compared with controls (Fig. 7). The number of medium-sized myelinated fibers with a diameter of 1.2–2.2 μm significantly decreased in the rats of group (iii) fed group #4 food at 1 year (Table 3).

The spinal cord in each group was not remarkable. The configuration and volume of the white matter and gray

matter were well preserved in each group, and the topographic lamination of the spinal neurons was well developed. The number of spinal anterior horn cells was preserved, and no Bunina bodies or skein inclusions were observed in the spinal neurons.

DISCUSSION

Epidemiological evidence relating to the pathogenesis of parkinsonism–dementia complex and amyotrophic lateral sclerosis of Guam

The maximum annual incidence rate of ALS among the Chamorro population of Guam between 1945 and 1955 was reported to be about 60–70 per 100 000 for men and 30–40 per 100 000 for women; that of PDC in Guam between 1950 and 1970 was about 60 per 100 000 for men and 20 per 100 000 for women.[18,19] The annual incidence rate of ALS was quite different among the villages, ranging from 0 to 250 per 100 000 of the population.[20]

The mortality rate of PDC among the Chamorro people on Saipan, a northern island of Guam, whose genotypic composition is similar to that of the Guam Chamorro population, is strikingly low, suggesting the existence of an environmental risk factor.[21] Filipino migrants to Guam are susceptible to the disease, further supporting an environmental over genetic etiology.[22,23] A longitudinal case-control study has revealed that the spouses, from other places, of affected individuals are exposed to an increased risk of developing the disease, which also strongly implicates environmental factors.[24] The incidence rate of Guam PDC has been decreasing since 1965, especially among men, but remains at about 10–25 per 100 000 (last estimated for the period of 1980–1990).[18,19,25] It has been reported that 5-year incidence rates for PDC have steadily declined since the 1950s and 1960s, and the temporal trends in incidence rates are far less striking for PDC than for ALS on Guam.[25] These findings suggest that environmental factors in combination with possible genetic risk factors predispose the inhabitants of the island to Guam PDC and may account for its decreasing incidence in recent years.

Mg deficiency over two generations and parkinsonism–dementia complex of Guam

A recent paper reported that, among the Chamorro population, the ingestion of biomagnified cycad toxins through consumption of flying foxes leads to an increased risk for ALS–PDC on Guam.[26] On the other hand, the findings reported in the present study lead to the conclusion that continuous Mg deficiency (one-fifth of the normal concentration) over generations induces exclusive degeneration of the dopaminergic neurons in the substantia nigra with slight atrophy of the cerebral cortex and Ammon's horn in rats at 1 year of age, and may support the Mg hypothesis in the pathogenesis of PDC on Guam.[2,3,27] The degree of loss of neurons in the substantia nigra, and low bodyweight were more evident in the Mg-deficient group (food group #4) than in the Mg- and Ca-deficient group (food group #6). These findings show that a solely Mg-deficient diet is more hazardous to nigral neurons than a diet that is deficient in both Mg and Ca. This alteration was not observed following low Mg intake after birth or before 1 month postnatally. These findings indicate that nigral neurons will only degenerate if a low Mg intake is continued from the fetal and newborn periods through to the prime of life. For the pathogenesity of Al in degeneration of nigral neurons in rats, experiments on rats over generations using high-Al diet should be performed.

In the present study, the decrease in number of nigral dopaminergic neurons and effects on the mitochondria, rough endoplasmic reticulum, free ribosomes, and nuclear DNA occurred at 1 year in the group (iii) animals fed group #4 food (group (iii) #4). Since the findings were not observed in the normal-aged rats, even at 2 years and 6 months, these findings were considered to be different from those of aging. As described earlier, Mg has been considered essential for the functioning of the respiratory chain in mitochondria, adequate concentration of ribosomes, and nucleic acid synthesis.[1] Thus, the findings observed here may be explained simply in terms of Mg deficiency (Fig. 8). These findings were not evident at 6 months after birth in group (iii) #4, indicating that they occurred after 6 months. In addition, it has been established that Mg induces ferrimagnetization of the tissues,[27] and it has been reported that cellular iron levels are correlated with selective dopaminergic neuron loss in the substantia nigra in Parkinson's disease.[28] Further, Mg inhibits the spontaneous and iron-induced aggregation of α-synuclein.[29] Although α-synuclein-immunopositive Lewy bodies were not observed in the present study, further studies are necessary to elucidate both the precise mechanism underlying the neural degeneration observed and the mineral contents within the brain cells in Mg-deficient rats.

Nuclear membrane indentation of neurons has been reported in polyglutamin diseases, and is considered to be a finding of neuronal degeneration.[30,31] The relationship between nuclear membrane indentation and positive-TUNEL observed in the present study is obscure, and no apoptotic body was found in the present study. Regarding the way of death of the nigral neurons, some neurons may die through apoptosis in rats fed low-Mg food.

Medium-sized myelinated fibers in the pars compacta was significantly reduced in number in the 1-year old group (iii) #4 (continuous Mg deficiency over the generations

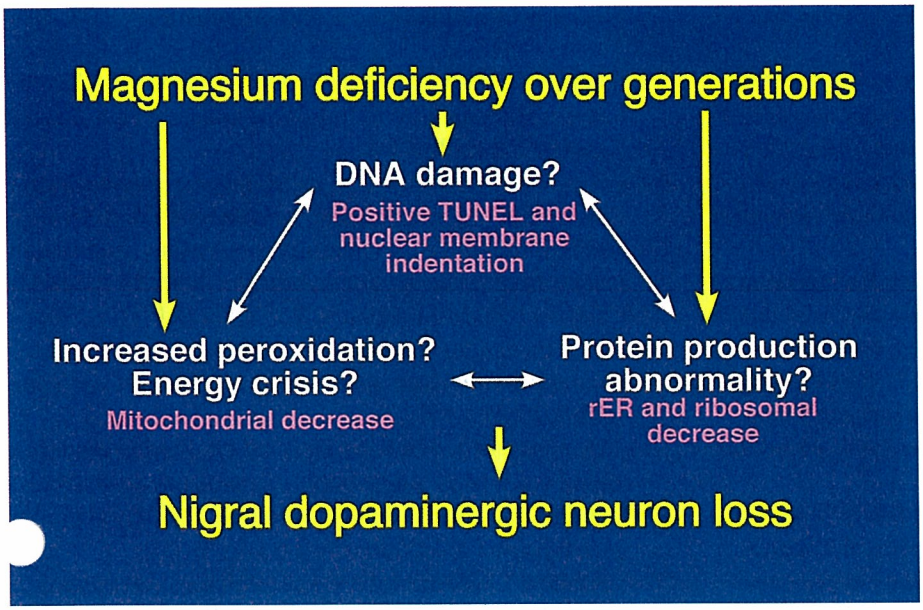


Fig. 8 Probable mechanism for nigral dopaminergic neuron loss by Mg deficiency in the present study.

until 1 year after birth of the pups). This finding indicates a loss and/or shrinkage of the nigro-striate and/or striato-nigral fibers[32] in the rats.

Serum Mg concentration decreased in rats fed a low-Mg diet, but that of the patients with PDC or ALS on Guam have been reported to be at normal levels.[33,34] Further study is necessary for elucidation of this discrepancy regarding the pathogenesis.

Pathological differences between amyotrophic lateral sclerosis and parkinsonism–dementia complex of Guam

Motor neurons, such as anterior horn cells, were unremarkable in our study, although the substantia nigra exhibited a significant loss of dopaminergic neurons. Regarding the identification of PDC and ALS on Guam, the present authors advocate that ALS on Guam is basically different from PDC, based on the findings that the neurofibrillary tangles observed in ALS patients are merely a background feature that is widely distributed in the population.[35] In addition, declining trends in incidence rates are far less striking for patients with PDC than for ALS on Guam.[25] These findings altogether might indicate that the pathogenesis of ALS on Guam is different from that of PDC, as we have reported previously.[35]

CONCLUSION

Significant loss of dopaminergic neurons was identified exclusively in the substantia nigra in 1-year-old rats that had been exposed continuously to a low Mg intake (one-fifth of the normal level) over generations. The present study suggests that low Mg intake over generations may be involved in the pathogenesis of substantia nigra degeneration in humans.

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Appendix I Number of examined rats in each experimental group

Groups from exposure period	Groups of food	Gender	Examined no. rats
i	#1	Fetus & New born	8
		Male	4
		Female	4
	#2	Fetus & New born	8
		Male	3
		Female	7
	#3	Fetus & New born	9
		Male	5
		Female	6
	#4	Fetus & New born	13
		Male	4
		Female	4
	#5	Fetus & New born	7
		Male	4
		Female	7
	#6	Fetus & New born	8
		Male	6
		Female	5
	#1	Fetus & New born	8
		Male	4
		Female	6
	#2	Fetus & New born	8
		Male	5
		Female	6
	#3	Fetus & New born	9
		Male	10
		Female	8
	#4	Fetus & New born	13
		Male	10
		Female	16
	#5	Fetus & New born	7
		Male	6
		Female	6
	#6	Fetus & New born	8
		Male	4
		Female	6
iii	#1	Fetus & New born	8
		Male	3
		Female	7
	#2	Fetus & New born	8
		Male	6
		Female	6

Appendix I *Continued*

Groups from exposure period	Groups of food	Gender	Examined no. rats
	#3	Fetus & New born	9
		Male	12
		Female	6
	#4	Fetus & New born	13
		Male	26
		Female	32
	#5	Fetus & New born	7
		Male	5
		Female	5
	#6	Fetus & New born	8
		Male	3
		Female	6
iv	#1	Male	8
		Female	5
	#2	Male	7
		Female	8
	#3	Male	4
		Female	6
	#4	Male	5
		Female	6
	#5	Male	4
		Female	10
	#6	Male	5
		Female	5
v	#1	Male	7
		Female	8
	#2	Male	7
		Female	8
	#3	Male	7
		Female	8
	#4	Male	9
		Female	17
	#5	Male	7
		Female	8
	#6	Male	7
		Female	7
Normal	Normal	Fetus & New born	122
		Male	45
		Female	65
			Total 827

Stroke, in press.

1. Author Information Page:

Fate of disseminated dead neurons in the cortical ischemic penumbra: Ultrastructure indicating novel scavenger mechanisms by microglia and astrocytes

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2. Acknowledgments and Funding Page

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3. Title Page

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