REDUCED HISTONE LEVELS INDUCED BY "REELER" AND "WEAVER" MUTATIONS IN THE MOUSE CEREBELLUM

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SUMMARY: Five major protein bands present in the polyacrylamide gel electrophoresis pattern of normal cerebellum are apparently absent or decreased in amounts in both reeler and weaver mutant cerebellar tissues. All five bands were identified as histones and the deficiencies related to the decreased cerebellar cellularity produced by both mutations. These results, therefore, rule out an earlier suggestion that two of these proteins are granular cell specific proteins (1). Preliminary evidence for high levels of F1 histone in the nuclei of cerebellar cells, which appear to be reduced in the reeler syndrome, is presented.

INTRODUCTION

The reeler (rl) and weaver (wv) syndromes result from recessive neurological mutations in mice which, in the homozygous condition, produce abnormal organization of the cerebellum. In reeler mutants, post-mitotic cells fail to migrate to their correct destination, causing defective laminar organization. This is most marked in the cerebellum, where a disarrayed structure with a reduced granular cell layer develops (2-4). The defect in weaver homozygotes is exclusively cerebellar and results in complete loss of the cerebellar granular cells (5-7). These cells are by far the most numerous cell type in the cerebellum and are the smallest neurones known, consisting almost entirely of a large highly heterochromatic nucleus (8).

Previously Mallet et al. (1) have reported the absence of two major proteins from the cerebellum of homozygous weaver mutant mice. In the experiments described here, the major protein components of the two mutant cerebella are compared to those of normal littermates, and also to the protein components of other brain regions. The major deficiencies are shown to be identical in both mutants and to be due to greatly reduced levels of the five nuclear histones, as a result of the decreased number of granular cells produced in both syndromes. Similar effects are seen in other regions of the brain which show lower cell density than the cerebellum.

MATERIALS AND METHODS

Animals: Stocks of C57/BL strain mice heterozygous for the reeler mutation (rl/+) and for the weaver mutation (wv/+) are maintained at the National Institute for Medical Research. Homozygous reeler (rl/rl) and weaver (wv/wv) mutant mice were obtained from heterozygous matings. In both cases the mutant mice in a litter were recognised by the behavioural defects and changes in gross cerebellar structure characteristic of each mutation. Mutants and littermate controls were used at 15/16 days after birth.

Subcellular fractionation of cerebellum: All operations were performed at 4°C. Cerebella derived from control 15-day old mice were weighed, homogenized in 20 volumes of homogenizing medium (0.25 M sucrose, 25 mM KCl, 4 mM $MgCl_2$ and 50 mM Tris-HCl buffer pH 7.4) using 20 strokes in a motor-driven teflon-glass homogenizer. The homogenate was centrifuged at 850g for 10 min. in a Sorvall centrifuge to yield supernatant A and a crude nuclear pellet. The latter was resuspended in nuclear wash solution (0.32 M sucrose, 3 mM $MgCl_2$, 0.25% Triton-X100 and 1 mM Tris-HCl buffer pH 6.5), re-homogenized briefly and again subjected to low-speed centrifugation to yield a relatively clean nuclear pellet. After one further low-speed centrifugation (to remove residual nuclei and unbroken cells) supernatant A was centrifuged at 12,000g for 15 min. to yield supernatant B and a crude mitochondrial pellet. This was re-homogenized in homogenizing solution and a mitochondrial pellet prepared by re-centrifugation. Supernatant B was again subjected to centrifugation at 12,000g before centrifugation at 105,000g for 2 hrs. to yield a ribosomal pellet and supernatant C, termed the soluble protein fraction.

Partial purification of F1 histone: F1 histone was preferentially and completely extracted from nuclear pellets of cerebellar and hippocampal homogenates by homogenization and extraction twice with 10 volumes of high salt medium (1 M KCl, 50 mM $NaHSO_3$, 2 mM phenyl methyl sulphonyl fluoride (PMSF) and 20 mM Tris-HCl buffer pH 7.4). The first extraction was overnight, the second for 4 hrs. The supernatants obtained by centrifugation at 105,000g for 1 hr., were combined and protein precipitated by addition of an equal volume of 40% trichloroacetic acid (TCA).

Pure histone samples: Samples of pure pig thymus histones purified in Dr. E.W. Johns' laboratory (Chester Beatty Research Institute) and of pure calf thymus total and purified F1 histone fractions prepared in Dr. E.M. Bradbury's laboratory (Biophysics Laboratory, Portsmouth Polytechnical College) were a gift to Dr. D. Rekosh (National Institute for Medical Research), from whom they were obtained.

Alkaline phosphatase incubations: Aliquots of the pure histone samples were incubated with alkaline phosphatase overnight as described by Sherod *et al.* (9) with the addition of 2 mM PMSF to all solutions. Casein and vitellogenin were used as positive controls for alkaline phosphatase activity.

Preparation of samples for electrophoresis: Small tissue slices of cerebellum or hippocampus were completely solubilized by boiling in electrophoresis sample buffer, as were pellets derived from cerebellar fractionation or TCA precipitation of protein samples. Soluble protein samples were dialysed against sample buffer and boiled briefly before use.

Gel electrophoresis: Slab gel electrophoresis in 7.5%, 8% and 12.5% polyacrylamide gels containing the "SDS-disc" buffer system of Maizel (10) and Laemmli (11) was used. Proteins were stained with Coomassie Brilliant Blue. Parathyroid hormone (molecular weight (MW) 9,500), cytochrome c (11,700), ribonuclease (13,700) and globin (15,500) were used for MW determinations of protein

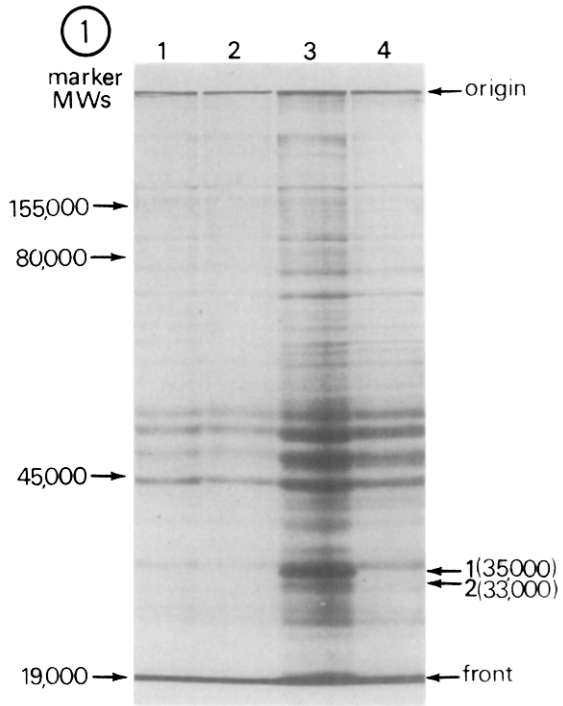

Fig. 1: Total protein electrophoretic profiles of cerebellar and hippocampal tissues derived from a homozygous reeler mutant and littermate control.
Similar wet weights of tissue were completely solubilized by boiling in sodium dodecyl sulphate/mercaptoethanol-containing sample buffer and aliquots containing approximately 200 μg protein were electrophoresed into a 7.5% polyacrylamide gel. 1- control total hippocampal proteins; 2- reeler total hippocampal proteins; 3- control total cerebellar proteins; 4- reeler total cerebellar proteins.

bands 3,4 and 5 (see below). Chymotrypsinogen (25,700), carbonic anhydrase (29,000), carboxypeptidase A (34,600), glyceraldehyde phosphate dehydrogenase (36,000) and enolase (41,000) were used for MW determinations of bands 1 and 2 (see below).

RESULTS

When thin tissue slices derived from reeler cerebellum and littermate controls were solubilized in sodium dodecyl sulphate-containing sample buffer and electrophoresed on 7.5 or 8.0% polyacrylamide gels, two major protein components of the control cerebellum were found to be missing from the mutant electrophoresis pattern (bands 1 and 2, see Fig. 1). The apparent MW's of these two protein bands estimated from calibration curves for these gels were 35,000 and 33,000,

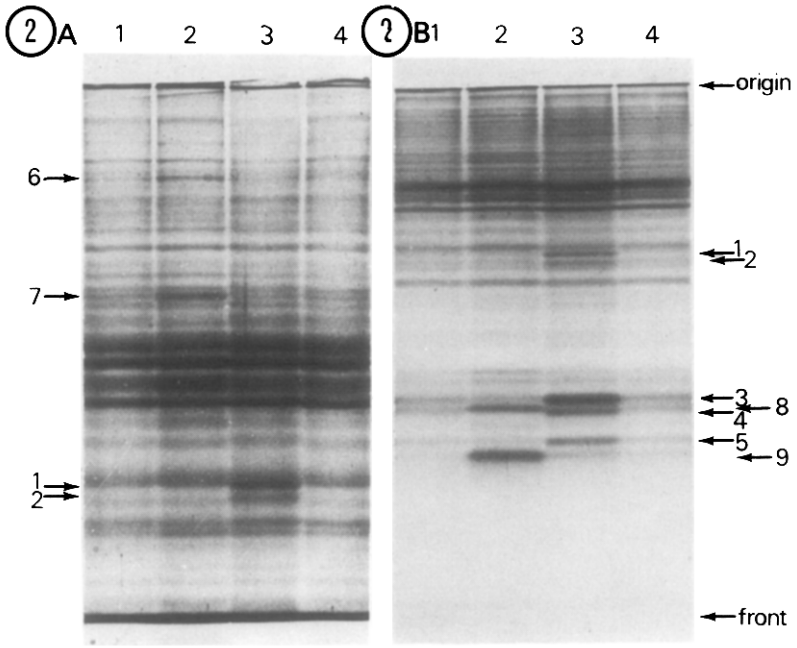

Fig. 2A and 2B: Comparison of reeler and weaver total cerebellar proteins with control cerebellar and neocortical tissue proteins.
Sample preparation and other details as in Fig. 1.
2A: electrophoretic profiles on 8.0% polyacrylamide gel.
1- normal littermate (rl/+ or +/+) neocortical total proteins; 2- weaver cerebellar total proteins; 3- normal littermate (wv/+ or +/+) cerebellar total proteins; 4- reeler cerebellar total proteins.
2B: electrophoretic profiles on 12.5% polyacrylamide gel.
Samples as for 2A.

respectively. Electrophoresis on 12.5% gels revealed three further bands of apparent molecular weights 13,000, 12,000 and 10,500 (bands 3,4 and 5, respectively of Fig. 2B) which were present in control cerebellum but were present in much reduced amounts in mutant samples (see Fig. 2B).

Total protein electrophoretic profiles of three other brain regions were also examined (olfactory lobe, hippocampus and neocortex) and in all three cases both control and mutant tissues yielded similar electrophoresis patterns to the mutant cerebellum with respect to these bands. That is, bands 1 and 2 were missing and bands 3,4 and 5 were very reduced in amount (see Figs. 1 and 2). This suggested that these proteins were cerebellar specific or, at least, greatly enhanced in the cerebellum, as opposed to other brain regions.

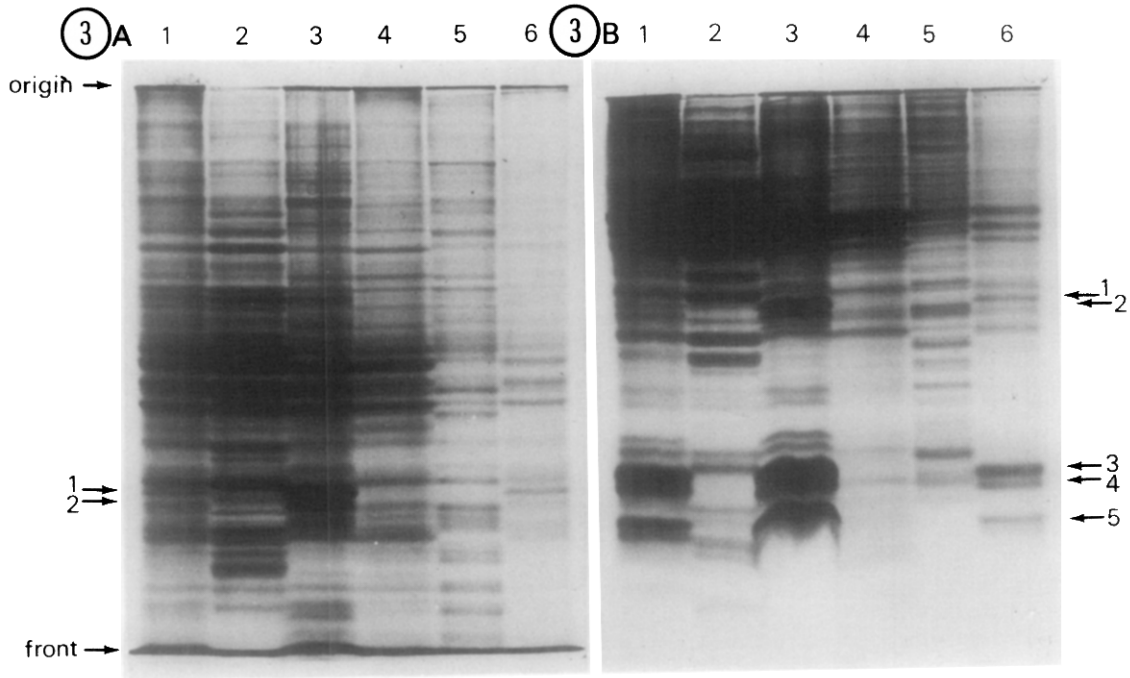

Fig. 3A and 3B: Subcellular localization of band 1-5 proteins. Electrophoretic profiles of subcellular fractions derived from 15 day normal cerebellum using 8% (Fig. 3A) and 12.5% (Fig. 3B) polyacrylamide gels. 1- total homogenate proteins; 2- soluble proteins; 3- nuclear pellet proteins; 4- mitochondrial pellet proteins; 5- ribosomal proteins; 6- proteins of a cerebellar tissue slice. Samples contained 100-200 μg protein. All 5 bands are confined to the nuclear fraction.

Previously Mallet et al. (1) have reported the absence from weaver cerebellum of two major protein components of molecular weights 31,000 and 29,000. A comparison of total weaver cerebellar proteins with total reeler cerebellar proteins was therefore performed which revealed noticeable similarities in the electrophoretic patterns. Marked increases in several minor bands of the normal cerebellum were regularly seen in weaver samples (see bands 6,7,8 and 9, Figs. 2A and 2B) which were without parallel in the reeler cerebellum, but the two mutant patterns were found to be identical in lacking band 1 and 2 proteins and containing very decreased levels of band 3,4 and 5 proteins. It seems certain, therefore, that bands 1 and 2 are the two protein bands described by Mallet et al. even though there is a difference in the apparent molecular weight estimations of approximately 4,000 between these results and those of Mallet

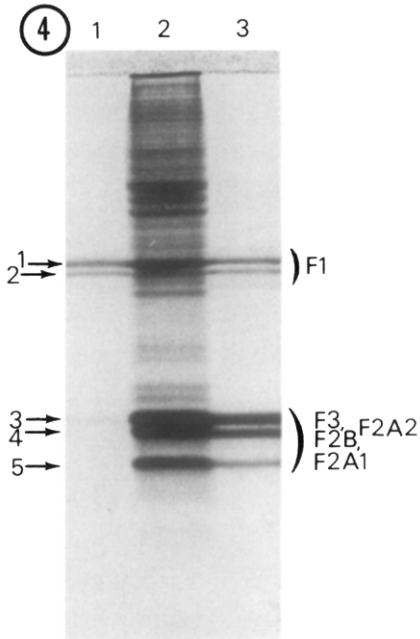

Fig. 4: Identification of bands 1-5 as histones by co-electrophoresis on 12.5% polyacrylamide gel with purified histone fractions.
1- purified calf thymus F1 histone fraction (10 μg); 2- approx. 100 μg cerebellar nuclear fraction proteins (see Fig. 3B); 3- purified pig thymus total histones (20 μg).

et al. (1). This discrepancy may be due to differences in the two electrophoresis systems and in the electrophoretic behaviour of the molecular weight markers used.

Subcellular fractionation of cerebellum from normal 15-day old mice revealed that all 5 bands were entirely confined to the nuclear fraction (see Figs. 3A and 3B) forming major components of the nuclear protein electrophoretic pattern. The identification of these bands as major nuclear proteins suggested that some of them might be histones, particularly bands 3,4 and 5, the MW values for which are similar to the known MW's of the 4 major histones - F2A1, F2A2, F2B and F3 (MW's of 11,300, 14,500, 13,700 and 15,300, respectively (12)) Parallel electrophoresis with various purified histone fractions was therefore performed (see Fig. 4). This showed convincingly that the lower MW bands corresponded to the 3 bands produced by a preparation of the 4 major histones and also, surprisingly, that bands 1 and 2 corresponded to the two bands produced by purified F1 histone in this electrophoresis system.

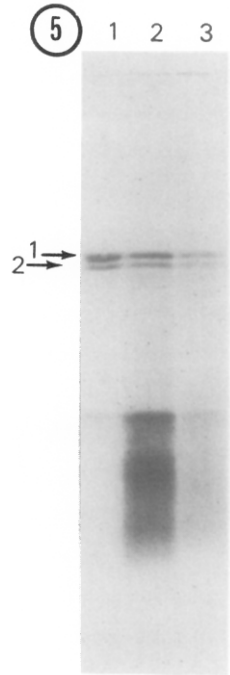

Fig. 5: Quantitation of F1 histone content in 16 day weaver cerebellum and in normal littermate cerebellum.
F1 histones (bands 1 and 2) were extracted from weaver and control cerebellar nuclei with 1 M KCl in the presence of 2 mM PMSF and 50 mM $NaHSO_3$. 1- purified calf thymus F1 histone protein (5 μg); 2- 1 M KCl extract of cerebellar nuclei derived from normal littermate; 3- 1 M KCl extract of weaver cerebellar nuclei derived from same wet weight of tissue as 2. Planimetry of stained absorbance profiles for bands 1 and 2 showed that the F1 content of the weaver cerebellum was 36% that of the control tissue on a tissue wet weight basis.

The similarity of MW of the four major histones could easily account for their incomplete separation in this electrophoresis system. However, the doublet of bands of MW 35/33,000 produced by F1 histone is unexpected since, although F1 histone is heterogeneous, being composed of up to five variants in some tissues (13), these variants all have a similar MW of 21,000 (11). A single protein band of much higher electrophoretic mobility would therefore have been anticipated. To determine whether this doublet of anomalous MW was due to phosphorylation of the F1 histone variants to two different levels, samples of F1 histone were dephosphorylated overnight using alkaline phosphatase (see Methods). Casein and vitellogenin were also dephosphorylated as positive controls for enzyme activity. This treatment did not alter the mobility or band profile of

the F1 histone. It must be concluded therefore that this unusual electrophoretic mobility and band complexity is as a result of peculiarities in the amino acid composition (particularly the high lysine content) of the F1 histones.

F1 histone is a universal component of all nuclei and thus, having established electrophoretically that bands 1 and 2 are F1 components, their complete absence from any of the tissues investigated seemed highly unlikely. Protein fractionation studies on the normal cerebellum had shown that band 1 and 2 proteins could be quantitatively extracted and considerably purified, without proteolytic degradation, by high salt extraction of the nuclear fraction in the presence of PMSF and sodium bisulphite. High salt extracts of weaver and reeler cerebellar nuclei and hippocampal tissue nuclei were therefore prepared. A reduced F1 content in the two mutant cerebella (see Fig. 5) and in other brain regions, as exemplified by the hippocampus, was thus established. Quantitation of the F1 contents of these various tissues by planimetry of the stained F1 absorbance profiles was carried out. This revealed that the F1 contents per wet weight of tissue for the 16 day weaver and reeler cerebella tissues were 29-36% (2 measurements) and 22-30% (3 measurements) respectively of littermate control values. That of 16 day hippocampus was 16% of the F1 content of a similar wet weight of cerebellar tissue from the same animals.

DISCUSSION

The four main histones (F2A1, F2A2, F2B and F3) perform a major structural function in the nucleus, being present in strictly equimolar amounts (12) and thus the total quantity of these proteins would not be expected to vary per diploid nucleus. The cerebellum contains more cell bodies per volume than any other region of the brain, the major cell population being the granular cells (8) which contain large heterochromatic nuclei surrounded by very little cytoplasm. Both reeler and weaver mutations decrease the cerebellar cell density by depleting the number of granular cells and thus it seems reasonable to assume that the decreased levels of bands 3,4 and 5 (i.e. the four main histones) seen in reeler and weaver cerebellum and in other brain regions is a reflection of the decreased cellularity (that is number of cell bodies (nuclei) per tissue wet weight) in these various situations.

F1 histone has regulatory as well as structural functions (14) and its stoichiometry as compared to the amounts of the other four histones is less well defined (12). Since it proved relatively easy to extract the F1 protein from

nuclei with a high salt medium, the relative quantities of this protein in reeler and weaver cerebella and in the hippocampus as compared to the normal 16 day littermate cerebellum were determined. This initial quantitative data on the F1 histone is interesting in that it does not correlate well with the cellularity differences suggested by gross histological examination of these various tissues. Thus, a six-fold higher cell content of the cerebellum as compared to the hippocampus is not anticipated. Similarly, the weaver mutation, which results in almost complete granular cell ablation, might be expected to result in a greater decrease in cerebellar cellularity (and therefore lower F1 content) than the reeler mutation, in which a detectable granular cell layer still exists. These unexpected findings thus suggest that cerebellar cell nuclei contain greater quantities of F1 histone than nuclei in other brain regions and in particular that the reeler mutation results in a decreased cerebellar F1 nuclear content. Correlation of the F1 tissue contents with those of the four major histone species should resolve these various possibilities.

This increased nuclear F1 content would particularly apply to the cerebellar granular cells, since these form the major cerebellar cell population and are the cell type specifically decreased by the two mutations studied. Whatever the final outcome in terms of granular cell F1 stoichiometry, it seems clear that the original identification of the two F1 protein bands as granular cell specific proteins by Mallet et al. resulted from the very high ratio of nuclear to cytoplasmic proteins in these virtually cytoplasm-free cells.

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