Ultrastructural and Morphometric Studies of Purkinje Cells of Brindled Mouse after Administration of Cupric Chloride

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Abstract. The mitochondrial and dendritic changes in Purkinje cells, which developed transiently in cupric chloride treated brindled mice, were investigated chronologically with light and electron microscopy. Both changes occurred predominantly in the anterior lobe of the cerebellum. The maximal mitochondrial changes coincided with dendritic changes, suggesting that these alterations were causally related. In the focally swollen dendrites there were disruption of neurotubules, abnormal mitochondria with electron-lucent or electron-dense matrix and large lamellar bodies. Quantitative analysis of the dendritic spine revealed significant differences in the spine area and synaptic length between the brindled mice and normal littersmates.

Key Words: Brindled mouse; Cerebellum; Copper; Dendrites; Kinky hair disease; Morphometry; Purkinje cell.

INTRODUCTION

The brindled mottled is an allele of X-linked mottled mutant mice. Hemizygous males, MO<sup>b</sup>/y, exhibit homologous metabolic defects in copper homeostasis. Copper accumulates in certain organs and is deficient in other organs including the brain. The clinical features in the mouse mutant are similar to those observed in Kinky hair disease (KHD) (1, 2). Hemizygous mice usually die before the 15th–16th postnatal (PN) day with extensive neuronal degeneration in the brain, and mitochondrial alterations are prominent morphological features in the degenerating nerve cells (3–5). Intraperitoneal injections of cupric chloride (CuCl<sub>2</sub>) twice on PN days seven and ten improve clinical symptoms and prevent neuronal degeneration in the cerebrum of these mutant mice (6). The activity of the copper dependent enzyme dopamine β-hydroxylase is low in both KHD and MO<sup>b</sup>/y but with CuCl<sub>2</sub> treatment, the decline of this enzymatic activity can be prevented (7). In these treated mutants, mitochondrial changes are far less pronounced in the cerebrum but become conspicuous, though transient, in the cerebellum (6).

Golgi impregnations reveal an improved arborization of Purkinje cell dendrites in CuCl<sub>2</sub>-treated hemizygous mice on PN day 15, but transient focal swellings in dendritic branches develop thereafter (8). Focal dendritic swellings are a feature of Purkinje cell abnormalities in KHD (9, 10). Therefore, we investigated the ultrastructural features of Purkinje cells in CuCl<sub>2</sub>-treated hemizygous mice during the course of development of these dendritic abnormalities.

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Fig. 1. These diagrams of the cerebellum indicate the distribution of Purkinje cells with clear vacuoles in MO1/a at various ages. These vacuoles correspond to abnormal swollen mitochondria and subsided spontaneously after day 94. The areas used for the quantitative analysis are indicated in the cerebellum at days 20, 50 and 210.

We also compared the area and synaptic length of the dendritic spines of Purkinje cells in CuCl₂-treated hemizygotes and in normal littermates at different ages, since some quantitative differences of these structures have been well documented in pathological conditions affecting the cerebellum (11–14).

MATERIALS AND METHODS

Nine hemizygotes, MO1/a, and nine normal littermate mice were used for the ultrastructural study. The day of birth was considered as day 1. All hemizygotes received intraperitoneal injections of 10 μg/g body weight (BW) of CuCl₂ on days seven and ten (6). One each of the hemizygotes and normal littermates were perfused through the heart with 2.5% glutaraldehyde in 0.1 M phosphate buffer at days 20, 32, 50, 62, 70, 80, 94, 110 and 210. The cerebellum was sagittally sectioned, post-osmicated and processed for embedding in epoxy resin according to a procedure described previously (3).

One micrometer (μm) thick sections of the cerebellum were stained with toluidine blue and examined by light microscopy. The selected areas of the anterior lobes were thin sectioned, stained with uranyl acetate and lead citrate and studied with an electron microscope.

For the morphometric study, one each of 20-, 50- and 210-day-old mice (hemizygotes, heterozygotes and normal littermates) were used. The outer half of the molecular layer in the ventral lobule of the culmen (15) as indicated in Figure 1 were thin sectioned. Random electron micrographs were taken at 12,000 magnification and photographically enlarged three times. The cross sectional area of spiny branchlets of Purkinje cells and the length of synaptic contact of dendritic spine and parallel fibers were measured by computer microscope.

The perimeters of synapses were digitized on a TALOS digitizing tablet which computed cross-sectional areas. Synaptic length measurements also were obtained with the TALOS tablet,
and the digitized data were processed with the aid of an Interdata 7/32 computer. FORTRAN programs were generated to compute the frequency distributions (in user-defined gradients), means and standard deviations (SD) of postsynaptic areas and synaptic lengths contained in the file. Statistical inferences on the differences between the mean spine cross sectional areas and the synaptic lengths of control heterozygotic and hemizygotic groups at each age were derived using a t-test analysis.

RESULTS

Light Microscopy

In the 20-day-old hemizygous mouse, the most pronounced abnormality in the cerebellum was well-defined clear vacuoles in the perikarya and dendrites of Purkinje cells. These vacuoles corresponded to the markedly swollen, abnormal mitochondria at the ultrastructural level (4, 5). Using these vacuoles as an indicator, we determined the distribution and degree of Purkinje cell lesions. Vacuolated Purkinje cells were confined to the anterior and middle lobes where dendritic abnormalities were most pronounced on Golgi-impregnated sections (8). Their numbers increased gradually after day 20, peaked on day 32 and gradually decreased thereafter. No vacuolated Purkinje cells were observed after day 94 in hemizygotes. These sequences are illustrated in Figure 1.

In the cerebellar white matter, axonal spheroids and myelin debris were conspicuous features, particularly in areas adjacent to the deep nuclei around days 50–70. However, these changes also subsided gradually, although rare spheroids were still detected in the granular cell layer even after day 110.
In control animals, no vacuolated Purkinje cells or axonal spheroids were observed at any age.

Electron Microscopy

In the somata and dendrites of Purkinje cells of the 20-day-old hemizygous mouse, there were numerous abnormal mitochondria. Many mitochondria were markedly enlarged in size with an electron-lucent matrix and short peripherally located cristae, which corresponded to vacuoles at the light microscopic level (Fig. 2). There were other types of abnormal mitochondria with relatively electron-dense matrix with many cristae (Figs. 3, 4). In the latter type of mitochondria the cristae were often tubular or vesicular in appearance (Figs. 3, 4) and their size and shape varied considerably. The mitochondrial changes were identical to those observed in the cerebrum and cerebellum of hemizygotes without CuCl₂ treatment (4, 5).

Cisterns of granular or agranular endoplasmic reticulum were frequently found in close proximity to these mitochondria (Figs. 3, 4). When mitochondria were clustered, a flattened agranular cistern was often found in apposition with two mitochondria. Stacks of parallel, regularly spaced cisterns with intercisternal electron-dense matrix were conspicuous in some neurons. Normal hypolemmal cisterns were seldom observed. Instead, several stacked agranular cisterns with electron-dense intercisternal matrix were observed in the subplasmalemmal region. When agranular cisterns were arranged parallel with the perinuclear cistern, a similar electron-dense intercisternal matrix was observed.

These changes in the mitochondria and endoplasmic reticulum were most pronounced at day 32 and gradually became less conspicuous thereafter. The enlarged mitochondria with electron-lucent matrices were only rarely encountered on days 94 and 110, but mitochondria with dense matrix were still observed at day 110 although their dimensions were generally smaller than those observed in the younger hemizygotes. No abnormal mitochondria were found at day 210.

An electron-dense, ovoid, intracytoplasmic inclusion was observed in some Purkinje cell somata after day 32. The inclusion was composed of stacks of electron-dense linear profiles with patchy areas of electron lucency (Fig. 2). At higher magnifications 10 nm filaments with cross bridges were identified readily in these electron-lucent areas.

In the molecular layer of hemizygotes, focal swellings of dendritic branches were conspicuous features from day 20 to 50. Abnormal mitochondria, as described above, were encountered frequently. Sometimes they clustered together and occupied a considerable area (Fig. 4); at other times they were scattered randomly (Fig. 3). Stacked, flattened, agranular cisterns of various lengths were frequently observed adjacent to mitochondria or immediately adjacent to the plasmalemma (Figs. 3, 4). These agranular cisterns were often composed of regularly spaced layers of flattened cisterns of about 40 μm width, with an electron-dense intercisternal matrix of about 35–40. Their length could reach up to 5 μm. On some occasions, more than 40 cisterns formed large lamellar bodies (Fig. 3). The neurotubules were less concentrated and oriented somewhat haphazardly in the swollen portion of the dendrites but were arranged normally in the proximal and distal portions (Fig. 3). Spines of the Purkinje cell spiny branchlets made asymmetrical synapses with parallel fibers (Fig. 5), and even in the swollen portion of the dendrites no morphological abnormalities were noted in the spine structure. Naked spines were not observed.

In the granular cell layers of hemizygotes, focally swollen axons were frequently observed. They contained numerous microtubules neurofilaments, scattered agran-
Fig. 3. Swollen portion of a Purkinje cell dendrite with lamellar bodies which are composed of flattened agranular cisterns; a close association with mitochondria and plasmalemma is evident (arrows). Bar: 1 μm. Inset: Higher magnification of a portion of the lamellar body indicated by *. There are flattened cisterns and an electron-dense intercisternal matrix. 32-day-old MOυ/y. Bar: 1 μm.

ular cisterns and mitochondria. In the glomerular synaptic complexes, central axons contained only a few abnormal mitochondria. The mitochondria of granule cells and Golgi type II cells appeared normal. The ultrastructural features of Purkinje cells in the control littermates were identical to those previously reported (16).
Fig. 4. Clustered abnormal mitochondria and many stacked, flattened cisterns of agranular reticulum (arrows) in the distal dendrite of a Purkinje cell. Bar: 1 μm. Inset: Higher magnification of the area indicated by *. 32-day-old MOv/y. Bar: 1 μm.

these control Purkinje cells hypolemmal cisterns were conspicuous and no lamellar bodies were found.

Morphometric Analysis

The cross sectioned areas of the dendritic spines of spiny branchlets of Purkinje cell dendrites and the lengths of synaptic contact of these spines with parallel fibers were measured. The latter was determined by measurement of the length of post-
synaptic thickening. As summarized in Table 1, spine area and synaptic length decreased with age in the littermate controls and heterozygotes while in hemizygotes the spine areas increased from day 20 to 50 but showed a marked decrease on day 210, although the length of synaptic contact decreased with age. The mean value of the areas in the hemizygote at day 50 was approximately 45% larger than that of age-matched normal littermates, but at day 20, there was no significant difference in the value between hemizygous and normal littermates. The mean value of the area in the 210 day old hemizygote, however, was approximately 27% smaller than the area observed in the age-matched normal littermates. The mean values of the synaptic length in the 20-, 50- and 210 day old hemizygotes were about 11%, 23% and 10% longer than those observed in the age-matched normal littermates. The heterozygotes showed intermediate values in both spine area and synaptic length.

DISCUSSION

Neuropathological changes were still observed in the cerebellum of the hemizygous mice even after the administration of CuCl₂. These changes consisted of abnormal mitochondria, electron-dense neuronal inclusions, and focal swellings of the Purkinje cell dendrites and axons. Such changes were basically the same as those found in a few long surviving MO₄²⁻/⁻/y (5) but were less pronounced. No necrotic Purkinje cells were found in the CuCl₂-treated MO₄²⁻/⁻/y. As shown in Figure 1, the distribution of abnormal mitochondria corresponded well with the observed dendritic abnormalities of Purkinje cells (8). The most extensive and numerous mitochondrial change also occurred simultaneously with the most pronounced focal swelling of dendritic branchlets (8). These results strongly suggest a “causal” relationship between mitochondrial changes and focal swellings of the dendrites. Since swollen, abnormal
<table>
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<th>Postnatal age</th>
<th>Day 20</th>
<th>Day 50</th>
<th>Day 210</th>
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<tr>
<td><strong>Area (μm²)</strong></td>
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<td>Normal littermate</td>
<td>0.15 ± 0.06 (n = 137)</td>
<td>0.13 ± 0.05 (n = 124)†</td>
<td>0.12 ± 0.04 (n = 114)†</td>
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<td>Hemizygote</td>
<td>0.16 ± 0.07 (n = 137)</td>
<td>0.19 ± 0.07 (n = 113)*</td>
<td>0.10 ± 0.08 (n = 105)*</td>
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<tr>
<td>Heterozygote</td>
<td>0.14 ± 0.07 (n = 127)</td>
<td>0.15 ± 0.06 (n = 168)*</td>
<td>0.11 ± 0.03 (n = 66)</td>
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<tr>
<td><strong>Synaptic length (μm)</strong></td>
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<td>Normal littermate</td>
<td>0.35 ± 0.09 (n = 137)</td>
<td>0.31 ± 0.09 (n = 137)†</td>
<td>0.29 ± 0.09 (n = 114)†</td>
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<tr>
<td>Hemizygote</td>
<td>0.39 ± 0.10 (n = 137)*</td>
<td>0.38 ± 0.10 (n = 113)*</td>
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</tr>
<tr>
<td>Heterozygote</td>
<td>0.34 ± 0.11 (n = 127)</td>
<td>0.33 ± 0.09 (n = 168)</td>
<td>0.31 ± 0.09 (n = 66)</td>
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Value: mean ± SD.

* P < 0.05 when the values of hemizygotes and heterozygotes were compared with those of the age-matched normal littermates using the t-test.
† P < 0.05 when the values of the 50-day-old, or the 210-day-old normal littermates were compared with those of the 20-day-old normal littermates using the t-test.
mitochondria tend to cluster, we initially hypothesized that these mitochondria merely mechanically obstructed the flow of various organelles within dendrites and thus caused focal stagnation. However, as noted in Figure 3, focal swelling occurred with disarray of the microtubules without mechanical obstruction by mitochondria. Similar microtubular disarray has been reported in the varicose dendrites of cerebral cortical pyramidal neurons in patients with neurobehavioral disorders and attributed to cytoskeletal disorganization (17). Therefore, these changes have described may be due to associated abnormalities in cytoskeletal components in the neurons in this mutant mouse, although the possibility that some of the findings may be due to mitochondrial dysfunction cannot be ruled out.

Another conspicuous feature was the presence of stacked, parallel flattened cisterns of agranular endoplasmic reticulum, which were most pronounced in the swollen portions of the dendrites. Herndon (18) first described these cisterns as lamellar bodies. They have also been observed in normal as well as pathological conditions (19-30) in Purkinje cells and other neurons. The functional significance of lamellar bodies is not well understood. However, lamellar bodies are composed of stacked cisterns of endoplasmic reticulum, a structure known to change its morphology in response to altered cellular function or injuries (31-33). In our experiment, these bodies are found in Purkinje cells only during the periods of recurring dendritic swelling in CuCl$_2$-treated M0$\text{v}/y$.

Lamellar bodies were conspicuous in some abnormal conditions of cerebellar organization (29, 34, 35). Large lamellar bodies similar to those in the dendrites in CuCl$_2$-treated hemizygotes have been reported in the dendrites of Purkinje cells in rabbits reared in an electric field (29). In these rabbits, smaller lamellar bodies were observed in the soma of Purkinje cells and other neurons. In neurons with lamellar bodies, the profiles of endoplasmic reticulum were also reduced and hypolemmal cisterns were absent. Our observations in CuCl$_2$-treated hemizygote mice were in complete agreement with the foregoing observations in rabbits. Therefore, these lamellar bodies may be an indicator of disturbed Purkinje cell function.

The electron-dense inclusions found in Purkinje cells of hemizygous mice were identical to those found in the thalamus of aging mice and humans (36, 37), CuCl$_2$-treated brindled mutant (38) and other pathological conditions in mice (39). As previously noted, these inclusions were morphologically related to neurofilaments (39). Therefore, the presence of these inclusions in Purkinje cells may support the possible presence of a disturbed cytoskeletal system in this mutant mouse.

In the cerebellum of a patient with KHD, some of the Purkinje cells had naked dendritic spines without making synaptic contact with parallel fibers (27). However, naked spines were not identified in hemizygous mice.

In a previous quantitative analysis of the dendritic spines in the cerebella of mice treated with cytosine arabinoside (13), increased synaptic length and spine area were observed. Since the number of granule cells decreased in these cerebella, dendritic spine changes were interpreted as a result of compensation for the decreased number of synaptic contacts. However, the results of the present study indicate that loss of granule cells may not be the only factor which induces an increase in such structures. The PN interval, 20 to 50 days, corresponds to a period of extensive mitochondrial and dendritic changes in hemizygous mice. Thus, the normal metabolic circuitry of Purkinje cells when disturbed by intrinsic factors, may also cause such spine and synaptic changes. The presence of giant dendritic spines in malnourished rats may support our contention (11, 12). The decrease of spine area in hemizygous mice at day 210 is difficult to explain, since dendritic and mitochondrial changes subsided.

by this age. In our previous study on CuCl₂-treated hemizygous mice older than six months of age, we found morphological features suggestive of premature aging (38). Therefore, decreased spine area in hemizygous males may be the result of accelerated aging. This hypothesis may be supported by the data in Table 1 which show a gradual decrease in spine area with age in both normal and heterozygous littermate mice. Further chronological investigations on the dendritic spine area in normal mice are currently underway to obtain a more precise answer to this intriguing question.

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