METHYLALOXYMETHANOL-INDUCED ABERRANT PURKINJE CELL DENDRITIC DEVELOPMENT

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ABSTRACT

Purkinje cell dendrites develop with a specific orientation and relationship to related neurons and glia. Their dendritic spine postsynaptic membrane specialization may, in turn, require a permanent presynaptic contact by parallel fibers. To determine whether changes in the surrounding cells influence the normal development of the cerebellar Purkinje cell dendrites and spine specializations, destruction of the differentiating cell layer was induced in the postnatal mouse by administration of methylaloxymethanol acetate (MAM) (0.05 μl/gm body weight) at day zero. The Purkinje cells were examined by light and electron microscopy on the tenth postnatal day. The midsagittal surface area of the cerebellar vermis in treated animals was reduced by an average of 60%. MAM-induced granule cell depletion and Purkinje cell dislocation were observed by light microscopy. When compared to controls, examination following Golgi impregnation revealed random orientation of Purkinje cell apical poles, and multiple primary dendrites of reduced length with few branches, branchlets and spines. Vertical processes of Golgi epithelial (Bergmann) cells were obliquely directed, reduced in length and complexity in MAM-treated mice. Ultrastructural examination revealed naked Purkinje cell dendritic spine specializations in both groups. Although necrotic debris persisted in astrocytes and macrophages, degenerating presynaptic terminals were not found. This study suggests that permanent presynaptic contact by parallel fibers is not essential for spine development. Astrocytic reactions to injury, in association with the reduced folial expansion, may have contributed to the observed abnormalities and disorientation of the Purkinje cells. The data suggests that Purkinje cell dendritic development may be strongly influenced by changes in surrounding cells.

INTRODUCTION

The cerebellar cortex is an excellent site for the study of pathological alterations because of its histological homogeneity; the existing broad knowledge of its normal development; and the established delineation, morphologi-

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cally and functionally, of its synaptic architecture. In the postnatal mouse cerebellum, Purkinje cell dendrites develop with a specific orientation and relationship to adjacent growing neurons and Golgi epithelial (Bergmann astroglial) cells. The influence of the latter structures on the development of the Purkinje cell dendrites and their spine specializations has not been fully established. However, some studies have indicated that the differentiation of dendritic spine specializations located on the tertiary Purkinje cell dendrites may not require a permanent presynaptic contact by parallel fibers. For example, when parallel fiber reduction is secondarily produced by methylazoxymethanol glucoside (cycasin) or methylazoxymethanol acetate (MAM) (11, 12, 18) such naked Purkinje cell dendritic spine specializations have been observed. Similarly, the granule cell depletion seen in viral infection with feline panleukopenia (10, 22); the genetic mutants, Weaver (13, 34, 36) and Staggerer (15, 34); x-irradiation (2, 3); or tissue culture (20, 31) has been shown to result in naked Purkinje spine formation. These and other dendritic alterations have also been described in a number of human disorders (14, 16). Although it has been suggested that parallel and climbing fibers are important for Purkinje cell spine development (8, 21), the autonomous development of spine specializations in tissue culture suggests that neither the granule cell parallel fiber or climbing fiber contacts are an essential factor (20, 28, 31).

Other developmental alterations in Purkinje cell dendrites have not been consistently associated with reduction in parallel fibers in various experimental models. Shorter, thicker, Purkinje cell dendrites with fewer secondary and tertiary branches and branchlets, have been observed in cats and ferrets infected with feline panleukopenia (10), and rats x-irradiated at birth (2). A search of the literature reveals no theories suggesting the possible cause for these particular Purkinje cell dendritic alterations. On the other hand, Woodward et al. have suggested that the reduced folial expansion associated with granule cell depletion may have led to Purkinje cell dendritic abnormalities following postnatal MAM treatment of rats (39).

Because of the presence of naked Purkinje cell dendritic spine specializations in many models, factors affecting the development and durability of these and related elements are frequently questioned. This study attempts to determine in what ways the development of Purkinje cell dendrites, their spine specializations, and Bergmann astroglia are influenced by the destruction of the external differentiating cell layer. Ten day Swiss albino mice, Webster strain, were treated with MAM on the first postnatal day. The earlier electron microscopic characteristics of the reported aberrant synapses as well as alterations of the Purkinje cell dendrites and Bergmann astroglia following postnatal MAM treatment were specifically addressed.

**MATERIALS AND METHODS**

Random selection of members of six litters of newborn Swiss albino mice were divided into two groups. Each group was examined, weighed, marked

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1 Webster strain purchased from Spartan Animal Research Inc. Haslett, Michigan.
for identification, and injected with MAM (0.05 μl/gm body weight) or saline (0.05 μl/gm body weight). Both groups were housed in plastic shoebox cages; the cage environment was maintained with control of room temperature, humidity and standard light-dark cycle. At 10 days of age the mice were reweighed, and physically examined. Nembutal (0.05 mg/gm body weight) was used to anesthetize 16 mice from the two groups: the eight MAM-injected that had survived, and eight selected from the saline group. After exposing the heart by thoracotomy, heparin (300 units/animal) and sodium nitrite (0.01 ml/gm, 1%) were injected into the left ventricle. The mice were then sacrificed by a modified perfusion-fixation technique utilizing 0.5% paraformaldehyde, 1% glutaraldehyde in a 0.12 M phosphate buffer with 0.02 mM calcium chloride added (27). Three cerebellums from each group were used for Golgi impregnation in order to assess the Purkinje and Bergmann astroglial cells. A morphometric evaluation of sagittal sections of the vermis was made using the modified technique of Weibel et al. (38). Vermis sections from half of each cerebellum of the remaining five mice of each group were postfixed in 10% buffered formalin, embedded in paraffin, sectioned, and mounted slides stained in routine fashion with Harris hematoxylin-eosin (H&E) or Luxol fast blue-cresyl violet (LFB-CV) stains for light microscopy (32). Vermis sections from the other half of each cerebellum were postfixed for two hours in 2% osmium tetroxide using 0.12 M phosphate buffer with 3.5% dextrose as diluent. After rinsing in 0.1 M sodium acetate, they were stained en-bloc with 0.5% uranyl acetate for 30 minutes at 4°C. Graded alcohols were used for dehydration and the sections were embedded in Epon-araaldite. Sections of one micron thickness were stained with 1% toluidine blue for light microscopy. Thin sections (600-900 Å) were stained with lead citrate (1-2 minutes) and uranyl acetate (30-90 minutes) (30, 37). The Purkinje cell dendrites, granule cell parallel fibers, and other cellular elements and their synapses were qualitatively assessed with a Philips 201 electron microscope.

RESULTS

Physical Examination: There was no significant weight difference between the two groups at day zero (1.7 grams av.). Prior to perfusion on day ten, there was a significant weight difference between the groups (sig: .005 level); the control group weighed an average of 6.19 ± 0.58 grams, while the experimental group’s average weight was 5.22 ± 0.84 grams. As previously reported (7, 17) both groups had equal general activity, climbing abilities, and righting abilities. At day zero and ten, both groups exhibited head and body tremors. At day zero both groups were hairless; but at day ten all of the control group had smooth white hair, while only one of the experimental group exhibited hair growth. As is normal for this age group, all of the mice positioned their legs laterally at day zero. But at day ten, three of the control group were maturely positioning their feet flat with legs tucked under, while the other five continued to position their feet laterally. In general, the three control group members with the mature foot position seemed better developed.

than their mates. All of the mice of the experimental group were still immaturely positioning their feet laterally and seemed less developed when compared to control mice (sig: .05 level).

Comparative gross examination of the cerebellum and brain revealed a reduction in cerebellar size for the experimental group (Fig. 1). The size reduction was confirmed by measuring the surface of sagittal sections of the vermis. Five animals of the control group had an average vermis surface area of $4.91 \pm 0.52 \text{ mm}^2$; whereas, eight animals of the experimental group had vermis sagittal surface areas of $1.98 \pm 0.51 \text{ mm}^2$. This 60% vermis surface area reduction in the experimental group is significant at the .005 level.

Light microscopic examination of many areas of the cerebellum from the experimental animals showed that the external differentiating cell layer and the granule cell layer could not be specifically identified (Fig. 2, 3). In the vermis of the experimental group, Purkinje cell somata were randomly located within the molecular and internal granule cell layers. Their dendritic trees were partially visible in both molecular and granule cell layers of the sagittal plane (Figs. 4, 5). Qualitatively, both groups at day ten seemed to have Purkinje somata of equal size. The Purkinje cell apical poles of the experimental group were randomly oriented (Fig. 5). Cells of the external differentiating cell layer were totally absent or severely diminished in the experimental group. Compared to the normal cerebellar internal granule cell layer, many lobules of the experimental animals appeared to have fewer granule cells (Figs. 2, 3, 4, 5). The differentiated granule cells of both groups appeared equal in diameter. Golgi impregnation studies of the experimental group revealed many massive processes protruding from each Purkinje cell soma exhibiting characteristics of primary dendrites, with diameters larger than normal (Figs. 6, 7, 8). There were fewer secondary and tertiary dendritic branches and branchlets. And these dendrites had a three dimensional orientation as compared to the two dimensional orientation of the normal Purkinje cell (Fig. 6). Comparatively, the dendrites of the experimental group were 30–40 $\mu$m long, whereas, those of a normal 10 day Swiss albino mouse would be 60–80 $\mu$m. Because of the reduced number of secondary and tertiary branches and branchlets of the experimental group, each dendritic arborization contained fewer than normal spines. In the experimental mice: the lateral processes with warty excrescences located on the vertical processes of the Bergmann astrocytes were greatly reduced or absent; the lengths of the vertical processes appeared to be reduced, and thus the Bergmann astrocyte somata were located nearer to the pial-glial membrane when compared to normal (Figs. 9, 10, 11). Engulfment of necrotic cellular debris by these cells was observed.

Ultrastructural examination of the molecular layer of control mice revealed parallel fibers, Purkinje cell dendrites, and Bergmann astroglial processes (Fig. 12). Numerous mature parallel fiber-Purkinje cell contacts surrounded by a Bergmann astroglial matrix were also observed (Fig. 12). In some areas of the molecular layer in experimental mice the parallel fibers were absent and the Purkinje cell dendrites had naked spine specializations
Fig. 1. A comparison of areas and shapes of sagittal sections through the cerebellar vermis of eight 10 day mice injected with MAM (top two rows) and five 10 day mice injected with saline (bottom two rows) shows a 60% reduction in surface areas secondary to MAM-induced differentiating cell loss. × 6.5.
Fig. 2 and 3. Two sagittal sections from 10 day control (Figure 2) and MAM-treated mice (Figure 3) show the differences between the cerebellar vermis morphology. Note the reduction in size, lack of an external differentiating, granule and Purkinje cell layers as well as the simplicity of folia in the MAM-treated mouse cerebellar vermis. The loss of cells following MAM-treatment is not always this complete. Paraffin embedded, 10 μm sectioned, hematoxylin and eosin stained. × 22.

Fig. 4. The random array of Purkinje cell somata (PC) in the molecular and reduced internal granule cell layers in a 10 day MAM-treated mouse cerebellum (Figure 5) contrasts sharply with the orderly arrangement of the external differentiating cell (EL), molecular (ML), Purkinje cell (PL) and internal granule cell layers (IGL) in control mice (Figure 4). Also notice the absence of a portion of the external differentiating cell layer as well as disorientation of the Purkinje cell apical poles in Figure 5. Epon-araldite, 1 μm sectioned, toluidine blue stained. × 390.
Fig. 6. This is a montage of a Golgi impregnated Purkinje cell soma and its single dendritic tree in a saline-treated mouse cerebellum. A through focus examination of this tissue section revealed a single dendritic tree with a main primary dendrite and remnants of one or possibly two other primary dendrites passing from the Purkinje cell soma. Three Purkinje cell somata are present (PC); one is impregnated and two others are not (small arrows). Spines are observed on branches of dendrites (box). External differentiating cell layer, EL. Rapid Golgi impregnation technique, 75 μm sectioned. × 360.

Figs. 7 and 8. Two Purkinje cell somata from MAM-treated mice with many massive primary dendrites (arrows) exhibit fewer secondary and tertiary branches and branchlets with spines (box). The disorientation of two Purkinje cell somata (PC) and their dendrites in 10 day MAM-treated mice is illustrated. Notice the variability in the distance of Purkinje cell somata (PC) from the meninges (Me) and their dendritic distribution in three dimensions: Axon, AX. Rapid Golgi impregnation technique, 75 μm sectioned. × 360.

Figs. 9, 10, and 11. Normal Golgi epithelial (Bergmann astroglial) cells (GEC) give off lateral processes with warty excrescences (arrows) on the vertically directed processes as they pass towards the pial-glial membrane (Figure 9). In the MAM-treated mice, the lateral processes are either greatly reduced in complexity or virtually absent (Figures 10 and 11). The lengths of the vertical processes in cerebells of MAM-treated mice (Figures 10 and 11) appear to be reduced when compared to the normal (Figure 9). Rapid Golgi impregnation technique, 75 μm sectioned. × 418.
Fig. 12. This electron micrograph from the middle of the molecular layer from a 10 day control mouse illustrates the relatively electron dense Purkinje cell dendrite (Pc d) surrounded in part by Bergmann astroglia (GL) and parallel fibers (pf). Notice the numerous parallel fiber-Purkinje cell dendritic spine synapses (PS) each surrounded in part by a Bergmann astroglial...
surrounded by a glial matrix (Figs. 13, 14). Naked Purkinje cell dendritic spines with postsynaptic thickenings were also identified in each of the control group mice (Figs. 15, 16, 17).

Necrotic cellular elements persisted within the external differentiating cell layer and molecular layer; and they appeared to be engulfed by astrocytes or differentiating cells as previously reported (18). However, no evidence of degeneration of presynaptic terminals was seen in either group.

DISCUSSION

The weight gain, tremor, growth of hair, opening of the eyes, and the proper positioning of the feet reveal the gradations of maturity in Swiss albino mice. Therefore, in this experiment at 10 days, the experimental mice appeared more immature than their control littermates. Histological changes within the cerebellum in experimental mice reflect a specific action on the dividing cells in this location, but may be partly attributed to the effects of the agent on growth and development.

Sagittal sections of the vermis in eight experimental and five control Swiss albino mice at 10 days of age when morphometrically measured, revealed a 60% reduction in the sagittal area of the vermis in the experimental mice. Less reduction of surface area was seen in a similar experiment (39). The variability of the reductions in the midsagittal area of the vermis appears to be due to the dosage and sequence of MAM injections.

Not only was there a reduction in the cross sectional area of the vermis at 10 days in the experimental mice, but the normal histological architecture was altered. The observed dislocation of Purkinje cell somata within the molecular and internal granule cell layers may be explained in a number of ways. For example, elimination of external differentiating and internal granule cells interrupts the development of the external differentiating cell layer and precludes the folial expansion that provides sufficient area for the normal Purkinje cell layer to develop (3, 10, 29, 39). Norman (25) hypothesized that Purkinje cell disorientation observed in cases of congenital cerebellar hypoplasia is correlated with the absence of granule cells or their abnormal migration. He also implicated the role of astrocytes in the misalignment. Recently, Jones and Gardner (19) suggested that the glial reaction has a more

matrix. Uranyl acetate en-bloc stained, Epon-araldite embedded, grids stained with uranyl acetate and lead citrate. × 7650.

Figs. 13 and 14. Purkinje cell dendrites (Pc d) with naked spines (NS) surrounded by a glial matrix (GL) are observed in the molecular layer of 10 day MAM-treated mice. Naked spines have postsynaptic thickenings (arrows) contacted by Bergmann astroglial matrix (GL) rather than parallel fiber presynaptic varicosities. Uranyl acetate en-bloc stained, Epon-araldite embedded, grids stained with uranyl acetate and lead citrate. 13. × 17,000. 14. × 25,500.

Figs. 15, 16, and 17. Naked Purkinje cell dendritic spines with postsynaptic thickenings surrounded by Bergmann astroglial matrix were occasionally observed in all 10 day control mice. These figures illustrate naked spines (NS) with postsynaptic spine specializations (arrows) surrounded by a glial matrix (GL) with parallel fibers (pf) in the vicinity. No differences from naked spines seen in MAM-treated mice are apparent. Figure 17 illustrates a naked Purkinje cell dendritic spine cut in cross section surrounded by an astroglial matrix next to another spine that is in synaptic contact with a parallel fiber varicosity. Uranyl acetate en-bloc stained, Epon-araldite embedded, grids stained with uranyl acetate and lead citrate. 15. × 22,500. 16. × 45,000. 17. × 30,000.
definitive role than absence or abnormal migration of granule cells, because
of the rapidity with which the misalignment occurs. They attributed the
Purkinje cell disorientation to the swelling of the closely associated astro-
cytes.

A plausible explanation for the random orientation of Purkinje cell apical
poles observes in our 10 day experimental mice has been offered by Altman
and Anderson (2). They observed that while the growth of the Purkinje cell
apical pole (the initial portion of primary dendrites) and dendrite appears to
be autonomous, the orientation of the apical pole may depend upon the
presence and location of the external differentiating cell layer. Destruction of
the external differentiating cell layer destroys this orientation (2).

In the absence of parallel fibers in an agranular cerebellum, dendritic
growth may spread from several early Purkinje cell somatic projections (39).
More than one Purkinje cell process may develop into a primary dendrite with
secondary and tertiary branches (39). In this research, deletion of the parallel
fibers resulted in the presence of Purkinje cell somata with many massive
primary dendrites. The dendrites that were present in experimental mice
were distributed in three dimensions rather than the normal sagittal plane.
It has been suggested that the shapes of the Purkinje cell dendritic branches
depend upon the positions of the cell bodies within the cerebellar cortex and
on the orientation of the apical pole (1, 2, 33, 39). We concluded that the three-
dimensional random orientation of the developing Purkinje dendrites
appeared to be due to the absence of the differentiating granule cells. Study of
the Purkinje cell dendrites over time may reveal progression toward normal
with maturity (2, 39).

As a result of MAM treatment, the growth and development of the Purkinje
cells appear to correspond to the maturity observed in six to eight day control
mice (23). The reduction in length of the Purkinje cell dendrites, the reduced
dendritic spine development, as well as the multiple primary dendrites
observed in 10 day experimental mice reflects abnormal maturation within
the cerebellar cortex. The permanence of some of these changes has been
suggested by previous studies. The abnormal maturation and development of
the Purkinje cell dendrites may result from the same phenomena responsible
for Purkinje cell dislocation. Destruction of the external differentiating cell
layer may interrupt the folial expansion, thereby providing less area for the
expansion of the Purkinje cell dendrites. The swelling of the Bergmann fibers
places further space limitations upon developing Purkinje cells and their
growing dendrites. The Purkinje cell dendrites are thus prevented from
achieving their full length and arborization by these two factors. The dendri-
tic arborization must be studied at later time intervals to determine if the
abnormally developed Purkinje cell dendrites persist or if they continue to
grow and develop into fairly normal dendrites.

With the reduction of external differentiating and internal granule cells in
experimental mice, changes are observed within the Bergmann cells and
their processes which may affect the development of closely related Purkinje
cells. Bergmann cells were observed within the outer portion of the inner granule cell layer giving off vertical processes that pass in an abnormally oblique course towards the pial-glial membrane. The lengths of the vertical processes appeared to be reduced and the lateral processes or warty excrescences, were greatly reduced or virtually absent in 10 day experimental mice. In 22 day old homozygous Weaver mice, the Bergmann fibers in the upper one-third of the molecular layer exhibited the typical cytoplasmic excrescences. The Bergmann fibers often passed in an oblique direction towards the pial-glial surface and did not show the straight and perpendicular orientation characteristic of these fibers (35). Their observations of Bergmann fibers were thus similar to those examined in this study of 10 day experimental mice. In the present study, it was difficult to assess whether the changes observed in the Bergmann fibers were direct or indirect effects of MAM treatment. Engulfment of necrotic debris by Bergmann fibers was also demonstrated within the external differentiating cell layer and has been previously reported by several investigators in both normal and experimentally induced cell necrosis (9, 19, 24). However, degenerating terminals were not identified.

The presence of naked Purkinje cell dendritic spine specializations surrounded by a Bergmann astroglial matrix has raised many questions pertaining to their development. Past investigators have assumed that the formation of pre- and postsynaptic membrane specializations have been preceded by the contact between the pre- and postsynaptic elements (4, 5, 21). Since then, naked Purkinje cell dendritic spine specializations have been shown to differentiate in the absence of parallel fibers. Among the pathogenetic factors leading to such aberrant synaptic development are radiation (2), viral infection (10, 22), gene mutations (13, 15, 34, 35), or MAM (11, 12, 18). Sotelo (36) suggested that not only postsynaptic densities, but presynaptic vesicular grids can apparently develop independently. Hirano and Dembitzer (15) also concluded that the dendritic spine specializations may be induced by some type of general stimulus. Cultures of mouse cerebellum incubated in the presence of MAM have produced Purkinje cell dendrites with naked spine specializations (6, 28). Besides the appearance of naked Purkinje cell dendritic spine specializations in pathologic conditions, naked postsynaptic sites have been reported in the normal differentiating olfactory bulbs (26) and in 14 day mice cerebella (21: bottom right figure of plate 8, page 815). Electron microscopic studies of cultures of normal mouse cerebellum have also revealed naked Purkinje cell dendritic spine specializations (6, 20, 31).

In the present study, the demonstration of naked Purkinje cell dendritic spine specializations surrounded by a Bergmann astroglial matrix, in both experimental and control 10 day mice, shows that the hypothesis that presynaptic contact is necessary for postsynaptic element development is limited. Finally, the absence of presynaptic terminal degeneration in either group of mice strengthens the hypothesis that Purkinje cell dendritic spine specializations do not require permanent presynaptic contact by parallel fibers for their development.
SUMMARY AND CONCLUSIONS

As in previous studies, the usefulness of MAM as an aid in the understanding of normal and abnormal cerebellar differentiation was documented (6, 11, 12, 17, 18, 19, 28, 33, 39). Dislocation of Purkinje cell somata was shown to be accompanied by random orientation of their apical poles and abnormalities of the Purkinje cell dendrites previously undescribed in 10 day MAM-treated mice. These alterations may have resulted from interruption of the folial expansion due to the paucity of external differentiating and internal granule cells (39). An additional important factor appeared to be the changes in the intimately related Bergmann astroglia themselves such as reduction in complexity and length of lateral processes and their abnormal orientation. Moreover, the swelling of the Bergmann fibers in response to injury may have placed significant constraints on the Purkinje cell somata and dendrites which led to both abnormal orientation and development (19). No evidence was found to suggest that postsynaptic spine specializations result from degeneration of presynaptic terminals in this or previous studies of the early postnatal period (19). Moreover, it was demonstrated that such differentiation occasionally occurs in the absence of disease. It was concluded that Purkinje cell dendritic development is influenced by changes in surrounding cells and that the factors leading to spine specializations remain to be clarified.

BIBLIOGRAPHY