

ULTRASTRUCTURAL CHANGES IN THE MITOCHONDRIA OF CEREBELLAR PURKINJE CELLS OF NERVOUS MUTANT MICE

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ABSTRACT

The maturation of cerebellar Purkinje cells of normal and nervous (*nr/nr*) mutant mice has been studied by light and electron microscopy. In the mutant, 90% of Purkinje cells selectively degenerate between postnatal days 23 and 50. Losses are greater in lateral than medial regions. Other cerebellar neurons appear normal. The first morphological abnormality recognized is the presence of rounded mitochondria in perikarya of some Purkinje cells of the mutant at 9 days after birth. By 15 days, all *nr/nr* Purkinje cells contain spherical mitochondria and begin to deviate from the normal maturational sequence. Elaboration of the extensive dendritic tree halts midway and newly formed axon collateral fibers degenerate. In the perikaryon, the basal polysomal accumulation and climbing fiber-somatic spine synapses are sometimes abnormally retained. Cisternae of the Golgi apparatus and rough endoplasmic reticulum cease to form aligned stacks, and decrease in number, while polysomes dissociate into free ribosomes. These changes are progressive, culminating in cell death. Although every *nr/nr* Purkinje cell demonstrates spherical mitochondria, some cells survive the critical period, retain a near-normal complement of organelles, and reacquire normal-appearing mitochondria. The disorder appears intrinsic to Purkinje cells since all major classes of synapses were identified before cell death.

INTRODUCTION

Nervous (*nr*) is an autosomal recessive mutation in mice which results in a selective degeneration of Purkinje cells in the cerebellum (Sidman and Green, 1970). Purkinje cells were found to be present in normal numbers in affected mice on the 15th postnatal day but were distinguished from those of littermate controls with the light microscope by the presence of numerous prominent bodies, tentatively identified as mitochondria, in the apical cytoplasm. By 60 days an estimated 90% of the Purkinje cells in the cerebellar hemispheres and more than 50% in the vermis had disappeared. No other class of neurons in the brain was recognized

to be similarly affected. The present descriptive electron microscope study initiates a search for the mechanism by which a single gene selectively modifies this neuronal population.

MATERIALS AND METHODS

The *nr* mutation was discovered and has been maintained in the Balb/cGr strain. The *nr* locus is in linkage group XVIII (Sidman and Green, 1970), which is on chromosome 8 (Committee on Standard Genetic Nomenclature for Mice, 1972). Affected mice were obtained from either *+nr* × *+nr* or *+nr* ♂ × *nr/nr* ♀ matings. Both sexes of affected mice are fertile but progeny rarely result from *nr/nr* ×

nr/nr crosses. Since no suitable genetic marker is known for the *nr* locus, nervous mice were identified in segregating litters with confidence only by behavioral criteria after the 14th postnatal day. At earlier ages it was necessary to rely on the observation that mutant pups generally are smaller than littermates and to verify the classification by the change in mitochondrial morphology to be described. Controls were either behaviorally normal littermates (+/+ or +/*nr*) or Balb/cGr mice known to be homozygous wild type (+/+) at the *nr* locus. Cerebellar cortex was examined from 23 mutants and 18 controls at postnatal ages from 9 days to 10 mo, and from two putative, but unverifiable, nervous pups, aged 7 and 8 days.

Anesthetized mice were injected intraperitoneally with 100 U of heparin and then were perfused through the heart for at least 20 min with several hundred ml of warm fixative (1% paraformaldehyde, 1.25% glutaraldehyde, and 0.02% trinitroresol in 0.1 M phosphate buffer, pH 7.3, saturated with CaCl₂ [Karnovsky, 1965]). Then the skull was opened and the whole head was immersed in fixative for 12–18 h at 4°C. The cerebellum was dissected out and cut in half along the midline. Each half was cut into three blocks, one half in the coronal plane and the other in the sagittal. (The sagittal blocks correspond roughly to the vermis, the junctional region of vermis with hemisphere, and the lateral hemisphere.) The blocks were rinsed in buffer, post-fixed with 1% OsO₄ in buffer for 2 h at room temperature, dehydrated with ethanol, and embedded in Epon 812.

1 μ m sections were cut with glass knives from the six blocks prepared from each animal. The sections were stained with alcoholic toluidine blue and examined with the light microscope. To establish the time course and magnitude of cell loss, the Purkinje cell lamina was traced in at least two sections 30 μ m apart from each of the sagittal blocks with the aid of a Wild drawing tube (Wild Heerbrugg Instruments, Inc., Farmingdale, N. Y.) at a magnification of $\times 34$ and the length of the lamina was determined with a map measurer. All the Purkinje cells in the lamina were then counted at a magnification of $\times 400$ and the sum divided by the length to give a relative measure of the number of cells present. These values were determined for three control (23, 112, and 300 days) and 11 nervous (23, 26, 29, 37, 42, 50, 63, 102, 105, and 112 days) mice.

Areas chosen for electron microscopy were primarily, but not exclusively, from the culmen and declive of the midline sagittal block. Thin sections were cut with a diamond knife on a Porter-Blum MT-1 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.), picked up on Formvar-coated slot grids (1 \times 2 mm), stained with uranyl acetate and lead citrate, and examined with a Siemens Elmiskop 1A.

Use of the slot grids allowed a complete and systematic examination of the cortex, layer by layer. Whenever more than one series of sections from a particular block was studied, they were separated by a minimum of 30 μ m, so that different Purkinje cells were observed. The criteria used to identify classes of synaptic endings within the cerebellar cortex are similar to those of previous workers (Larramendi, 1969; Chan-Palay and Palay, 1970; Chan-Palay, 1971).

Certain aspects of mitochondrial morphology were quantitated to allow a somewhat more accurate description of differences between nervous and normal Purkinje cells. Measurements of the diameter of mitochondrial profiles were made on photographic prints of micrographs taken at magnifications of 8,000–13,000 and then enlarged three times. Each of the averaged values represents a minimum of 25 profiles. The percent cytoplasmic volume occupied by mitochondria was determined at 12 days for mutant and control mice, and at 15 days for a control, by a modification of the methods of Loud (1968). 30 randomly selected Purkinje cells from each of the 12-day animals and 20 from the 15-day control were photographed at $\times 3,000$ and then enlarged photographically to $\times 9,000$. A square grid was superimposed twice in different orientations over each picture, the number of intersections lying over mitochondria and cytoplasm were counted, and a percentage was calculated from the ratio.

OBSERVATIONS

Timing and Extent of Cell Loss

Fig. 1, in which the number of Purkinje cells per mm of Purkinje lamina is plotted as a function of age, presents the time course of cell loss. At 23 days almost the normal number of cells is present in nervous mice, but the number falls sharply during the next 2 wk. Nearly all of the cells that will degenerate have done so by 50 days, leaving approximately five Purkinje cells per mm in the vermis, three in the vermis-hemisphere junction, and one in the hemisphere (compared with the normal values of 25 in the vermis, 26 in the vermis-hemisphere, and 34 in the hemisphere). The extent of the loss is greater in lateral than in medial regions of the cerebellum. In a span of only a few weeks, nervous mice almost 90% of their Purkinje cells.

Morphological Maturation of Normal Purkinje Cells

The sequence of maturation of Purkinje cells in the mouse has been studied with the electron

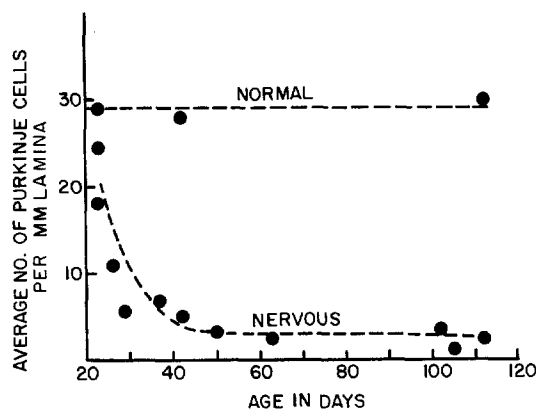


FIGURE 1 The number of cells along 1 mm of the Purkinje cell lamina (averaged for three regions of cerebellum) is shown as a function of age. The scatter represents both individual variation among mice and limitations of the method of measurement.

microscope by Larramendi and his co-workers (review by Larramendi, 1969) and by Meller and Glees (1969). Essentially it differs only in timing from that described for other species (Smith, 1963; Kornuth et al., 1967; Mugnaini, 1969; Altman, 1972). The observations on the development of normal Purkinje cells made in this study are similar to those of previous workers and will only be summarized to allow comparison with the development of nervous Purkinje cells.

At postnatal day 7, Purkinje cells are irregular in shape. The nucleus usually lies basal in the cell, and above it is a large, prodendritic expansion filled with many mitochondrial profiles, well-formed Golgi complexes, and scattered cisternae of rough endoplasmic reticulum mixed with numerous free polysomes. By 9 days, the perikaryon has begun to round up, and the prodendritic expansion appears to have been transformed into a primary dendrite. Cisternae of rough endoplasmic reticulum may be scattered lateral to the nucleus and several stacked cisternae are usually present above the nucleus (Fig. 2) which now lies eccentric with respect to a large basal collection of free polysomes. During the next week the collection of polysomes is infiltrated by mitochondria and increasing numbers of the polysomes become associated with membranes, forming aligned stacks of rough endoplasmic reticulum. During the same period, both the nucleus and the perikaryon continue to enlarge and the perikaryon gradually assumes the adult flasklike shape. Usually by 15 days, the ma-

ture cytoplasmic architecture has been achieved, characterized by mitochondria, Golgi complexes, and numerous stacks of cisternae of rough endoplasmic reticulum distributed concentrically around a large central nucleus and interspersed with free polysomes (Fig. 4).

This modification in the distribution of organelles in the perikaryon is accompanied by the construction of both an enormous apical dendritic arborization and a complex axon collateral plexus. The development of the dendritic arborization may be analyzed in 1 μ m toluidine blue-stained sections, since major dendritic processes are easily distinguished in the molecular layer and smaller dendritic profiles are visualized through their content of intensely stained mitochondria. At 7 days, the dendritic arbor is very primitive with only stubby processes arising from the prodendritic expansion, but by 9 days a clearly defined primary dendrite with several major branches has been formed. The arbor is rapidly elaborated and at 19 days the lacy network of mitochondria-filled processes in the molecular layer has almost reached the adult extent (Fig. 9). These observations are consonant with an earlier Golgi analysis which demonstrated that the major growth of the arborization is completed by 15 days (Meller and Glees, 1969). The same study indicated that the axon collateral plexus begins to form in the first postnatal week in the mouse. The construction of the collateral plexus may, therefore, antedate that of the dendritic tree. In this study, typical axon collateral endings were not identified with the electron microscope until the 12th postnatal day and during the next 2 wk they became increasingly common.

While these extensive internal changes are occurring, four different types of axons are making synaptic contact with the Purkinje cells. Climbing fiber synapses with Purkinje cells are easily identified at 7 days, the earliest age studied. Initially, the majority of such synapses are found on somatic spines, but on subsequent days, climbing fiber contacts are seen more often on the surfaces or spines of dendritic processes, and by 19 days, very few climbing fiber-somatic spine synapses are observed. As these contacts become less frequent, basket cell axons form synapses directly on the soma. Parallel fiber synapses on Purkinje cell dendritic spines, which will come to be the most numerous synapse in the cerebellar cortex, are present at 7 days and greatly increase in number

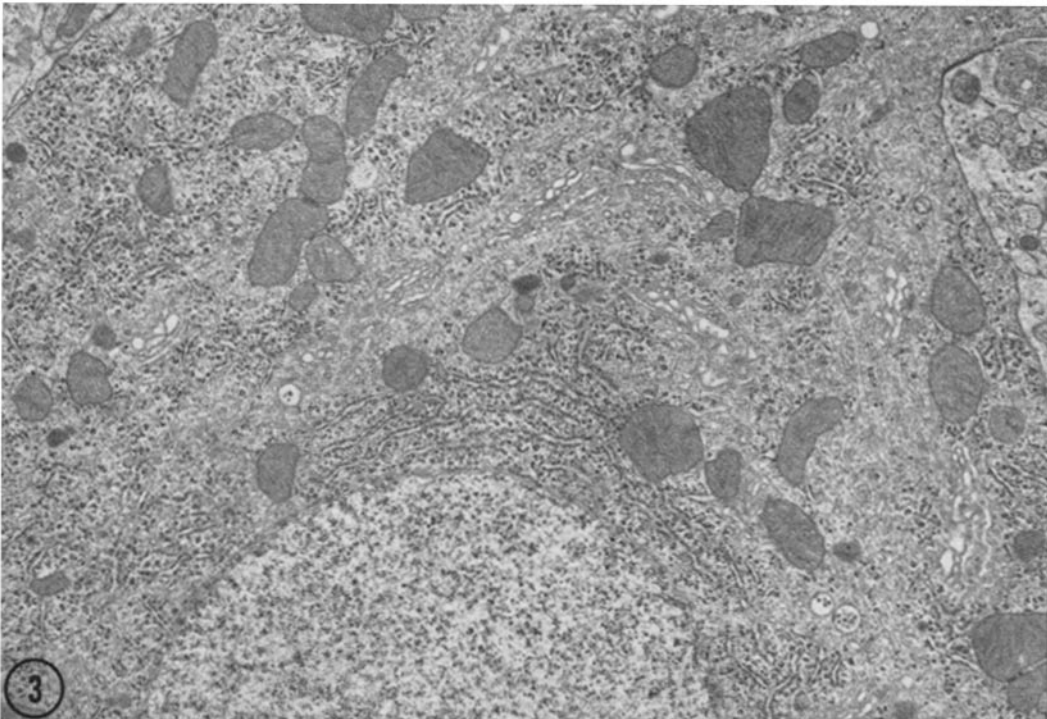
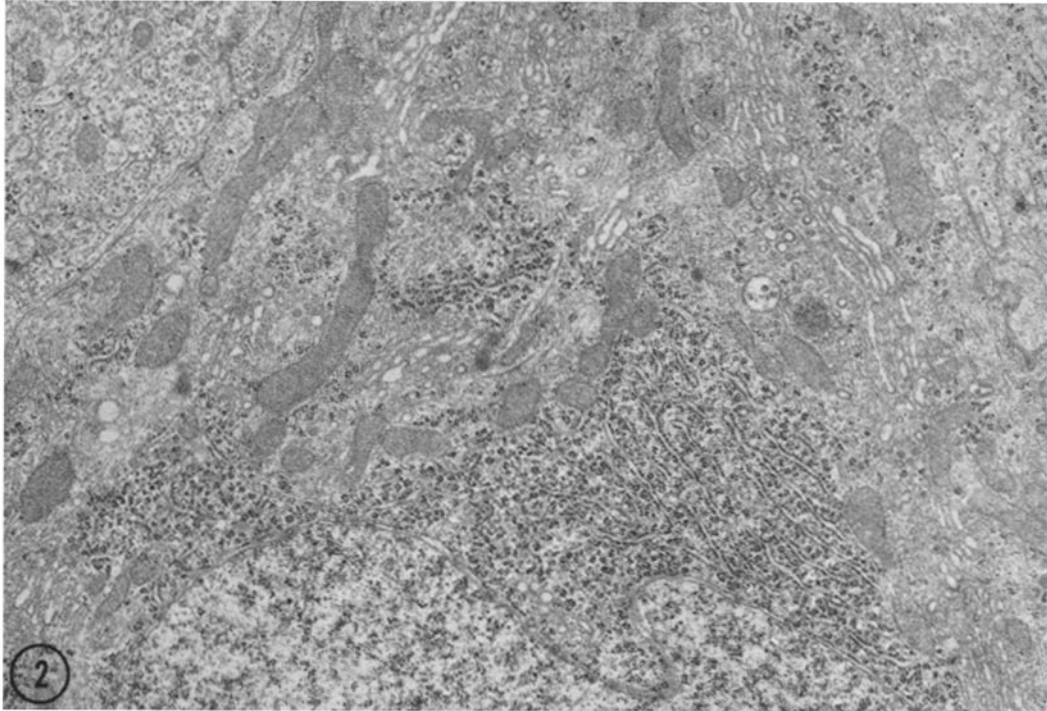


FIGURE 2 Supranuclear region of 9-day normal Purkinje cell. Elongate mitochondrial profiles are prominent. $\times 9,000$.

FIGURE 3 Supranuclear region of a 9-day nervous Purkinje cell. Almost all mitochondrial profiles are becoming rounded. $\times 9,000$.

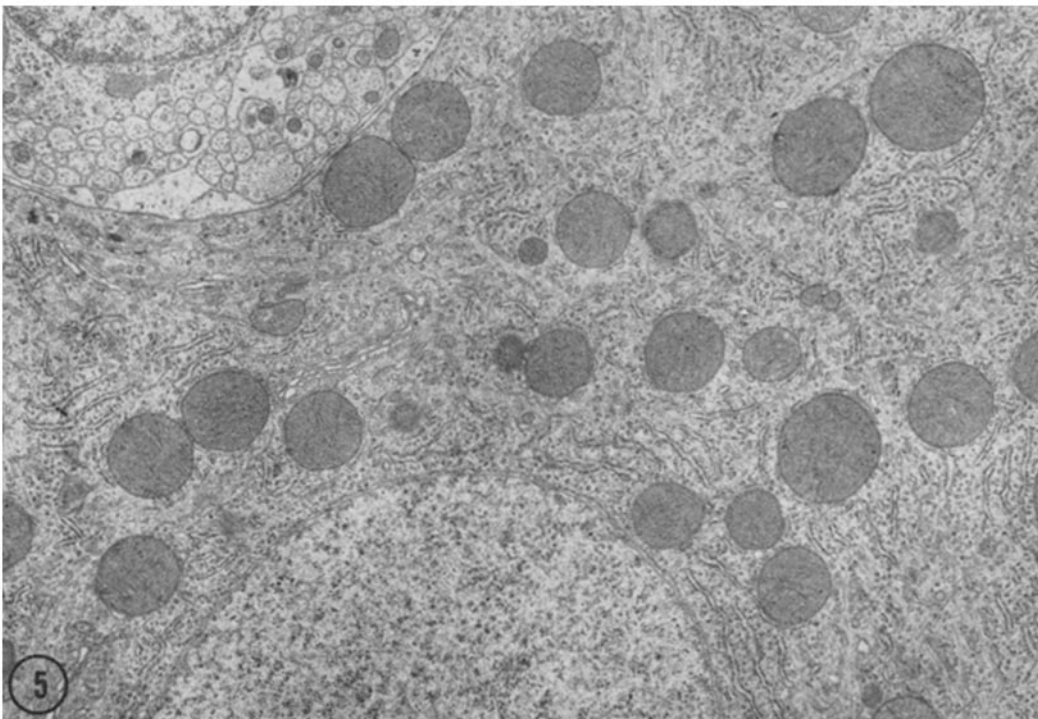
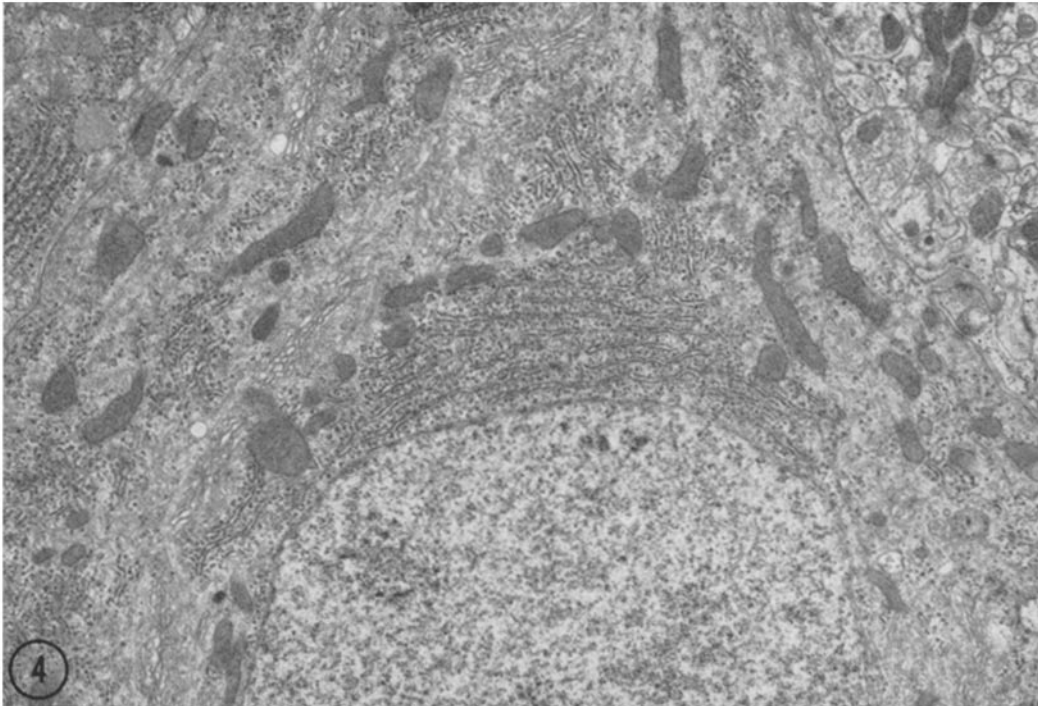


FIGURE 4 Supranuclear region of a 15-day normal Purkinje cell. Stacks of rough endoplasmic reticulum, Golgi complexes, and elongate mitochondrial profiles are seen. $\times 9,000$.

FIGURE 5 Supranuclear region of 15-day nervous Purkinje cell. Some stacking of the rough endoplasmic reticulum and Golgi cisternae are present but the most striking organelles are the spherical mitochondria. $\times 9,000$.

as the arbor grows. Stellate cell axons are first identifiable on dendritic processes at day 12.

At all ages, the shapes of mitochondrial profiles in thin sections through the perikarya of normal Purkinje cells range from long and ellipsoidal to small and circular (average diameter, $0.42\ \mu\text{m}$). The interpretation most consistent with the appearance of the profiles is that the mitochondria are long, sinuous cylinders randomly oriented within the cell body. The dendritic arbor also contains elongate mitochondria oriented longitudinally in the processes. Ten such mitochondria in the primary dendrite of a 15-day normal mouse had an average length of $3.96\ \mu\text{m}$ and an average width of $0.50\ \mu\text{m}$. On the assumption that the mitochondria are cylinders, each one has an approximate volume of $0.78\ \mu\text{m}^3$. The mitochondria themselves possess no unusual characteristics (Fig. 15). The matrix is moderately dense and may contain a few granules. Both lamellar and tubular cristae are present. Often a cisterna of rough endoplasmic reticulum, lacking ribosomes on the side facing the mitochondrion, lies closely associated. Flattened smooth cisternae, resembling subsurface cisternae, may appear intercalated either between two mitochondria or between a mitochondrion and the plasma membrane.

Maturation of Nervous Purkinje Cells

Gradual gradients in developmental timing across the cerebellar cortex exist normally for Purkinje cells; for example, at a given age cells in the vermis are more mature than those in the hemispheres. Although in normal mice there is relatively little variation in the stage of development demonstrated by adjacent cells, in nervous mice striking variations do exist. The following description of maturation and its timing reflects that of the majority of Purkinje cells.

Initially, development within the perikaryon parallels that observed in normal cells. At 9 days the nucleus lies above a normal basal accumulation of polysomes. Aligned cisternae of rough endoplasmic reticulum are present above the nucleus. Other scattered cisternae, Golgi complexes, mitochondria, and free polysomes fill the apical cytoplasm (Fig. 3). During the next 10 days the perikaryon apparently continues to grow normally and the cell body usually acquires the adult flasklike shape. The nucleus also increases appropriately in volume and diameter but maintains a uniformly rounded contour without the usual deep indenta-

tion (Figs. 9, 10). The basal polysomal collection, which in control Purkinje cells has invariably been dispersed by 15 days, is often retained in part in nervous cells (Fig. 10). The increase in the number of membrane-bound polysomes and in the degree of stacking of cisternae of rough endoplasmic reticulum is less obvious than in normal cells (Fig. 5). By 19 days, some nervous Purkinje cells have clearly entered into the sequence of pathological changes which will ultimately terminate in the degeneration of the cell (Fig. 6). The flattened sacs of the Golgi complexes appear to fragment, then swell into round, empty vesicles, scatter in clusters rather than lie in stacks, and finally disappear. Cisternae of rough endoplasmic reticulum decrease in number, also cease to form stacks, and instead are usually found closely associated with mitochondria and frequently appear filled with an amorphous material. The patterned groups of polysomes are replaced by free ribosomes (Fig. 17). Autophagic vacuoles may develop and mitochondria often appear enveloped by isolated cisternae of rough endoplasmic reticulum. Even after extensive cytoplasmic changes, the nucleus remains relatively unaffected. At the end of its foreshortened life, the cell abruptly condenses and the debris is removed by phagocytic cells. Although this sequence of changes appears relatively constant, the initiation of the disorganization and depletion of cytoplasmic membrane systems which precede cell death is not synchronous among adjacent cells. At 29 days, a cell just beginning to fail may be present in a region of the cerebellum from which other Purkinje cells have already disappeared.

At all ages Purkinje cells are present which retain a normal though reduced complement of Golgi complexes, endoplasmic reticulum, and polysomes (Fig. 7). In mature nervous mice, the perikarya of the few remaining Purkinje cells can be distinguished from normal cells only by the unusually numerous lipofuscin granules (Fig. 8). It seems likely that these survivors are the cells whose membrane systems have remained relatively intact throughout.

Nervous Purkinje cells begin normally to construct a dendritic arbor. At 9 days a primary dendrite with several major branches is evident and the smaller processes, marked by mitochondria, occur in appropriate numbers. In normal cells, additional large branches and a myriad of finer processes would appear during the next 2 wk but in nervous mice there is a paucity of mitochondria-laden proc-

esses at 12 days and 3 days later they are virtually absent. The dendritic arborization of nervous Purkinje cells apparently never attains the extensive and intricate pattern of branching observed in normal animals. Before the cells have begun to degenerate, the tree typically consists of a main dendritic trunk which extends to the upper reaches of the molecular layer with relatively few branches (Fig. 10). In adult nervous mice, major dendritic branches and mitochondria-filled processes are present external to the perikarya of the rare surviving Purkinje cells but even these appear more limited in extent than their counterparts in control mice. Although only a few Golgi impregnations have been studied, they confirm the impression of a reduction in the amount of dendritic branching. The branches which are present, however, are appropriately encrusted with spines.

During the period in which developmental aberrations of the dendritic arbor become manifest, typically degenerative changes become apparent also in the Purkinje cell axon collaterals. Very rarely at 9 days, but then with increasing frequency, segments of swollen axons which contain microtubules and larger membranous tubules in a dark, sometimes fibrillar axoplasm are seen in the cerebellar cortex. (The characteristic morphology of these profiles permits recognition of ingrowing collateral fibers in nervous mice before the formation of the synaptic boutons which are necessary for their identification in normal animals.) Usually the collateral profiles in nervous mice fail to demonstrate synaptic specializations, but some do achieve synaptic contacts and contain small collections of flattened vesicles clustered near discrete membrane densities. After 12 days, a small number of normal axon collateral boutons are seen, some of which may degenerate later (Fig. 11).

As nervous Purkinje cells are inadequately elaborating their dendritic and axonal collateral components, they do receive all the major classes of syn-

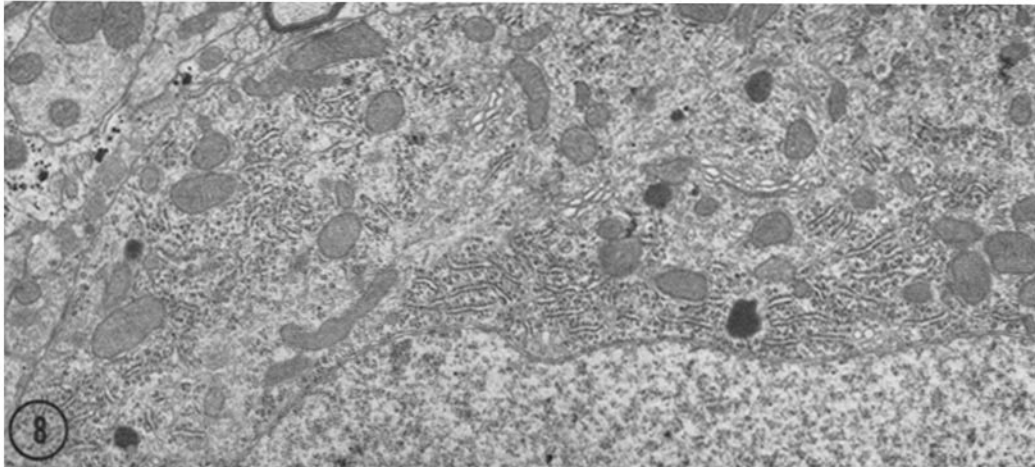
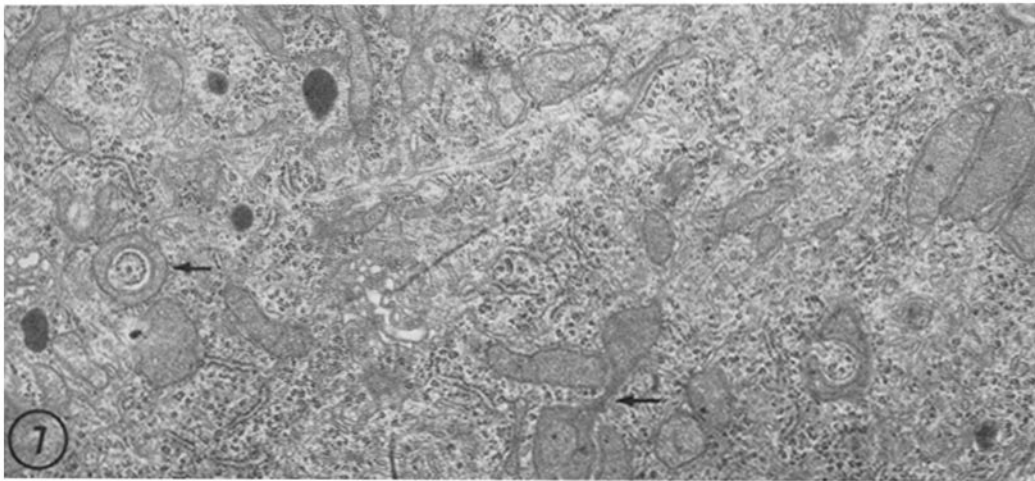
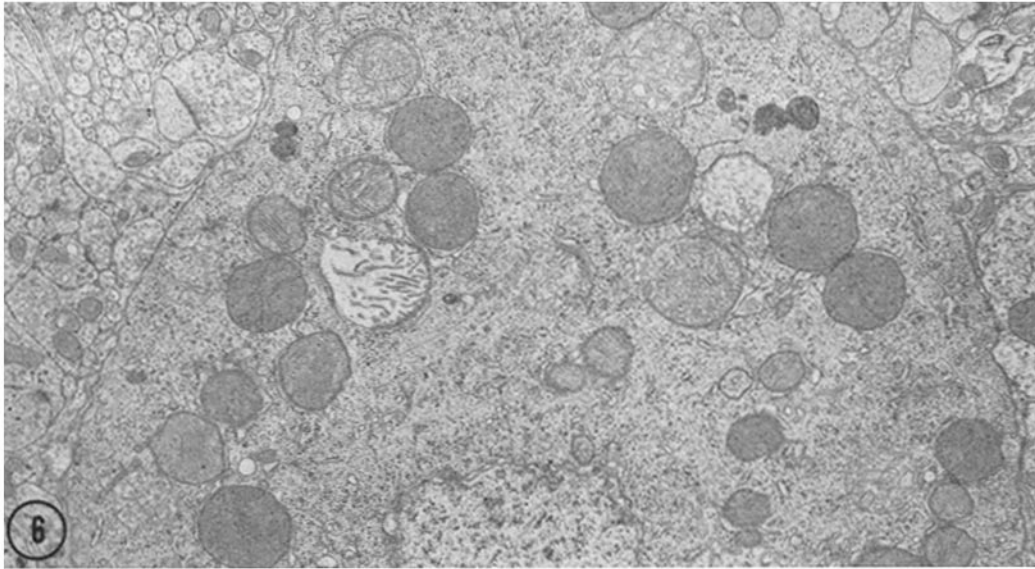
aptic contacts. At 9 days, normal climbing fiber synapses are found primarily on somatic spines. A few are also present in the lower reaches of the dendritic tree and during the next 2 wk increasing numbers of climbing fiber synapses are seen on dendritic processes (Fig. 11). Although the climbing fiber-somatic spine synapses have largely disappeared from the somata of all normal and many nervous Purkinje cells by 19 days, they tend to be maintained by those nervous cells which show signs of pathology relatively early. Characteristic synapses are made on the somata of all nervous Purkinje cells by basket cell axons and these remain intact until the final stages of degeneration (Figs. 6, 7, 12). Parallel fiber synapses are present at 9 days on normal-appearing dendritic spines and can be seen much later still in synapsis with condensed fragments of dendrite (Fig. 13). Stellate axons are also observed on dendritic processes from 12 days on but are less common because of the reduced arbor. No quantitative analysis of synaptogenesis has been made but nervous Purkinje cells appear to receive a normal complement of synaptic contacts at the appropriate time.

Although extensive aberrations exist in perikaryonal, dendritic, and axonal development, the most characteristic morphological difference between nervous and normal Purkinje cells concerns the shape of the mitochondria. At 9 days, the cerebellum of a nervous mouse can be clearly distinguished with the light microscope from that of a control littermate by the presence of discrete, rounded, densely stained mitochondria in the perikarya of some Purkinje cells. The cells which contain rounded mitochondria are found in groups whose occurrence seems to be independent of position in the folium and proximity to capillaries. The frequency of affected cells decreases laterally across the cerebellum and in most regions of the hemisphere all of the Purkinje cells appear normal at this age. Cerebellar cortex from two younger

FIGURE 6 Supranuclear region of a 19-day nervous Purkinje cell. Rounded mitochondrial profiles, some appearing to be healthy and others clearly degenerating, are present in a perikaryon otherwise virtually devoid of cytoplasmic membranes. $\times 9,000$.

FIGURE 7 Perinuclear region of a 23-day nervous Purkinje cell. Numerous bizarre mitochondrial profiles are seen (arrows). $\times 9,000$.

FIGURE 8 Perinuclear region of a 10-mo nervous Purkinje cell. Elongate mitochondria, abundant rough endoplasmic reticulum, and lipofuscin granules are present. $\times 9,000$.



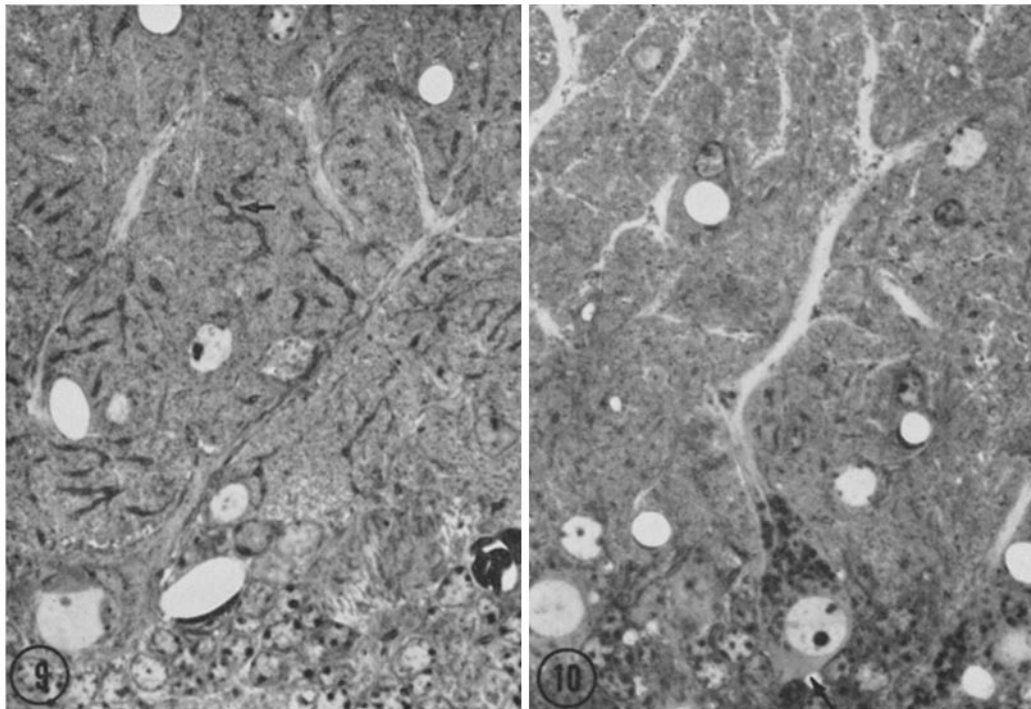


FIGURE 9 Somata and part of dendritic arbor of 19-day normal Purkinje cell. Arrow indicates mitochondria-filled processes. 1 μ m section.

FIGURE 10 Somata and part of dendritic arbor of 19-day nervous Purkinje cell. Small dark profiles of dendritic processes are missing while the soma is filled with spherical mitochondria. Clear region (arrow) retained basal polysomal mass. 1 μ m section.

mice, chosen for their small body size, was examined with the light and electron microscopes and was found to be indistinguishable from that of control mice. Possibly neither animal was actually a nervous mouse. The presence, however, at 9 days of relatively unaffected areas in the hemisphere, which lags behind the vermis developmentally, suggests that change in mitochondrial shape in the second postnatal week may be the first morphological indication of the mutant phenotype.

Examination of nervous Purkinje cells with the electron microscope at 9 days verifies the variation in mitochondrial shape observed with the light microscope. Some cells retain a normal complement of small, circular, and long, ellipsoidal profiles while others display almost exclusively rounded profiles, with an average diameter of 0.97 μ m (Fig. 3). In other respects the appearance of the mitochondria is normal. Numerous tubular and lamellar cristae and a few granules are present in a matrix of nor-

mal density. The relationships with vesicles of both the rough and smooth endoplasmic reticulum are identical to those observed in normal Purkinje cells.

Most Purkinje cells contain rounded mitochondria by 12 days (Fig. 12). The profiles remain somewhat diverse in shape, but if not circular they are uniformly shorter and thicker than those in control cells. A greater percentage of the perikaryonal area appears to be occupied by mitochondrial profiles in nervous cells than in control cells, although numerically fewer profiles seem to be present in a given section. Fig. 14 presents an analysis of the percent cytoplasmic volume occupied by mitochondria and shows the average mitochondrial volume for nervous Purkinje cells to be 1.5 times that for normal cells. Mutant cells do demonstrate greater variability, however, and some values fall within the normal range.

At 15 days, large, circular mitochondrial profiles are present in all Purkinje cells. To eliminate

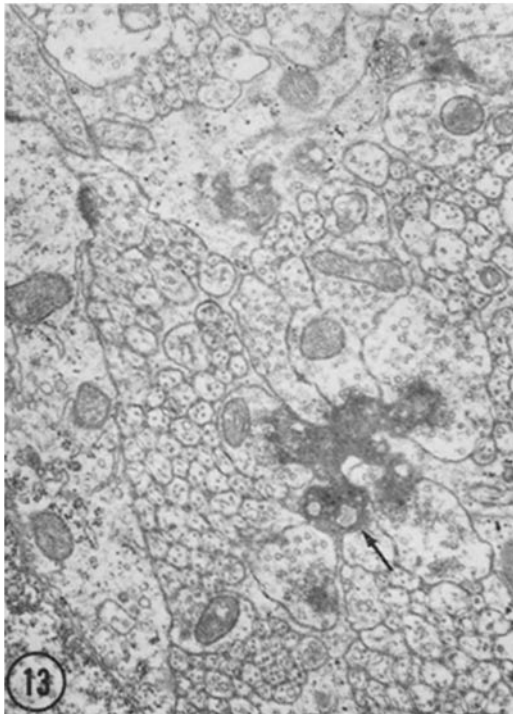
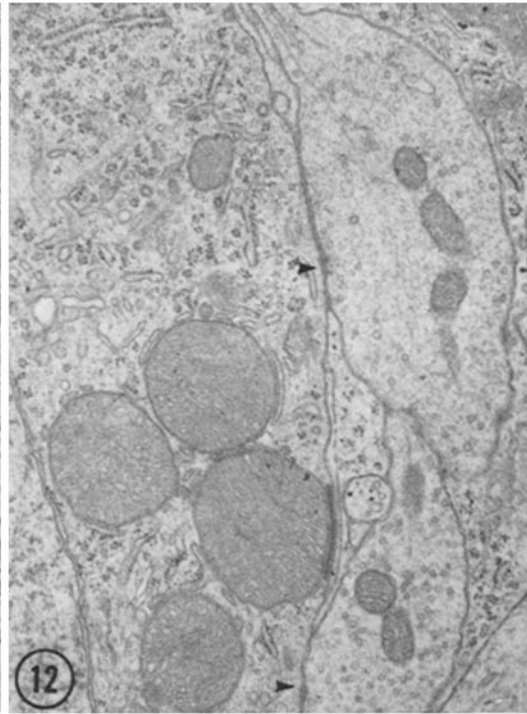
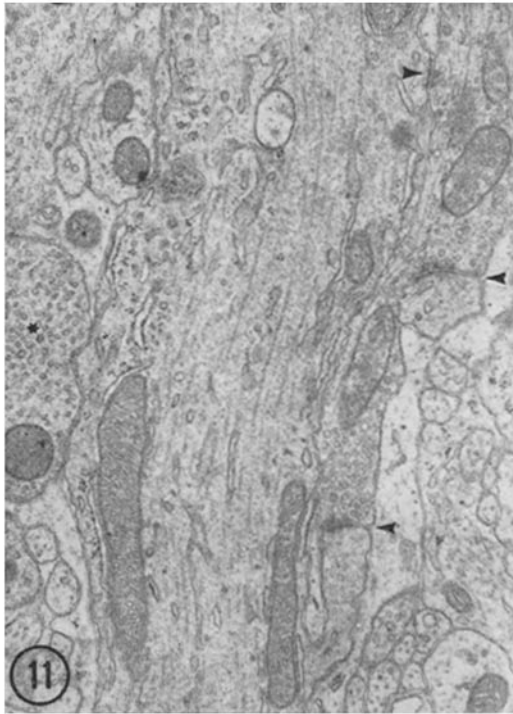


FIGURE 11 Dendritic segment of 15-day nervous Purkinje soma shown in Fig. 5. A climbing fiber makes several contacts with the trunk and spines (arrowheads). (*), normal axon collateral. Polysomal clusters and elongate mitochondria are present. $\times 18,000$.

FIGURE 12 Perikaryon of a 12-day nervous Purkinje cell. Basket cell axon contacts are indicated by arrowheads. Note round mitochondrial profiles with numerous tubular cristae. $\times 18,000$.

FIGURE 13 Debris from Purkinje cell dendrite in the molecular layer of 26-day nervous mouse. Synaptic specializations of parallel fibers are still present (arrow). $\times 18,000$.

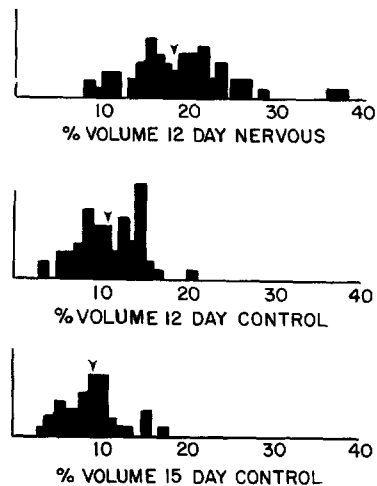


FIGURE 14 A plot of the percent perikaryonal volume occupied by mitochondria in 12-day nervous and normal and 15-day normal mice. Each block represents a single count. Arrows indicate median values. The 15-day percent is included to indicate the apparent decrease in mitochondrial volume in normal mice.

the possibility of biased selection, 110 consecutive Purkinje cells along two folia in the vermis were examined. Although approximately one in six of these cells will escape degeneration, each one contains large rounded profiles. The majority of these profiles are perfectly circular with an average diameter of $1.17 \mu\text{m}$ and are best interpreted as sections through spheres. If one assumes that they are spheres, then each mitochondrion has a volume of about $0.74 \mu\text{m}^3$. A few cells (less than 5%) contain, in addition, a significant number of small circular and ellipsoidal profiles. These latter are likely to represent not only tangential sections through spherical mitochondria but also sections, perpendicular to the long axis, through mitochondria that have retained an elongate shape.

As sweeping changes in the appearance of the cytoplasmic membrane systems begin to occur, the internal appearance of the mitochondria becomes clearly different from that in normal Purkinje cells. Increases in the length and number of cristae are apparent and in some mitochondria the cristae occur as closely stacked lamellae, some of which may be continuous across the mitochondrion (Fig. 6). In other mitochondria, cristae may appear as a random tangle of tubules (Fig. 16). The matrix often seems slightly denser than usual. At 19 days those cells with initial changes in cytoplasmic membrane systems show also a wide spectrum of

pathological modifications of mitochondria. In some mitochondria both the inner and outer membranes are intact but the amount of inner membranes is definitely increased (Fig. 6). In others, discontinuities exist in the outer membrane and angular cristae enclose a swollen inner compartment (Fig. 17). Still other mitochondria have become surrounded by additional membrane with the resultant formation of an autophagic vacuole. Ultimately, as the Purkinje cells degenerate, only dark ghosts of mitochondria remain faintly visible in the cellular debris.

The characteristically large, circular mitochondrial profiles appear to be confined to the Purkinje cell perikaryon. Occasionally, they are observed in myelinated axonal segments or in the primary dendritic stalk, but they are not seen beyond the first branch. The mitochondrial profiles in the major dendritic processes remain elongate until about 19 days (Fig. 11). Then numerous spheroidal profiles appear which are much smaller than those of the perikaryon and which may result from fragmentation of mitochondria already present within the processes (Fig. 10). In general, from 12 days on, fewer mitochondria seem to be present in the major branches and, as noted earlier, the small dendritic processes that they normally would mark are missing.

In the rare Purkinje cells which remain in the adult nervous mouse, mitochondrial profiles are not large and spheroidal as in earlier stages, but instead they are similar, if not identical, to those of normal Purkinje cells (Fig. 8). Very rare exceptions exist and these cells seem to have postponed, rather than circumvented, degeneration. Purkinje cells which contain mitochondrial profiles apparently in transition from large spheres to long cylinders are first evident at 23 days (Figs. 7, 18). Unlike mitochondrial profiles of normal or most nervous Purkinje cells, these profiles suggest no ready transition into a three-dimensional solid of simple form, but instead suggest a complex process of mitochondrial elongation and possibly division. Some mitochondria contain complex arrangements of inner membranes (Fig. 7). Other mitochondria, clearly separated from each other by closely intercalated cisternae of smooth endoplasmic reticulum, lie in spherical aggregations (Figs. 7, 18). Still other elongate mitochondrial profiles with extremely attenuated midregions resemble dumbbells or doughnuts (Fig. 7). Invariably, cisternae of endoplasmic reticulum are present in close association.

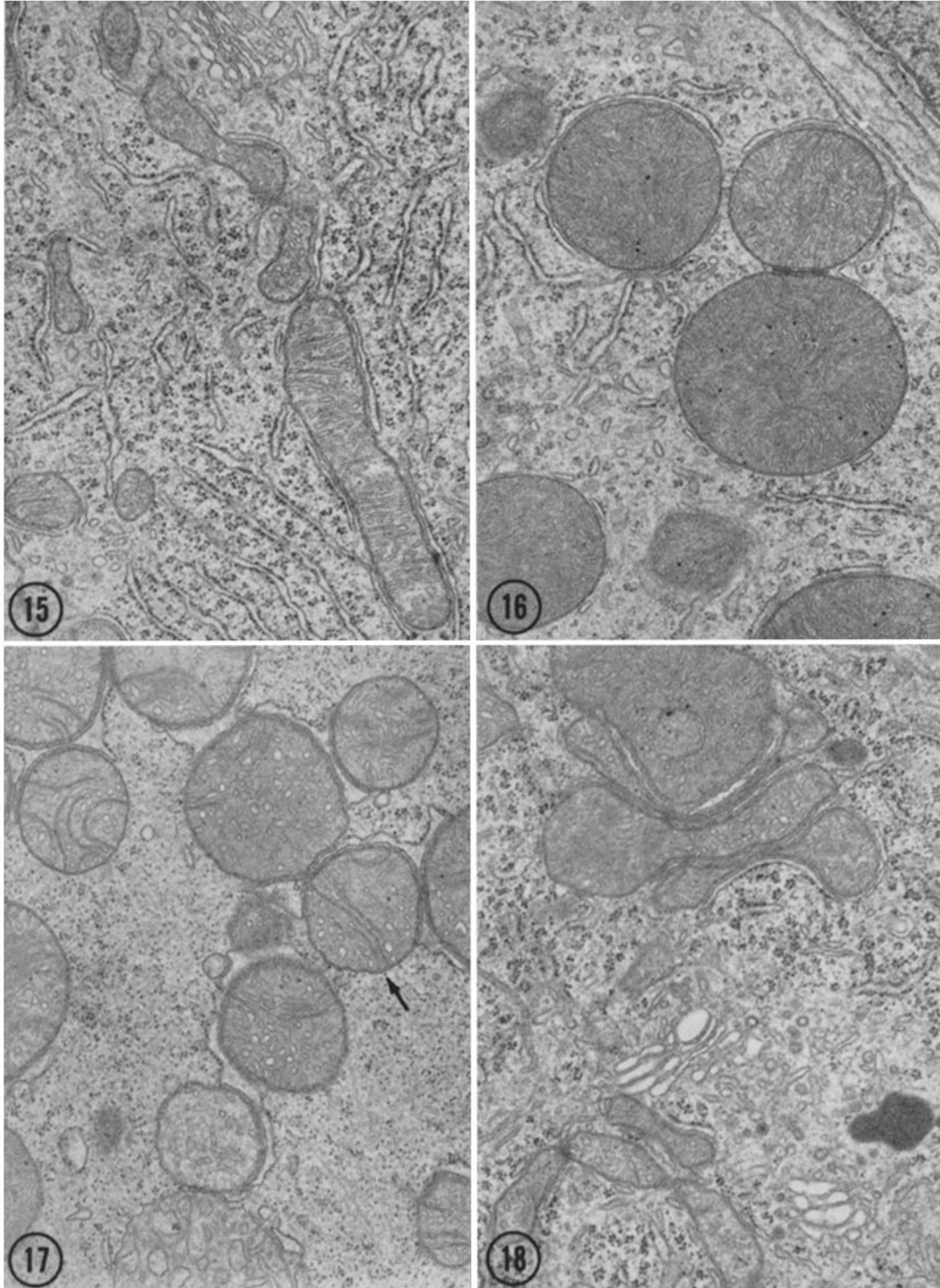


FIGURE 15 12-day control Purkinje cell perikaryon. The string of profiles at the left may represent a single mitochondrion. $\times 20,000$.

FIGURE 16 15-day nervous Purkinje cell perikaryon. Intramitochondrial granules and both tubular and lamellar cristae are seen. $\times 20,000$.

FIGURE 17 19-day nervous Purkinje cell perikaryon. Swelling has occurred, and some cristae extend across the mitochondrion (arrow).

FIGURE 18 23-day nervous Purkinje cell perikaryon. Recovering mitochondria still with unusual shapes are intercalated with cisternae of smooth endoplasmic reticulum. Numerous polysomal clusters and a small Golgi complex are present. $\times 20,000$.

Somewhat similar changes in mitochondrial morphology have been observed occasionally in other classes of large neurons in nervous mice (Landis, manuscript in preparation), but only Purkinje cells have shown the changes in cytoplasmic membrane systems and have gone on to die. Sidman and Green (1970) noted that in restricted regions of the cerebellar hemispheres in older nervous mice, many neurons other than Purkinje cells had disappeared. A similar loss has been noted in the course of this study, but it occurs later than the Purkinje cell loss, without the associated cytological changes, and as they suggested, is probably secondary.

DISCUSSION

The initial description of the nervous mutation by Sidman and Green (1970) indicated that the gene selectively causes the death of most Purkinje cells. One might have predicted that the mutant gene affects some unique characteristic of the Purkinje cell, perhaps a certain class of synapse. Instead, the first morphologically observable difference between nervous and normal Purkinje cells is expressed in an organelle common to all cells, the mitochondrion. There is presently no evidence of Purkinje cell abnormality before the observed rounding up of mitochondria and the cells do receive all classes of synaptic contacts at the correct time and in apparently normal numbers. The recently described noradrenergic input from the locus coeruleus (Olson and Fuxe, 1971; Bloom et al., 1971) has not been studied.

The change in mitochondrial shape is not necessarily a prelude to degeneration since all Purkinje cells demonstrate aberrant spherical mitochondrial profiles but not all go on to degenerate. Furthermore, a preliminary survey indicates that a similar mitochondrial abnormality occurs in some other postnatally maturing neuronal populations with no subsequent evidence of cell death (Landis, unpublished observations).

It is likely that the abnormal spherical mitochondria arise by conversion of the normal elongate mitochondria already present in the perikaryon. Intermediate shapes are seen and there is no evidence that elongate profiles degenerate as spherical ones appear. Moreover, there is a close correspondence between the volumes estimated for nervous spherical and normal elongate mitochondria. If the volume is unchanged during the transformation from a cylinder to a sphere, then changes

must occur in the surface area of the mitochondrial membranes. For example, the surface area of a cylinder with an axial ratio of 8:1 (as observed for normal mitochondria in the dendrite where alignment permits accurate determination of length) is about 1.7 times greater than that of a sphere of equivalent volume. The fate of the membrane made redundant by the shape change is unclear. Presumably superfluous inner membrane could fold inwards, and in the spherical mitochondria, the cristae do appear longer and more numerous than in the elongate mitochondria. No such obvious mechanism exists for the disposal of excessive outer membrane.

Approximately 10% of the Purkinje cells do not degenerate, despite the fact that at 15 days after birth, all demonstrate spherical mitochondria. At subsequent stages most cells display slowly progressive degenerative changes in rough endoplasmic reticulum, Golgi complexes, and polysomes, but a small percentage of Purkinje cells in nervous mice retain a near normal, albeit reduced, complement of cytoplasmic membrane systems. Presumably these are the cells destined to survive. Their spherical mitochondria apparently revert to the normal shape so that by 3 mo their somata are distinguishable from those of normal cells only by a slightly greater number of lipofuscin granules. During the reversion from spherical to normal elongate form, the mitochondria present a more complex series of morphological images than during the initial transformation. This difference may reflect in part the generation of additional mitochondria. In the recovery phase, mitochondrial profiles are invariably associated with cisternae of the endoplasmic reticulum. Similar apposition of membrane profiles to dividing mitochondria have been described during early liver regeneration (Claude, 1965) and in neonatal hepatocytes (Stempak, 1967). The significance of this association is unclear but biochemical studies have suggested that many mitochondrial proteins are synthesized outside of the mammalian mitochondrion (review by Wilkie, 1970). Isolated mitochondria, for example, do not make outer membrane proteins (Neupert et al., 1967) and transfer of newly synthesized proteins (Kadenbach, 1967 *a*), in particular cytochromes *c* and $a + a_3$ (Kadenbach, 1967 *b*, 1971), from microsomes to mitochondria has been demonstrated *in vitro*.

Changes in mitochondrial shape have been reported in a variety of experimental systems al-

though in none of these are the results entirely analogous to the changes in nervous mice. Spherical mitochondria of greatly increased volume have been induced in hepatocytes by essential fatty acid deficiency (Wilson and Leduc, 1963), riboflavin deficiency (Tandler et al., 1968), and cuprizone poisoning (Suzuki, 1969). Iron deficiency (Dallman and Goodman, 1971), cortisone treatment (Wiener et al., 1968), azo dye poisoning (LaFontaine and Allard, 1964), and breathing pure oxygen (Schaffner and Felig, 1965) induce smaller increases in volume, changes in the number of mitochondria, and acquisition of bizarre shapes. These manipulations take effect over a relatively long time, cause at least a threefold volume increase, and seem to involve either fusion or growth coupled with a failure of division. In contrast, the change in mitochondrial shape in nervous Purkinje cells occurs over the course of a few days and with no evidence of fusion or volume change.

Somewhat closer parallels are found in studies of insect fat body cells. In *Calopedes* mitochondria change from filamentous in the larvae to spherical in the adult (Larsen, 1970). This transformation, however, involves a complex sequence of events in which some larval mitochondria autolyze and others undergo several divisions to yield the adult complement of spheres. Wigglesworth (1967) reported that during severe and prolonged starvation of *Rhodnius* larvae, the filamentous mitochondria of the fat body first become short and rodlike and then spherical. After feeding, the spherical mitochondria become elongate again. Elongation may be prevented by anoxia. It seems clear that changes in either mitochondrial function or metabolic state are reflected in changed morphology and this may be true also for nervous Purkinje mitochondria.

Dumbbell-shaped profiles like those observed in recovering Purkinje cells have been described in regenerating hepatocytes (Claude, 1965), in hepatocytes recovering from cuprizone intoxication (Tandler and Hoppel, 1971), and in cells of rapidly growing *Neurospora* (Hawley and Wagner, 1967). Stempak (1967) has demonstrated in serial sections of neonatal rat liver that the dumbbells may be sections through disks. In general, these profiles are interpreted as mitochondria dividing by attenuation of the midpiece, although Wigglesworth described similar biconcave disks as a stage in mitochondrial elongation after feeding starved *Rhodnius* larvae (1967).

Some spherical mitochondria in the nervous mutant possess unusual arrangements of inner membranes that resemble forms previously described. Cristae which are continuous across the entire mitochondrion have been observed in liver cells under various abnormal metabolic conditions (Schaffner and Felig, 1956; Dallman and Goodman, 1971; Rohr et al., 1971; Tandler et al., 1969), in cardiac cells (Tandler and Hoppel, 1972), and in maturing insect fat body cells (Larsen, 1970). Such mitochondria have been termed "partitioned" by Tandler and have come to be interpreted as an early stage in mitochondrial division, to be followed by the formation of a furrow in the outer membrane and fission of the organelle. Many partitioned mitochondria are observed in both degenerating and recovering nervous Purkinje cells, but since no furrows were seen, these partitions may represent merely the infolding of excess internal membrane in the rounded mitochondria.

The conformational change from elongate to spherical may cause a loss of mitochondrial motility. In cinemicrographic studies of cultured cells (Lewis and Lewis, 1915) and of cultured nerve cells in particular (Godina, 1963; Pomerat et al., 1967), motile mitochondria are virtually always elongate and serpentine. Normally during the second postnatal week, large numbers of mitochondria, it is thought, are supplied by the Purkinje perikaryon to the growing dendritic arbor; presumably they migrate or are carried outward. Although no direct evidence is presently available to substantiate this postulated redistribution, ultrastructural observation of the perikaryon and growing arbor evoke a sense of outward flow (noted also by Altman, 1972). In cells of the mutant, the large spherical mitochondria are confined to the perikaryon and never appear beyond the initial dendritic segment. In addition, they occupy a greater percent of perikaryonal volume than in the normal cells, while the distal dendrites have a greatly reduced complement of mitochondria. These observations suggest that the change in mitochondrial shape has interfered with the outward movement of the mitochondria during the growth of the dendritic arbor. Mitochondria within the dendritic processes themselves retain an elongate form until quite late; only at 19 days do spherical mitochondria appear in any numbers in the arbor, and these profiles are much smaller than those in the perikaryon. They may arise from fragmentation of

mitochondria already present within the dendritic processes.

This study has not uncovered the primary action of the nervous genetic locus, but has delineated a series of morphological changes peculiar to a single cell population in the homozygous mutant animals. It is notable that these mitochondrial abnormalities first become evident when the Purkinje cell is attempting to build an enormous dendritic tree, elaborate a complex axonal plexus in both the cerebellar cortex and the deep nuclei, and enlarge and remodel its perikaryon. The unanswered question is whether the unusual change in mitochondrial shape is an expression of a gene-controlled metabolic abnormality that compromises the Purkinje cell at a time of great metabolic demand or whether it represents the response, possibly even a healthy response, of mitochondria attempting to meet the metabolic needs of a cell under some undefined gene-mediated stress.

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