Patterns of cerebellar foliation in recombinant inbred mice

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Wahlsten, D., and Andison, M. Patterns of cerebellar foliation in recombinant inbred mice. Brain Research, 1991, 557, 184-189.

Abstract:
Morphometric analysis of cerebellar foliation patterns at the midsagittal plane was done in the inbred strains BALB/cByJ, C57BL/6ByJ and their 7 recombinant inbred strains in order to assess possible major gene influences. The cerebellum was dissected away from the brainstem and weighed prior to histology so that measures of the depth of fissures and sulci could be related to overall size of the brain and cerebellum. Results for 177 mice revealed that many brains had extra sulci present within the central lobe, the culmen, the declive and the uvula, and that patterns within a genetically uniform inbred strain were highly variable. The measure of sulcus depth was continuous, showing no evidence of a normal versus abnormal dichotomy. Furthermore, the frequency and depth of extra sulci were greater in mice with larger-cerebella. Strain differences in size of the whole brain and cerebellum clearly resulted from several genetic loci. This was also true of the extra sulci, except for the declival sulcus which revealed a single gene influence. The gene symbol 'declival sulcus of cerebellum' (*dsc*) is proposed.
Key words: Cerebellum; Morphometry; Genetic analysis; Inbred strain; Single gene; Pattern formation

Article:
INTRODUCTION
The cytoarchitectonics of the mouse brain are de-scribed in the authoritative atlas by Sidman, Angevine and Taber Pierce" for the C57BL/6J inbred strain. The major structures identified therein can be found in most viable laboratory strains, with two noteworthy exceptions. The corpus callosum is grossly deficient or absent altogether in the BALB/c[17], DDN[12], I/LnJ[9] and 129[17] strains; and certain extra fissures or sulci are found in the cerebellum of almost all strains[2,3,7,11,13]. Whereas absence of the corpus callosum is an abnormal state, extra sulci in the cerebellum are so common that they cannot be considered pathological. It is not at all obvious which pattern of foliation is typical of mice and which is anomalous.

A severe neurological mutation such as weaver *(wv)* disrupts cerebellar foliation6.15 and also impairs repro-ductive fitness. Without special care by the researcher, such a gene would soon become rare or totally extinct. On the other hand, extra sulci in the cerebellum appear to be common and viable. Generally speaking, genetic polymorphisms will be maintained at quite high frequencies when they have little impact on reproductive fitness. Viable genetic variants should tell us much about development as well as evolution. Knowledge of the genetic bases for differences in cerebellar foliation may help to understand the origins of cell patterns in the brain.

It has been said that each inbred strain shows its characteristic pattern of foliation[7,13]. However, studies of reasonably large samples of mice have detected striking morphological differences among genetically identical animals[3,11]. This ‘incomplete penetrance’ of strain-specific genotype creates difficulties for the usual mendelian crossing studies which assess mode of inheritance from the percentage of abnormal animals in a group. The recombinant inbred strain method[1] is superior for genetic analysis because single locus inheritance can be perceived in the pattern of strain mean scores even when there is substantial variance within the strain, and

genetic linkage may also be determined. In this way a new (*Cfp-l*) on chromosome 4 which influences the form of culmen in the cerebellum has been discovered[11].

As the variation within a strain suggests, the genome is not the only determinant of cerebellar morphology. Neonatal thyroxine administration produces a smaller cerebellum with fewer folia[2,8]. Prolonged undernutrition impairs growth of the cerebellum[16,21] and large litter-size yields mice with smaller brains and cerebella[20]. It is reasonable to expect that a larger cerebellum will have more and deeper sulci. Nutritional and other environmental influences on cerebellum size vary by degree[5]. Consequently, there is no good reason to expect that in every mouse an unusual fissure or sulcus will be present or absent.

The present study examined cerebellar foliation in the Bailey recombinant inbred strains and their 2 progenitors using sufficient samples to evaluate variation within a strain. Continuous measures of cerebellum size as well as depth of fissures and sulci were analyzed.

Table I
Strain names, abbreviations, and sample sizes

Strain	*Letter*	*Sample size*
BALB/cByK	B	38
C57BL/6ByJ	C	11
CXBD/By	D	24
CXBE/By	E	26
CXBG/By	G	13
CXBH/By	H	17
CXBI/By	I	19
CXBJ/By	J	17
CXBK/By	K	12

M ATERI ALS AND METHODS

The inbred strains BALB/cByJ, C57BL/6ByJ and their 7 recombinant inbred strains (see Table I) were obtained from the Jackson Laboratory and bred at the University of Waterloo under standard conditions[17]. All mice used in this study were conceived and reared in the same laboratory. This is of some importance in a genetic analysis because differences between laboratories in breeding protocols and nutrition can affect brain size and incidence of abnormalities[17,18].

Histology

At 100 ± 1 days after birth, each mouse was anaesthetized with sodium pentobarbital and perfused intracardially with 0.9% sodium chloride followed by 10% neutral buffered formalin. After one week in fresh fixative, the brain was trimmed to a standard configuration and weighed. Then the cerebellum was removed by cutting the peduncles and weighed after blotting on a Nalgene work surface. The detached cerebellum was placed on a flat surface and cut in half vertically with a razor blade at or near the midsagittal plane. Another parasagittal cut was made about 3 mm from midplane, and then serial frozen 35 μm sections were cut from the midplane surface and stained with metachromatic thionin. If one surface did not yield suitable sections, the other half was sectioned and stained. A midsaggital section can best be recognized by the appearance of the cerebellar commissure, which is most compact at this region. Although the midplane usually cannot be specified precisely as present in one section and absent from adjacent ones, the architecture of the lobules and fissures does not change appreciably within 400 μm of midplane[14]. In 9 cases a suitable section for measurement was not obtained, and these brains were omitted from the analysis. The resulting data set represented a region at or very close to the midsagittal plane. Because all orientation and sectioning of the cerebellum was done without knowledge of the strain or sex of the mouse, any imperfection tended to increase variance within a group but would not bias strain differences.

Morphometry

A tracing of the midsagittal section at 40 × was made with a tracing device on a Leitz Dialux microscope. Outlines of the surface of the cerebellum, including all fissures and sulci, and the outer limit of the internal granule cell layer (GCL) were drawn. Some fissures were fairly obvious where two pial membranes were apposed, but the inner and outer limits of the fissure were often obscure. Furthermore, the smaller sulci could possibly be confused with the periarterial (Virchow—Robin) space where a blood vessel enters the cerebellum, especially in tissue where the meninges and superficial blood vessels were removed prior to histology. Generally this was not a problem because sulci extend through many sections, but it may have affected measurements in some cases. The depth of a fissure was measured along a curved line that followed the fissure where it was obvious and then extended inward, ending at the GCL, and outward, ending at a line drawn from the outermost points of the GCL on either side of the fissure (see Fig. 1). This definition of fissure depth permitted an unambiguous measure in every instance. Sulci were sometimes obvious but not as deep as fissures, whereas in many cases there was only a shallow depression in the GCL. In almost every instance such a depression occurred at the precise location where an obvious sulcus was often seen in other animals.

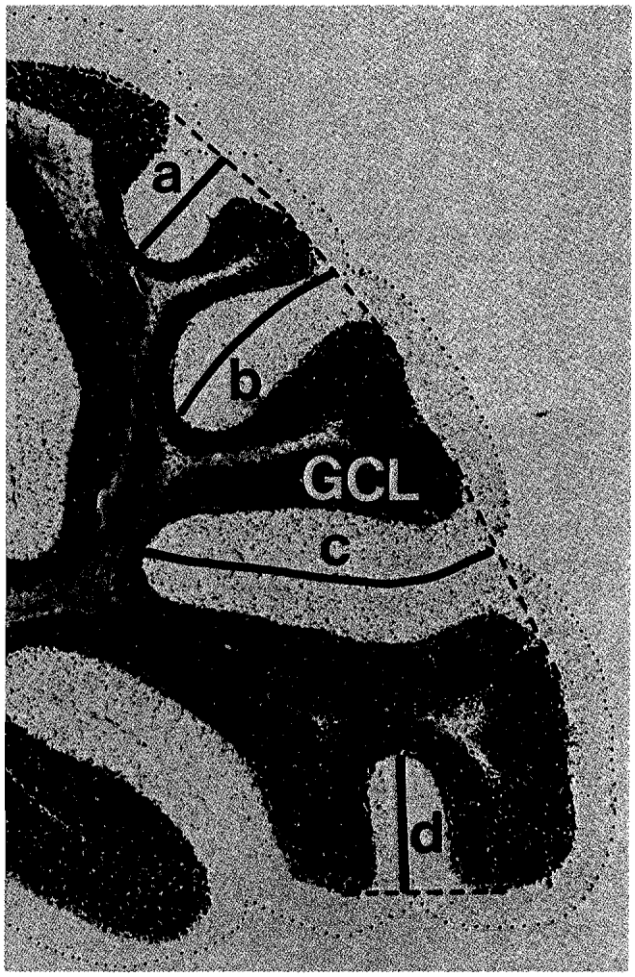


Fig. 1. Definitions of fissure and sulcus depths at the midsagittal plane. Wherever an indentation in the internal granule cell layer (GCL) occurred, a dashed line was drawn from the apex of the GCL on one side to the apex on the opposite side. Depth of a major fissure (b, c) was measured (heavy line) from this dashed line, then along the curved fissure, ending at the nadir of the GCL. Depth of a fissure or sulcus with no obvious midpoint (a, d) was the length of the heavy line from the nadir perpendicular to the dashed line spanning the apices.

Sulcus depth was measured along a perpendicular line extending from the line spanning the outermost limits of the GCL inward to the nadir of the GCL (see Fig. 1). All fissure and sulcus depths were measured with a graphics tablet and the Sigma Scan program from Jandel Scientific. A sulcus or nascent sulcus was considered to be present only if its depth exceeded 0.025 mm (or 1 film on the tracing). The person doing the tracings and measures (M.A.) did not know the strain of the mouse at the time.

TABLE II

Average depths (microns) for all mice and number absent out of 154 mice for all fissures (f) and sulci (s)

Structure	*Mean*	*S.D.*	*Number absent**
Intracentral s	60.3	59.9	113
Preculminate f	2146.2	292.3	0
Intraculminate s	175.9	219.6	74
ventral	157.4	221.7	
dorsal	18.5	39.5	
Primary f	2147.6	120.9	0
Declival s	93.4	96.6	85
Intercrural f	369.7	150.1	6
Prepyramidal f	725.3	93.7	0
Secondary f	1213.8	83.6	0
Uvular s, dorsal	28.3	57.3	142
Uvular s, ventral	321.3	209.4	24
Total depth	7318.7	764.5	

* Absent defined as fissure or sulcus depth less than 1% of total depth for all fissures and sulci in that brain.

RESULTS

Useful data were obtained from 177 mice. Among these, damage to the lingula and nodule at the base of the cerebellum was so frequent that data on sulci within these structures were not reliable. Depths of 12 fissures and sulci were measured (see Table II), but damage to one or more of these occurred for 23 mice. A complete set of data was available for 154 mice. There were no significant (a 0.05) effects of sex or interactions between sex and strain for any measure except body weight (males were heavier). Hence, data were combined for males and females.

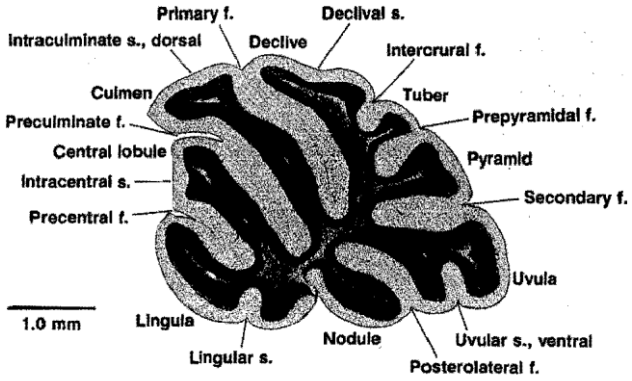
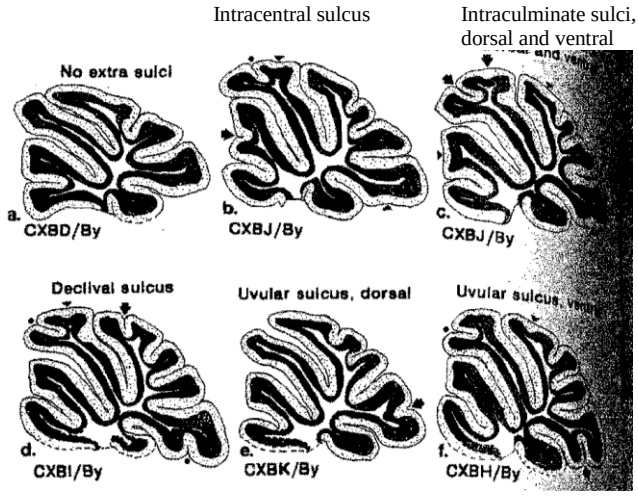


Fig. 2. The cerebellar vermis of a CXBE/By mouse at the midsagittal plane, showing the major lobules, fissures (f.) and sulci (s.).



Nomenclature

The cerebellum at the midsagittal plane consists of 3 major lobes separated by the deep primary and secondary fissures (see Fig. 2). Within each lobe are smaller lobules (lingula, central, culmen, declive, tuber, pyramid, uvula, nodule) separated by fissures. The names assigned to these lobules and fissures follow Sidman et al.[14]. They were apparent in every brain with the sole exception of the intercrural fissure, which was totally absent (depth less than 0.025 mm) in 6 mice and very shallow in several others (see Table II). Six other structures were observed *within* a major lobule. Most have been noted in one mouse strain or another,7,11,13, and they have generally been called 'fissures'. The ocation of these in the present data is described in Fig. 3, and the frequency distributions of their measured depths are given in Fig. 4. Consider the structure within the culmen. Four articles have named this the intraculminate fissure[3,7,11,13]. However, Fig. 4 reveals that in the large majority of cases the indentation in the GCL is quite shallow, although in some mice the structure is almost as deep as a bona fide fissure such as the prepuramidal. It is proposed here that an invagination of the GCL *within* a lobule be termed a sulcus. Thus, the intraculminate sulcus is named for its most common occurence rather than the most extreme instances. The precise location of the intraculminate sulcus varies somewhat from brain to brain, being relatively more dorsal in some and ventral in others (Table II and Fig. 3). The depths of the two were negatively correlated (Spearman r = -0.21, n = 177, P < 0.003), and the ventral sulcus was never present if the dorsal sulcus was deeper than 100 μm. Therefore, the two were combined for further analysis.

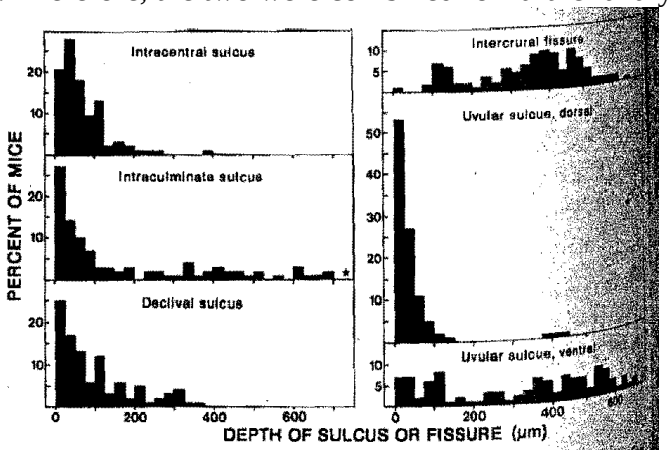


Figure 4. ***Frequently distributions of sulcus and fissure depth in μm for all 177 mice. Only those structures which sometimes had depth less than 25 μm are shown. For the intraculminate sulcus, the solid star indicates 3 mice with depth beyond 700 μm. For the ventral uvular suclus, the open star indicates one animal beyond 700 μm.***

Continuity, not dichotomy

Figure 4 demonstrates clearly that sulcus depth is a continuous measure. The skilled anatomist using a qualitative approach draws a mental line at a certain sulcus depth and regards anything deeper as a fissure. There is nothing inherently wrong with this, provided the observer does not know the group membership of any making the judgment. However, the qualitative approach lacks 2 advantages of the quantitative approach (i) The subjective cutoff point may be different for various judges, making comparisons between studies ambiguous. (ii) The dichotomy implies to the reader an all-or-none situation in the data which does not exist. This latter point is very important because deeper in vaginations which immediately catch the eye invariably are found at the precise locations of shallower sulci. This gives a tantalizing clue about the origin of the deeper structures.

Cerebellum size versus extra sulci

The data confirmed that mice with a larger cerebellum tend to have deeper fissures and more sulci. For the 154 animals with a complete data set, the sum of depths of all 12 fissures and sulci was positively correlated with cerebellum weight (Pearson r = 0.521). The percentage of total fissure and sulcus depth which was found in the extra sulci was also positively associated with cerebellum size (Spearman r = 0.398). If an extra sulcus is said to be present when its depth amounts to at least 1% of the total fissure and sulcus depth for that brain, mice with heavier cerebella tended to have more extra sulci (Pearson r = 0.450). Because these correlations with weight account for only about 20% of total variance in sulcus number and depth, there must be other important

influences on foliation in addition to the size factor. Nevertheless, the size factor is very important and should be taken into account in the genetic analysis of foliation. As indicated in Fig. 5, the CXBD/By strain had the smallest cerebellum and very few extra sulci, whereas the CXBJ/By and CXBH/By strains had the largest cerebella and many extra sulci. The progenitor strains, however, showed similar cerebellum size but a substantial difference in number of extra sulci.

Polygenic influence on size

As shown in Fig. 5, whole brain weight and cerebellum weight were polygenic. Means not significantly different by a Newman—Keuls' test with $\alpha = 0.05$ are connected by a solid line. If the 2 progenitor strains (B, C) differ at only one locus relevant to a measure, they and their recombinant inbreds should form 2 clusters of strain means. If they differ at only 2 relevant loci, there should be 3 or 4 clusters of means and no recombinant strain should occur outside the range of B and C means (provided B and C means differ). For both brain and cerebellum weights, several strains have a lower mean than C or a higher mean than B. The method described by Bailey' to estimate the lower limit of the effective number of segregating loci indicated 6.0 for brain weight, 5.2 for cerebellum weight, 5.5 for number of extra sulci, and 5.0 for percent of total depth in the intercrural fissure. Because overall size is polygenic and a larger cerebellum tends to have more and deeper extra sulci, a simple measure of sulcus depth will also tend to show a polygenic influence, unless each strain is prone to formation of a specific sulcus when cerebellum size is large.

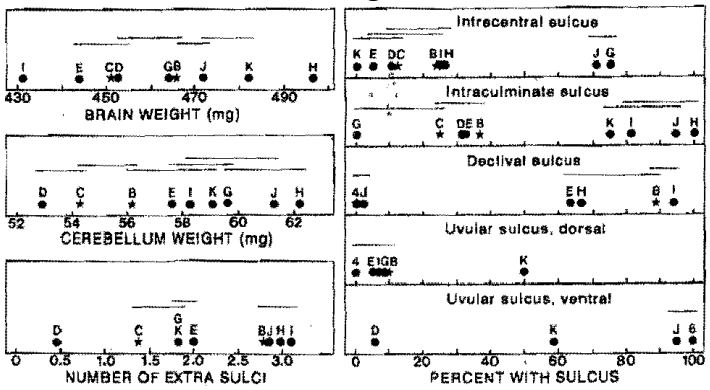


Fig. 5. Strain means for brain weight, cerebellum weight, number of extra sulci, and presence of specific sulci. Strain abbreviations are given in Table I. Progenitor strains are indicated by stars. Where several strains are tied at 0 or 100, the number of such strains is represented by 4 or 6. Strains not significantly different by criteria discussed in the text are joined by a solid line.

Strain-specific sulci

The depths in μm of the various fissures and sulci tended to be positively correlated (average r = +0,22) because of the general size factor, but correlations among percent of total depth for each fissure or sulcus were much lower (average r = —0.08). Defining an extra sulcus as one with a depth that is 1%, of more of total fissure and sulcus depth compensates to some extent for the cerebellum size factor. A simple ratio is reasonable when both measures are in the same units. With this definition, Fig. 5 shows that certain strains were much more likely to exhibit one sulcus rather than another. For example, CXBJ/By with a larger mean cerebellum size almost always had intraculminate and ventral uvular sulci but never a declival sulcusi. Strain G was prone to formation of an intracentral sulcus. For all but the declival sulcus, one or more recombinant strains showed a frequency of present or absent sulcus that was far outside the range from B to C parent scores, which is incompatible with single locus inheritance. For the declival sulcus the question is whether strains E and H form a different cluster from B and I. A Newman—Keuls test does not apply to a dichotomous measure. The proportion with an extra fissure in one group can be compared with another group using a z test, but this encounters problems with multiple, nonindependent tests. Matters are further complicated because the mean and variance of a proportion are closely related. To yield a fair approximation of a post hoc test of strain means, the 807o confidence interval for the proportion with an extra sulcus was determined for each strain, If the confidence intervals for 2 strains overlapped, they were not considered significantly different. This criterion yields lengths of lines joining strains which were similar to the lengths for weight measures using the

Newman—Keuls test (see Fig, 5). One might opt for a 90% or 95%0 confidence interval, but there is a hazard in using too strict a criterion for testing the number of genes. Because affirmation of a single locus requires that a null hypothesis not be rejected by the data, a stringent criterion for a Type I error (rejecting the null when it is true) reduces the sensitivity of the test, and an insensitive test is more likely to 'find' a gene. Use of the 80% confidence interval was adopted as a reasonable compromise. The lower limit of the interval for strain I was 81.3%, and the upper limit for strain H was 79.9%. Given the very large gap between the 4 high scoring strains and the 5 lacking the sulcus, these data are sufficient to support the hypothesis of single locus inheritance and reserve the gene symbol ‘declival sulcus of cerebellum’ (*dsc*). Like all interesting findings, this needs to be repeated by others similar methods.

Correlations among fissures and sulci

If the presence of the declival sulcus reflects an influence of one genetic locus, it is possible that this locus might be one of several affecting other fissure or sulci. Indeed, a relatively deeper (in terms of percent total depth) declival sulcus tends to be positively associated with a deeper intercrural fissure and ventral declival sulcus (both $r = 0.7$, $n = 154$) but shallower preculminate, central and secondary fissures (all $r < -0.5$). When the primary fissure is deeper the adjacent intraculminate and declival sulci tend to be shallower (both $r <$ and when the secondary fissure is deeper the nearby intercrural fissure and ventral uvular sulci also tend to be shallower ($r <$ —0.6). These data, especially the negative correlations, suggest that an event at one place in the cerebellum early in postnatal development may have an opposite effect elsewhere in the immature cerebellum. Genetic analysis of the earliest stages of foliation may reveal a somewhat simpler pattern of genetic influence and point more directly to the causal mechanisms.

DISCUSSION

The finding of single locus inheritance for the declival sulcus is novel, Peterson and Nowakowski[13] a1so examined the Bailey recombinant inbred strains but found that neither progenitor strain had a declival sulcus. This clearly contradicts the present results for BALB/cByJ. Whether this reflects their qualitative anatomical approach, which took account of only the deepest sulci, or their small sample size (6 BALB/c mice, 4 of other strains) is not certain. They did find the sulcus in CXBI/By, our highest frequency strain. In any event, the presence of the declival sulcus in 4 of our strains is beyond doubt. They claimed single locus inheritance for uvular sulcus 1, which is our ventral uvular sulcus, but our much larger sample size contradicts this. Their claim that cerebella *within* a strain 'were identical in their folial pattern' is at odds with the present study and two other recent reports[3,11].

Neumann et al.[11] provided strong evidence indicating a major gene, 'cerebellar folial pattern -1' *(Cfp-1),* which predisposes mice to a definite intraculminate fissure (or sulcus) typically seen in DBA/2J mice. The present study did not examine DBA/2J or strains derived from it, and we neither confirm nor deny their result. They found that *every* one of 16 DBA/2J cerebella had the intraculminate fissure, whereas our BALB/cByJ progenitor showed much lower penetrance for this characteristic using a presumably less stringent criterion. Hence, the discrepancy in mode of inheritance is not at all surprising.

When a major gene effect on foliation is observed, this does not imply that the morphogenetic process derives solely from one genetic program. The impressive variability within an inbred strain remains to be explained. Furthermore, the general pattern of results associated with a major gene can be modified by the genetic background of the strain, as occurs for the cerebellum with the reeler (*r1*) mutation[4]. Detecting a major gene effect helps to analyze an extraordinarily complex process of pattern formation by isolating one of many causal influences. Interactions among component processes are undoubtedly important for morphogenesis. Knowing the identity of one or more components helps to observe and understand the interactions.

Patterns of foliation must arise to some extent from the kinetics of cell proliferation in the external granular layer and migration past the Purkinje cells to the internal granular layer. It has been noted[10] that mitotic activity is greater in the external granular layer in major fissures than on the cerebellar surface of CBA mice. It is possible that extra sulci form in zones where there is unusually low granule cell number. Alternatively, it is

conceivable that each strain has certain locations along the Purkinje cell layer where, as granule cells accumulate in the internal granular layer, folding is most likely to occur. It is hypothesized that 2 distinct processes contribute to the formation of extra sulci: an overall size factor which generates pressure for invagination as the cerebellum grows larger, and genotype-specific break points where inward folding is most likely.

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