Inheritance of Retarded Forebrain Commissure Development in Fetal Mice: Results from Classical Crosses and Recombinant Inbred Strains

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Abstract:

Deficiency of the adult corpus callosum In BALB/c mice shows Incomplete penetrance and is clearly polygenic, whereas the defect In fetuses shows complete penetrance and a much less complex mode of inheritance. Retardation of the growth of the corpus callosum and the hippocampal commissure In the fetal mouse forebrain was expressed by a standard score (z) derived from body weight, such that a fetus with a score less than —2.0 was held to have commissures abnormally small for the body size. By this Index, almost all C57BL/8 fetuses were normal, whereas BALB/c fetuses in the body weight range 0.5 to 1.0 g were often 5 standard deviations below the expected value of 0.0. In classical crosses between C578L/6.1 and BALB/cWah, Inheritance of the Index of abnormality (z) was recessive, and about half of the fetuses In backcrosses to BALB/c were below —2.0. However, the distribution of scores was not bimodal. The results were consistent with a two-locus but not a single-locus difference between parent strains. Among the seven recombinant Inbred strains derived from the By strains of C57BL/8 and BALB/c, there were three or possibly four distinct clusters of strains, which also suggested two-locus inheritance and excluded a single-locus difference. Although substantial retardation of commissure growth was evident In fetuses, deficiency or absence of the corpus callosum In weanling and adult By recombinant Inbred mice was extremely rare in all strains except BALB/cByJ. These data confirm anatomical results showing that, in all but the most extremely retarded cases, the corpus callosum recovers from an obvious prenatal defect.

Article:

The deficiency or absence of the corpus callosum (CC) in the forebrain of the adult BALB/c mouse is produced by allelic differences at more than one autosomal locus,[18] but it is not known whether two, a few, or many loci are involved. The answer to this question will greatly influence the course of future work on the physiological bases of this interesting brain defect. Although anatomical aspects of the defect have been explored in the fetus[15,21] and in adult mice[13] at relatively macroscopic levels, virtually nothing is known about relevant gene actions at the molecular level. The many powerful techniques of molecular genetics probably will be fruitful only if the characteristic proves to be relatively simple genetically,[6] so that a few distinct developmental processes can be identified and manipulated. Alternatively, a genetically complex characteristic is best studied using morphometry to identity processes acting in development.[2]

A recent study[21] of prenatal CC development in BALB and normal fetuses uncovered two facts which facilitate further genetic analysis. Whereas serious CC defects occur in only 10% to 20% of adult BALB mice, almost every BALB fetus at 17 days after conception shows a distinct bulge in the fissure between the cerebral hemispheres and greatly retarded formation of the CC. The BALB heredity shows nearly complete "penetrance" before birth, but many fetuses are able to overcome this defect and form a CC when axons cross between the hemispheres via an unusual pathway. Complete penetrance should simplify genetic analysis and increase statistical power. Furthermore, adult BALD mice have deficits only in the CC, but BALB fetuses also suffer retarded formation of the hippocampal commissure (HC). The precise border between the CC and FIC is extremely difficult to perceive in fetuses,[7] but they can be conveniently measured as one large commissure in

early development for the purpose of genetic analysis.

Study of the BALB fetus also presents difficulties. Growth is very rapid[20] and nonlinear.[21] The rate of growth of the whole fetus differs between strains, shows heterosis, and is very sensitive to the maternal environment.[22] Individual differences in rate of development within a litter are quite large, sometimes amounting to a full day in degree of maturity. Consequently, it is essential to distinguish between a fetus having a very small CC merely because the whole organism is small for its chronological age and one which has a defect specific to the CC and a CC that is small relative to the rest of the brain or the body.[20] If all groups in a classical crossing experiment are assessed at the same chronological age, large differences in CC and I-1C sizes will be observed, but results will be confounded by group differences in the overall degree of maturity. Proportions of groups showing deficient CC will be very different for those that are generally immature and those that have entered the period of recovery from the hereditary defect.

Table 1. Sample sizes, ages, and index (z) of CC and HC retardation in fetuses from classical crosses

| ♀ | ♂ | n | Age | Index of CC + HC abnormality (z) | | |
				Mean	SD	t
BALB	× BALB	14	17.00	−4.43	0.87	−19.0[a]
C57	× C57	13	16.25	0.17	0.80	0.8
BALB	× C57	14	16.50	−0.54	1.95	−1.0
C57	× BALB	10	16.00	−0.53	1.31	−1.3
BALB	× F_1	25	16.50	−1.74	1.98	−4.4[a]
F_1	× BALB	13	16.00	−1.13	2.26	−1.8[a]
F_1	× F_1	48	16.00	−0.19	1.89	−0.7
BALB/cWah1	× F_1	40	16.50	−2.35	1.73	−8.6[a]
F_1	× BALB/cWah1	19	16.00	−2.60	1.38	−8.2[a]

[a] $P < .05$, one-tailed.

To solve these problems, data from a previous study[21] were used to derive equations for normal CC and HC growth in relation to the weight of the whole fetus, and an index of the degree of abnormality was constructed. Offspring from several classical crosses and recombinant inbred strains between BALB/c and C57BL/6 were then obtained within a fairly narrow range of body weights by extracting fetuses at different ages for different groups. Experiments were designed to test the hypothesis that the degree of retardation of CC and HC growth is the result of a single recessive gene.

Methods
Conditions of housing, feeding, and breeding have been described previously.[18] Procedures for extracting fetuses, histological processing of the brains, and measurement are elaborated in detail in a recent report.[21] Briefly, we checked females for vaginal plugs at four-hour intervals and isolated them as soon as a plug was found. At the appointed hour several days later, fetuses were removed surgically, immersed for 48 hours in Bouin-Duboscq fixative, embedded in paraffin, sectioned sagittally at 10 μm, and stained with hematoxylin and eosin. We anesthetized adult mice with a sodium pentobarbital overdose and perfused them intracardially with 10% formalin. We cut the brains sagittally and stained them with Sudan Black B to reveal myelinated tracts. The midsagittal section in fetal brains was identified by the location of the anterior cerebral artery, which is adjacent and dorsal to the CC in normal brains and is always present when the CC is absent We traced outlines of the CC and HC in a midsagittal section and determined cross-sectional areas with a digitizing tablet and the Bioquant morphometry program. All brain measurements were done without knowledge of the genotype, Data for fetuses were corrected for tissue shrinkage during histology by multiplying areas by 1.98, a value determined from a large sample of brains in a previous study[21] in which fine needles 1.0 mm apart pierced the midbrain prior to fixation.

Standards for Normal Development
Data for separate measures of CC and HC sizes have been published previously.[21] For this experiment we combined data on the same animals to derive a developmental index. We used mice of two groups known to have no CC or MC defects to construct the index for normal and abnormal development. Fetuses of C57BL/6J ($n = 71$) and B6D2F$_2$/J ($n = 92$) genotypes were obtained at 15.5 days to 18.0 days of gestation. The inbred C57 fetuses developed a little slower and were more variable than the F_2 hybrids, but no group difference in brain growth was evident once they were equated for body weights. A large sample of BALB/cWah mice was also

assessed with the index.

Classical Crosses

We used the inbred strains C57BL/6J and BALB/cWah, both maintained at the University of Waterloo, to produce fetuses from at least two litters of several different genotypes (Table 1). On the basis of previous studies[21,22] and preliminary tests, chronological ages for the extraction of each group were chosen to yield fetuses in the weight range from 0.5 to 0.75 g. The F_1 hybrids used to produce backcross and F_2 samples included both reciprocal crosses. However, there were no signs of cytoplasmic or sex chromosome effects, and data from reciprocal F_1 hybrid parents were pooled. There are actually four separate substrains of BALB/cWah, all derived in 1976 and 1977 from stock purchased from Carworth Farms. We derived most mice in this study from BALB/cWah2, but several litters in the crucial backcross groups were derived from BALB/cWah I, which recently has developed a relatively high frequency of CC defects in adults.

Recombinant Inbreds

We purchased the inbred strains BALB/ cByJ, C57BL/6ByJ, and their seven recombinant inbred strains from The Jackson Laboratory and mated them in Waterloo to produce fetuses from at least two litters per strain. We obtained BALB fetuses at 17,0 days, C57 at 16.5 days, C57 × BALB at 16.0 clays, and the seven recombinant inbreds at 17.0 days, at least initially. The development of strain CXBD/By proved to be very slow, whereas CXBE/By was relatively fast. Hence, we required additional litters of D at 17.25 and 17.5 days and of E at 16.5 days. We later studied adult mice from The Jackson Laboratory histologically at ages ranging from 83 to 240 days. We also perfused several litters produced at Waterloo for histology from 28 to 31 days after birth.
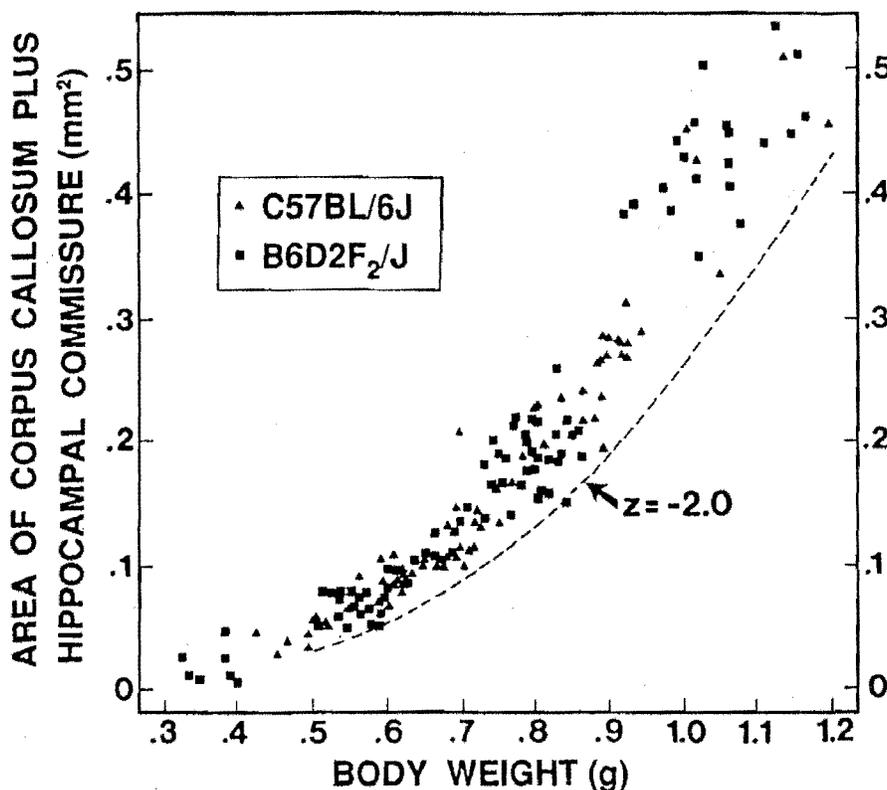

Figure 1. Scatter plot of the cross-sectional area of the corpus callosum and the hippocampal commissure at the midsagittal plane against body weight of C57BL/6 (triangles) and hybrid (squares) fetuses in the standard population of 163 fetuses. The dashed line indicates the value of the index of abnormality (z) two standard deviations below the expected value, derived as discussed in the text. There are fewer than 163 points plotted because several fetuses had almost identical values.

Results

Index of Abnormality

The dependent measure for all analyses of fetuses was the sum of the cross-sectional areas of the CC and MC at the midsagittal plane, henceforth referred to as CC + HC. This measure is shown in Figure 1 for 163 C57BL/6 and B6D2F$_2$ hybrid fetuses ranging from 0.3 to 1.2 g body weight. Quadratic regression on body weight yielded the equation

$$E(CC + HC) = 0.050 - 0.323X + 0.633X^2$$

for obtaining expected or predicted CC + HC from fetal body weight (X). The goodness of fit was $R^2 = 0.94$. The dispersion of points around the line of best fit was greater for heavier fetuses (Figure 1). To address this problem, the deviation of each score from the value expected on the basis of its body weight, E(CC + HC), was determined, and the square of this difference or residual was in turn predicted from body weight (X) using quadratic regression to obtain the curve

$$E(\text{squared diff.}) = 0.00231 - 0.00920X + 0.00940X^2$$

with a goodness of fit $R^2 = 0.25$. The expected standard deviation of the difference between observed and expected CC + HC is the square root of this quadratic expression. Thus, knowing the body weight (X) of a fetus, these equations can be used to determine the expected value of its CC + HC as well as the expected dispersion of scores of fetuses with the same weight around that mean value, From the expected mean and standard deviation, a simple index of abnormality can be constructed as a standard score (z) specifying the number of standard deviations that the actual CC + HC deviates from the value expected on the basis of body weight:

$$z = \frac{(CC + HC) - (0.050 - 0.323X + 0.633X^2)}{SQRT(0.00231 - 0.00920X + 0.00940X^2)}$$

If scores are approximately normally distributed about the expected value, then about 98% of all fetuses in a defect-free strain should occur within —2.0 and +2.0 standard deviations. Consequently, a fetus with a z score less than —2.0 would be judged to have a CC + HC value abnormally small for its body size or overall degree of maturity.

Values of the index are illustrated for four individual fetal brains in Figure 2, and values of the index for large samples of C57 and BALB fetuses from a previous study[21] are shown in the top panels of Figure 3. It comes as no surprise that C57 mice cluster nicely around 0.0, because they were part of the standard population. Results for BALB are more revealing. In striking contrast with the situation in adult mice, only one BALB/cWah 1 fetus was in the normal range, and most showed severe retardation of CC + HC. The distribution of z scores for the larger sample of BALB/ cWah2 had a similar shape. As evidence of the efficacy of the index, there was no significant correlation between z score and chronological age in the sample of BALB fetuses in the body weight range 0.45 to 1.00 g. The index was not as useful for larger BALB fetuses because many showed clear signs of recovery.

Classical Crosses
Diagrams of the midsagittal region of a normal C57 fetus and an abnormal BALB fetus matched for body weight are shown in Figure 2. The principal difference apparent at this location in the forebrain is the much smaller CC + HC of the BALB (Figure 2b), which in fact has no CC axons crossing midline at this time. The index of abnormality (z) did not differ significantly from 0.0 for the C57 parent strain or either F_1 hybrid, as shown in Table I. The BALB parent strain was far below the limit of normality (z = —2.0) but was no more variable than C57 or the value of 1.0 expected for a normal strain, which indicates "complete penetrance" for the z index in this study. All backcrosses to BALB were significantly below 0.0, and those crossed to BALB/cWah 1 were more abnormal than those crossed to BALB/cWah2. Although the F_2 hybrids were not significantly below 0.0 on average, they showed clear evidence of genetic segregation by virtue of increased variability, as did backcross groups. Because reciprocal F_1 hybrids did not differ between themselves or as parents of backcross and F_2 groups, they were pooled to obtain distributions of individual scores (Figure 3). The frequencies of fetuses with z scores less than —2.0 did not differ significantly from values expected on the basis

of Mendelian single-locus inheritance in backcrosses to either BALB substrain or for the F_2 hybrids (all $P>$ .05), although the test for the F_2 group was marginal (χ^2 3.39, P .066). However, the distributions provided no clear evidence of bimodality, and the majority of backcross fetuses occurred in a zone between the bulk of BALB and C57 fetuses.

Given the rather narrow range of z scores within each inbred parent strain, a simple single-locus effect should have produced clear bimodality with the present sample sizes. The results of this study are not entirely supportive of a single-locus difference producing retarded forebrain commissure development in BALB, but they certainly suggest a much simpler situation than for CC size in adults, wherein inbred BALB mice show extreme variation and frequencies of defective animals in back-crosses of C57 × BALB to BALB (about 1%) are far below predictions from a single-locus model.[18] They are entirely consistent with a simple two-locus model, whereas models asserting a large number of loci would seem to be ruled out by the appearance in backcrosses of many fetuses similar to BALB. However, discrimination between two- and three-locus models is not possible without the use of additional crosses.[5]
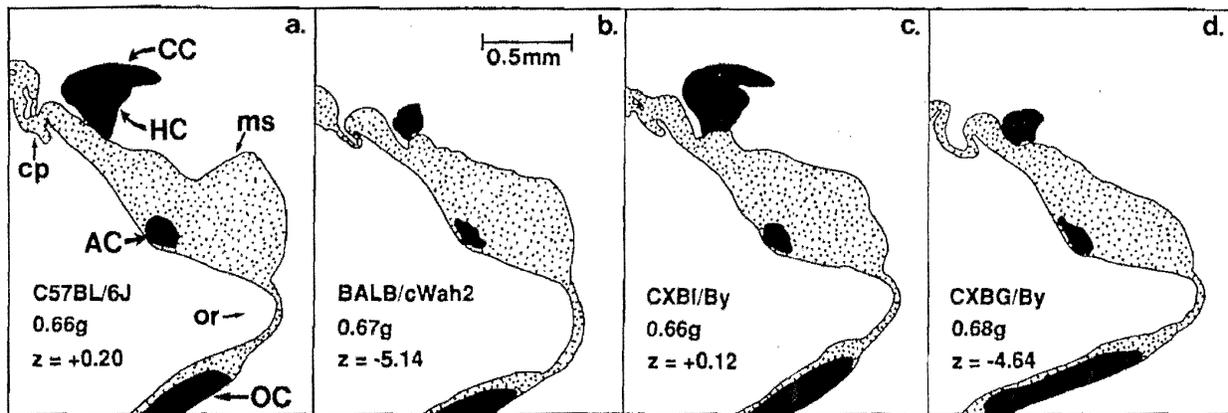

Figure 2. Diagrams of major structures in the forebrain at the midsagittal plane in four fetuses matched for body weight. Fiber tracts are solid black and neural tissue is stippled. The abundant blood vessels and the meninges are not shown in order to give a clear picture of the location of the commissures with respect to other neural structures. The approximate border between the CC and HC is shown by a dashed line. AC = anterior commissure; CC = corpus callosum; HC = hippocampal commissure; OC = optic chiasm; cp = choroid plexus of the third ventricle; ms = medial septum; or = optic recess. Anterior is to the right. (a) Normal C57 fetus at 16.25 days gestation age, where index of abnormality is close to the expected value of zero. (b) BALB/c fetus at 17.0 days gestation age in which CC + HC area is more than 5 standard deviations below the expected value. No CC is present at midplane (c) Recombinant inbred fetus at 17.0 days gestation age with normal CC + HC. (d) Recombinant inbred fetus at 17.0 days gestation age with severe abnormality very similar to the defect seen in BALB/c.
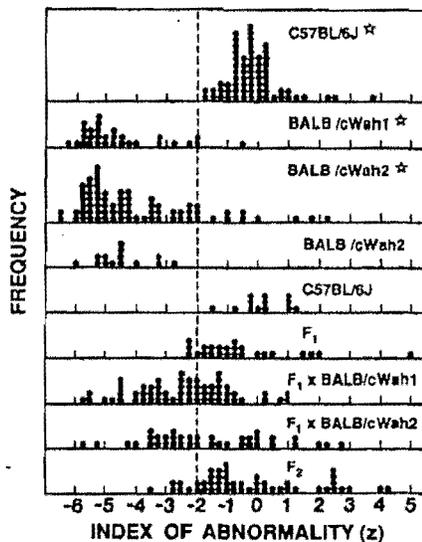

Figure 3. Frequency distributions of the index of abnormality (z) for fetuses in the body weight range 0.50 to 1.00 g. The three groups designated by a star are from a previous study.[21] The groups designated F_1 and F_1 were derived from BALB/cWah2, except for the backcross to BALB/cWah1, where the F_1 hybrid was from substrain 1. The dashed line indicates the lower limit of the region of normal variation in the standard population.
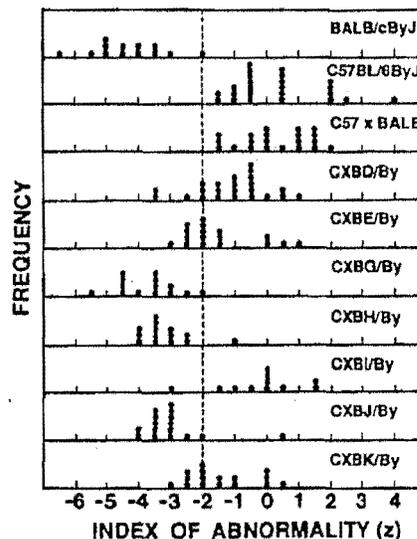

Figure 4. Frequency distributions of the index of abnormality (z) for fetuses from two inbred strains, their F_1 hybrid, and seven recombinant inbred strains. Dashed line indicates the lower limit of the region of normal variation.

Recombinant Inbred Fetuses

Further evidence was sought from the By strains and their seven recombinant inbreds. The BALB/cByJ strain is almost identical genetically with the BALB/cJ strain[14] from which BALB/cWah was derived,[18] and adults show CC defects very similar to those of BALB/cWah. Individual z scores for fetuses are shown in Figure 4. The effectiveness of the z index for the By strains is shown by the remarkable consistency of within-strain variability (Bartlett test, $P = .50$) and the close correspondence of the standard deviations (Table 2) to the expected 1.0. In a one-way analysis of variance on the 10 groups in Table 2, the pooled within-group standard deviation was 1.13, whereas the differences between group means were very large ($F = 35.9$, est. $\omega^2 = 0.70$, $P < .001$). Mean values of the index of abnormality were not significantly different from 0.0 for C57, the F_1 hybrids, or recombinant strain CXBI. The BALB fetuses showed severe abnormalities which were very similar to those of the BALB/cWah fetuses in the classical cross study. Recombinant strains CXBG, CXBH, and CX13.1 had several fetuses in the range for BALB, although H and J were clearly less severely affected than BALB. Strains CXBD, CX BE, and CXBK were significantly below 0.0 but had few abnormal fetuses. A Newman-Keuls post-hoc test on ordered group means revealed the following pattern:

$$\underline{C57 \ F_1 \ I} \quad \underline{D \ K \ E} \quad \underline{J \ H \ G} \quad BALB$$

Strains joined by a solid line were not significantly different at the $\alpha = 0.05$ level. The pattern suggests four groupings, with uncertainty about the group membership of strain CXBG, which conclusively rules out single-locus inheritance. The two clusters unlike either progenitor strain suggest two loci, and variation between extreme strains and all strains yields an estimate of 2.04 loci.[1]

Table 2. Results for fetuses of the By strains and F_1 hybrid

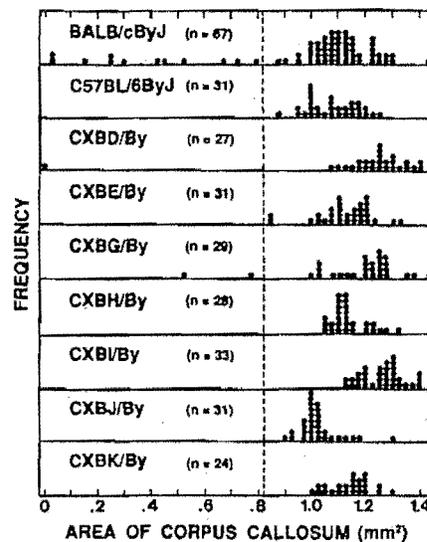| Group	n	Index of CC + HC abnormality (z)		
		Mean	SD	t
BALB	13	−4.23	1.15	−13 3*
C57	24	0.34	1.42	1.2
C57 × BALB	21	0.27	1.09	1.2
CXBD	23	−1.08	1.15	−4.2*
CXBE	17	−1.51	1.14	−5.4*
CXBG	14	−3.70	0.94	−14.8*
CXBH	14	−3.17	0.73	−16.2*
CXBI	11	−0.14	1.30	−0.4
CXBJ	16	−2 96	1.03	−11.5*
CXBK	16	−1.46	0.99	−5.9*

*P < .05, one-tailed.

Figure 5. Frequency distributions and sample sizes (n) of the cross-sectional area of the corpus callosum at the midsagittal plane for weanling and adult mice of two inbred strains and their seven recombinant inbred strains. The dashed line indicates the criterion for abnormally small CC, 0.82 mm².

It is important to note that several fetuses of strain CXBG showed severe defects that were indistinguishable from those of the BALB strain. Figure 2 compares fetuses from CXBI (Figure 2c) *and* CXBG (Figure 2d) that were matched for body weight and external morphological maturity. The C57 and CXBI fetuses are very similar, as are the BALB and CXBG. The anterior commissures are of similar size in all four fetuses in Figure 2, but the *HCs* of BALB and CXBG are obviously much smaller than those of the C57 and CXBI, and the CCs are not visible at all at the midsagittal plane. In both the BALB and CXBG fetuses the axons of the CC were present laterally but had not yet reached midplane, presumably because of the absence of a layer of glia-like subventricular cells, termed the "sling," that normally provides a substrate pathway to facilitate the crossing of CC axons between the hemispheres.[15,21]

Recombinant Inbred Weanlings and Adults

A recent study found that callosal axons are eventually able to cross to the opposite hemisphere and form a fairly large CC, despite severe defects of the sling, in almost all BALB fetuses and that only those with the most extreme retardation of development of structures at the midline of the telencephalon fail to form a substantial CC by the time of birth,[21] If the CC is absent or extremely small at birth, however, it will not recover and will be grossly abnormal in the adult. If this also holds true for the By strains, then Figure 4 leads to the prediction that CC anomalies in adult recombinant inbred mice should be very rare, with the possible exception of strain CXBG.

Individual values of the cross-sectional area of the CC at midline for the By strains are shown in Figure 5, Adult mice more than 90 days old showed no correlation between CC size and age, and their scores are plotted without change. Weanlings had CC areas averaging 74.6% of the adult mean, so their values were multiplied by 1.34 to compensate for immaturity and equate weanling and adult means.[19] According to criteria devised in a previous study,[18] adult mice approximately 100 days old have abnormally small CCs if the area is less than 0.82 mm^2 and the anterior-posterior length is less than about 2.9 mm. That is, a mouse with a long but thin CC is not abnormal in the same way that BALB mice are. The dotted line in Figure 5 indicates the criterion for deficient CC area. In this sample, every mouse below 0.82 mm^2 also had a CC length of less than 3.0 mm. About 18% of BALB/cByJ mice were abnormal, a frequency that is close to the value for BALB/ cWah2. There were two CXBG mice with small CCs, which corresponds nicely to the general predicted pattern. However, there was also one CXBD mouse with no CC at all. The rarity of this surprising event needs to be evaluated with a very large sample. For mice in the normal range, the relation between the average strain z scare in fetuses (Figure 4) and the mean CC size postnatally (Figure 5) was inconsistent.

Discussion

The large difference in CC anatomy between adult BALB/c and C57BL/6 mice is not a consequence of an allelic difference at a single autosomal locus,[18] and the same is true for the degree of retardation of CC and HC development prenatally. Having excluded single-locus inheritance, several possibilities can be evaluated for both adult and fetal situations:

1.   There may be one major locus exerting a deleterious influence on CC development, but its effects may be severe only when the genetic background at many other loci is homozygous because of inbreeding, thereby disrupting developmental homeostasis or buffering.

2.   There may be a large number of relevant loci with recessive inheritance at each, and a mouse must be homozygous for the BALB-type allele at a high proportion of loci in order to suffer defective development.

3.   There may be two loci with recessive inheritance at each, and a mouse homozygous recessive at both loci will show more severe defects than one homozygous recessive at only one locus.

The first hypothesis amounts to a form of epistatic interaction between a major locus and the genetic background. It clearly predicts that the frequency of defective CC will be far below single-locus values in segregating backcross[18] and F_2 groups but much higher and close to a Mendelian ratio in recombinant inbred strains, For adult CC, this hypothesis must be rejected because defects are quite rare in backcrosses and in the CXB/By recombinant inbreds. These seven strains have been inbred for over 70 generations,[1] which is sufficient to yield a high degree of genome purity and inbreeding depression. For retardation of fetal CC and HC development, the hypothesis must also be rejected because (1) the overall frequency of retarded CC + HC in backcrosses to BALB (about half) was quite similar to the frequency in the seven recombinant inbred strains (53 of 111 fetuses abnormal), and (2) the two progenitor and seven recombinant inbred strains showed four clusters, not the two which would result from single-locus inheritance.

The second hypothesis remains a good possibility for the inheritance of the adult but not the fetal defect, In contrast to the rare occurrence of CC defects in adults of backcrosses to BALB and recombinant inbred strains,

retarded CC + HC is very common in backcross and recombinant inbred strain fetuses. There must be many other physiological processes that become involved between *the* fetal period and adult maturity, and many of these probably differ between BALB and C57BL/ 6. The size of the adult CC is affected by early postnatal nutrition,[19,23] litter size,[4] enriched environment,[3,8] and other factors that govern whole brain growth. The CC is a very dynamic structure that loses a high proportion of axons after birth[9,10] but continues to grow by myelination for many months.[11,17]

A two-locus hypothesis fits the fetal data rather well, especially if one locus produces somewhat greater retardation than the other and the retarding influence at each locus is completely recessive. A backcross to BALB should then yield approximately equal proportions of mice homozygous recessive at both, neither, and either of the loci, which, combined with nongenetic variation, should yield a rather wide distribution of z scores with no distinct modes in a sample of moderate size. In a group of seven recombinant inbred strains there should be three or possibly four clusters. It might be possible to compare the two-loci hypothesis with the three-loci hypothesis by examining very large samples in each recombinant inbred strain, but the power of such a test would not be very great because there are only seven recombinant inbred strains. The great potential of the recombinant inbred method can be tapped better by searching for single-locus differences between pairs of strains that show less extreme phenotypic differences than the parent strains. For example, the two-locus hypothesis requires that the difference between BALB and CXBH, CXBE and C5781/6, and CXBJ and CXBK should be monogenic. Comparing fetal data in Figure 4 with strain distribution patterns for the seven CXB strains at many other loci, one locus with identity number 101 might be linked closely to Ly-6. The other locus would have identity number 30. Neither could be a pleiotropic effect of the hippocampal lamination defect (Hid) which also afflicts BALB/c.[12] Of course, linkage cannot be proved conclusively with only seven recombinant inbred strains,[1,16] and further evidence from crosses will be needed.

The recombinant inbred strain method is especially well suited to developmental studies of an anatomical system such as the CC and the midline of the telencephaton, It is expected that recombinant inbred strain differences at earlier stages of development should be simpler genetically[2] and that differences between closely spaced stages should be more likely to show monogenic inheritance. The method also can be utilized to study relations between CC development and behavior. Just as strain distribution patterns of known genes can be compared to a new phenotypic pattern to provide clues about linkage, so can patterns for measures of brain and behavior be compared to detect genetic correlations manifest through development.

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