The Effects of Intrauterine Position on the Degree of Corpus Callosum Deficiency in Two Substrains of BALB/c Mice

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Abstract:

Measures of several intrauterine position variables as well as an index of abnormality of fetal commissure development (z score) were obtained for fetuses of two substrains of BALB mice, BALB/ cWahl and BALB/cWah2, known to differ as adults in the proportion of animals exhibiting deficient corpus callosum (about 55% & 20% respectively). The extent of midline commissure development at embryonic Day 17.5 for most strain 1 fetuses was significantly reduced compared to strain 2 fetuses of the same chronological age. The two substrains also differed with respect to mean litter size and mean body weight (strain 2 > strain 1 for both measures). The ovarian and cervical positions for strain 2 uteri were found to be the most favorable for body and placental growth; no such differences were evident in strain 1. In strain 2, fetuses in the left uterine horns showed lower z scores (more retardation) than littermates on the right side, but this difference was not evident in strain I ; no other right/left differences were found in strain 2 which could help to explain the right side advantage. None of the other position variables either separately or in combination was found to be important in predicting the z score index. Tests for randomness failed to provide evidence for nonrandom distribution of severely affected fetuses. We suggest that nongenetic variability resulting from stochastic events early in development and intrinsic to the fetus may be responsible for only certain BALB fetuses within a litter exhibiting the callosal anomaly.

Article:

Introduction

The corpus callosum (CC) is the largest commissure joining the brain's two hemispheres and, as such, plays an important role in the functional integration of information. Indeed, the demonstration of the disparate functions of the two hemispheres resulted from the work of Sperry and Gazzaniga with "split-brain" patients whose callosa had been surgically severed because of severe and intractable seizures (Gazzaniga & Sperry, 1967). The callosum's role during the course of development is less well understood, although Elberger, in an elegant series of experiments, has demonstrated the importance of the integrity of callosal connections during development for the establishment of a normal visual system in the cat (Elberger, 1988).

For mice of the inbred strain BALB/c the callosum is severely malformed. As adults, a proportion of the animals displays a midsagittal CC area that is very much reduced compared to normal animals and, for a small number of animals, *no* callosal fibres cross midplane. Figure 1 compares midsagittal areas of adult callosa from several typical BALB litters with those from litters of two normal strains of mice. Previous work has shown that the anomaly is completely recessive, is caused by more than a single autosomal gene, and appears to affect males and females to the same degree (Wahlsten, 1982c). Examination of BALB fetuses revealed that almost all exhibit severe retardation of both hippocampal commissure (HC) and CC development when compared with normal fetuses of an equivalent develop-mental age (Wahlsten, 1987a). The problem appears to be with either the generation, proliferation, or migration of cells which normally would provide the scaffold for vanguard fibers crossing over into the opposite hemisphere (Silver, Lorenz, Wahlsten, & Coughlin, 1982). Virtually all

adult BALB mice have an HC of normal size and the CC of many is also in the normal range because pioneering CC fibers traverse midplane using a novel route dorsal to fibers of the young hippocampal commissure (Wahlsten, 1987a).

The expression of the callosal deficit is, to some extent at least, susceptible to environmental perturbations. For example, differences in breeding protocols among laboratories (Wahlsten, 1982a/1982b) can effect quite dramatic changes in the proportion of adult animals showing the defect. Recently, the technique of ovarian grafting was employed to test the hypothesis that the BALB maternal environment was somehow exacerbating an already unfavorable situation with respect to callosal development. The results of that study showed conclusively that, notwithstanding the fact that the brains and bodies of animals nurtured pre-and postnatally by F_1 hybrid mothers were significantly larger than those whose mothers were BALB, the proportion of animals showing defective callosa did not differ between the groups (Bulman-Fleming & Wahlsten, 1988). Yet, this result does not preclude the possibility of prenatal microenvironmental differences for animals *within* a litter which result from the differential placement of fetuses within the uterine horns. Note that differences in fetal development rate among inbred BALB/c littermates are substantially greater than among hybrid mice (Wahlsten, 1987a), which suggests that the maternal environment during CC formation may be important.

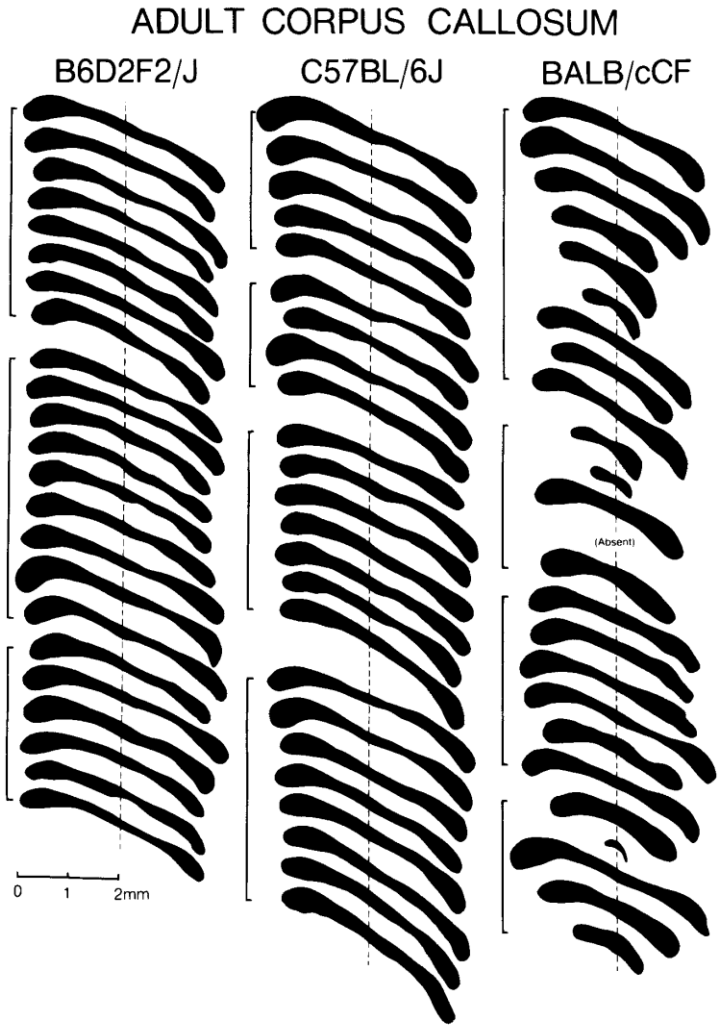


Fig. 1. Outlines of the corpus callosum at the mid-sagittal plane for three strains of mice 100 days after birth. Brackets indicate littermates. Dashed line passes through the middle of the hippocampal commissure for each brain. From Wahlsten (1987a). Reprinted with permission from Wiley-Liss, a division of John Wiley & Sons, Inc. Copyright, 1987.

The intrauterine position phenomenon, discovered by Clemens (1974), refers to the morphological, physiological, and behavioral differences among animals within a sex which are related to intrauterine placement next to fetuses of the same or opposite sex (Babine & Smotherman, 1984; Clark & Galef, 1988; Vomachka & Lisk, 1986; vom Saal, 1981, 1984). The various effects are thought to be mediated through

differences in levels of steroid hormones to which fetuses are exposed in utero. In addition to differences resulting from patterns of sex contiguity, fetuses can experience relatively more or less crowding within the horn, be placed nearer to the ovary or nearer to the cervix, and either in the left or the right uterine horn. These last three variables have also been demonstrated to influence adult morphology and physiology (e.g., Colombo & Giavini, 1975; Healy, McLaren, & Michie, 1960; Kalter, 1975; McLaren, 1963; 0 & Chow, 1987). Of special interest is the high frequency of embryo death and resorption in BALB/c mice. Proximity to nectrotic tissue could influence brain development.

In order to address the issue of correlations between intrauterine position variables and degree of CC deficit, two approaches are possible. Caesarian deliveries could be performed as close to full term as possible, fetuses could be marked to indicate their relative positions, and then fostered to surrogate mothers who had given birth within 48 hr. Animals could then be sacrificed as adults and the extent of adult callosal deficit correlated with variables of intrauterine placement. The second approach, and the one adopted for this study, is to sacrifice the mother and fetuses before full term, code the fetuses, record the position variables and then correlate these with the extent of the *fetal* callosal abnormality. The reasons why we chose this latter approach follow.

Surrogate fostering is indeed feasible and has been successfully accomplished in a past experiment in this laboratory (Bulman-Fleming, Wahlsten, & Lassalle, 1991); however, the BALB strain is not hardy, and results of pilot work for a different project suggested that the mortality rate would be quite high for Caesarean-delivered pups. Because the sequences of scores as well as the individual scores themselves were of interest, the loss of even one fetus from a small litter would have resulted in substantial data loss. Fortunately, during the course of extensive previous work with BALB fetuses and fetuses of other strains which do not exhibit the deficit (Wahlsten, 1987a; Wahlsten & Smith, 1989), a fetal index of abnormality for commissure size was developed which is predictive of adult CC size. These considerations prompted the use of fetuses to investigate the relationship between intrauterine placement and degree of callosal deficit in BALB.

In the experiment described here, we tested the hypothesis that some factor or combination of factors in the intrauterine environment of some BALB fetuses predisposes them *not* to be able to recover from the developmental handicap with which virtually all of them are burdened. In addition, the data were subjected to several statistical procedures in order to test whether, alternatively, placement of severely affected fetuses within the uterine horns is random. The subjects for this study were fetuses of two substrains of BALB/c mice (Wahlsten, 1989), BALB/ cWahl and BALB/cWah2, which differ as adults in expression of the callosal deficit *(55% &* 20%, respectively). It was expected that retardation of HC and CC formation would be more severe in BALB/cWahl and that any feature of the uterine environment which correlates with severity of CC defect within the BALB/ cWah2 strain would have a greater mean effect size in BALB/cWahl .

Methods

Animals

The BALB/cWahl and BALB/cWah2 substrains used to produce fetuses for this study were offspring of animals purchased from Carworth Farms in 1976 and 1977 and were maintained using full-sibling matings in Wahlsten's laboratory at the University of Waterloo (presently located at the University of Alberta). All female mice were between 8 and 16 weeks of age at the time of mating and were nulliparous. The animals were maintained under standard laboratory conditions which have been described previously (Bulman-Fleming & Wahlsten, 1988). Pregnancies were determined by the presence of a vaginal plug. Each female was separated and weighed on the day a plug was found, which was designated Day 0 (E 0) of pregnancy. On E 12 the females were weighed again, and those not pregnant were remated. A total of 23 litters of Wahl and 29 litters of Wah2 was collected, which comprised 116 and 220 fetuses, respectively.

Fetal Extractions

Between 1000 hr and 1200 hr on E 17, each pregnant animal was anesthetized with an overdose of sodium pentobarbital and fetuses were extracted from the uterine horns, weighed to the nearest 0.1 mg, and immersed in freshly prepared Bouin-Duboscq fixative. Placentas were also extracted. As one experimenter was performing

the actual extractions, another worker drew a diagram of the two uterine horns showing placement of fetuses and resorptions relative to the ovary and cervix.

After 48 hr in the fixative, placentas were freed of surrounding membranes, blotted, and weighed to the nearest 0.1 mg. The sex of each fetus was determined at this time by examination of internal sex organs. The fetus was then put in 70% ethanol, with one change of ethanol solution after 24 hr.

Histology and Fibre Tract Measurement

Heads of fetuses were embedded in paraffin and 10 μm sections were cut serially in the sagittal plane, then stained with hematoxylin and eosin. Tracings of the outlines of the CC and HC at the midsagittal section were then made for each fetus and areas were determined using an IBM PC/XT computer, a Hipad digitizing tablet, and the Bioquant morphometry software program (R & M Biometrics, Nashville, TN). Because of shrinkage resulting from our embedding procedure, each area was multiplied by a factor of 1.98 (Wahlsten, 1987a).

An Index of Abnormality

The midline area of the CC alone is inadequate as a measure of retardation of fiber tract development. A small CC area could result from retardation in *overall* growth of the fetus. Littermates of the same chronological age can differ by more than 1 day in measures of morphological age (Wahlsten & Wainwright, 1977). Also known is the fact that the early formation of the HC of BALB is retarded significantly (Wahlsten, 1987a). Furthermore, to distinguish the border between HC and CC in stained sections of fetal tissue is sometimes difficult. Consequently, Wahlsten and Smith (1989) developed an index of abnormality of the combined area of the CC and HC which eliminates the variability due to differences in overall development of the fetus and circumvents the need to distinguish the border between the two fiber tracts. The expected value of the combined midline area of the two tracts is determined for each fetus based on its body weight, accomplished by using a quadratic equation which predicts the combined area of CC and HC from the fetal body weight with great accuracy (goodness of fit, $R^2 = 0.94$). This equation was previously derived using a large group of C57BL/6J and B6D2F_2 fetuses, none of which ever exhibits the callosal anomaly (Wahlsten, 1987a). This expected value of the area CC + HC is given by the following expression:

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

in which E(CC + HC) is the predicted combined area (mm^2) of *CC* and *HC* for a fetus with body weight *X* (g). The dispersion of points about the line of best fit increases as body weight increases and, in fact, is also a quadratic function of body weight (Wahlsten & Smith, 1989). Because of this heteroscedasticity and the nonlinear relationship between body weight and CC + HC, to adopt the much simpler approach of using the untransformed CC + HC area as the dependent variable and body weight as a covariate in statistical analyses was not appropriate. If the squared difference between the expected value and the actual value of CC + HC (residual) is regressed on body weight and the square of body weight, the result is the following equation:

$$E(\text{squared difference}) = 0.00231 — 0.00920X + 0.00940X^2$$

The square root of the quantity on the right is the expected standard deviation of the difference between observed and expected values of CC + HC (Wahlsten & Smith, 1989). Using these two relationships, a standard score (z) can be determined which indicates the number of standard deviations by which a fetus' CC + HC value deviates from the mean (z = 0) of normal fetuses of the same body weight. The following formula was used to determine the z score index for each fetus:

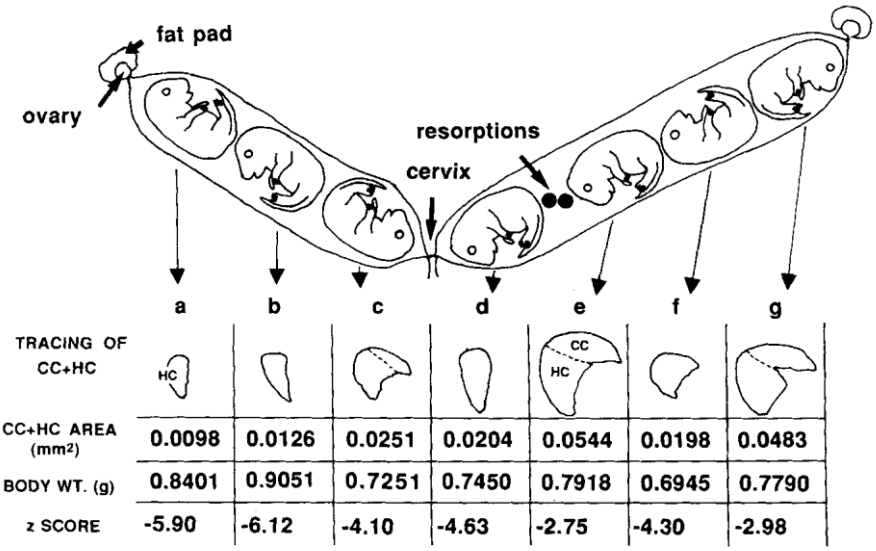
$$z = \frac{(CC+HC) - (0.050 - 0.323X + 0.633X^2)}{\sqrt{0.00231 - 0.00920X + 0.00940X^2}}$$

where CC + HC = actual combined area of the corpus callosum and hippocampal commissure, and *X* = body weight (g) of the fetus. Figure 2 is a diagram which includes the commissure tracings, body weight, and z score data for each fetus in a litter. Although the combined areas of CC and HC for fetuses *b* and *f* are not too

discrepant, *b*'s body weight is much larger than *f*'s, and the z score index for *b* is much lower (— 6.12 as opposed to — 4.3)

Data Analysis

The dependent measures in this study were the z score index, and the body and placental weights of the fetuses. The independent variables were the following: sex, direction of head, uterine (right or left) horn side, number of fetuses in horn, relative position in horn, number of resorptions in horn and next to fetus, proximity to males, and number of neighbors (1 or 2). The data were analysed using the SPSSx and SYSTAT (Wilkinson, 1986) statistical software packages. The litter mean or horn mean value was used as the unit of analysis when testing for differences between the strains or between sides, respectively (Abbey & Howard, 1973); however, the individual pup value was the appropriate unit for tests of intralitter variability.



	a	b	c	d	e	f	g
CC+HC AREA (mm^2)	0.0098	0.0126	0.0251	0.0204	0.0544	0.0198	0.0483
BODY WT. (g)	0.8401	0.9051	0.7251	0.7450	0.7918	0.6945	0.7790
z SCORE	-5.90	-6.12	-4.10	-4.63	-2.75	-4.30	-2.98

Fig. 2. Diagram depicting ventral view of a BALB/cWah2 mouse showing fetuses at embryonic Day 17.5 within the uterine horns. (Right side of diagram is animal's left side.) Note that the z score depends not only on the actual area of corpus callosum and hippocampal commissure (CC + HC) but also on the expected value of the CC + HC based on fetal body weight (i.e., compare fetuses *b* & *f*).

Results

Group Differences Between the BALB Substrains

Figure 3 shows the frequency distributions of the z score index of abnormality of combined CC + HC areas for BALB/cWahl (strain 1) and BALB/cWah2 (strain 2) fetuses. (The z score of 3.84 from one strain 2 fetus does not appear in Fig. 3.) The result of the Kolmogorov-Smirnov two-sample test, which compares the cumulative distributions of two samples (Siegel & Castellan, 1988), was highly significant ($\chi^2 = 48.83$, $df = 2$, $p < 0.001$) indicating that the distributions were significantly different. If the extent of the anomaly were more severe in strain 1 than in strain 2, the mode of the z score distribution (see Fig. 3) of strain 1 would be displaced to the left relative to strain 2's distribution. As is evident from Figure 3, this is not the case; apparently, fewer fetuses from strain 1 have z scores approaching a normal value, thus indicating that fewer animals will recover from the developmental defect.

Table 1 contains mean values of litter size, number of resorptions, body and placental weights, and z scores for the two BALB substrains. Differences between the lines on placental weights and number of resorptions were nonsignificant, although strain 1 had more resorptions than did strain 2 (2.4 vs. 1.9, respectively). The average litter size (not including resorptions) of strain 1 was significantly smaller than that of strain 2 ($t = 4.67$, $df = 50$, $p < 0.0001$), as were the *mean* body weight of strain 1 fetuses ($t = 4.08$, $df = 50$, $p < 0.001$) and strain l's mean z score ($t = 4.71$, $df = 50$, $p < 0.001$). The mean z score values in Table 1 are slightly discrepant from those in Figure 3 because the former values were calculated on the basis of litter mean values whereas the latter represent the mean of all fetuses in the group irrespective of litter membership.

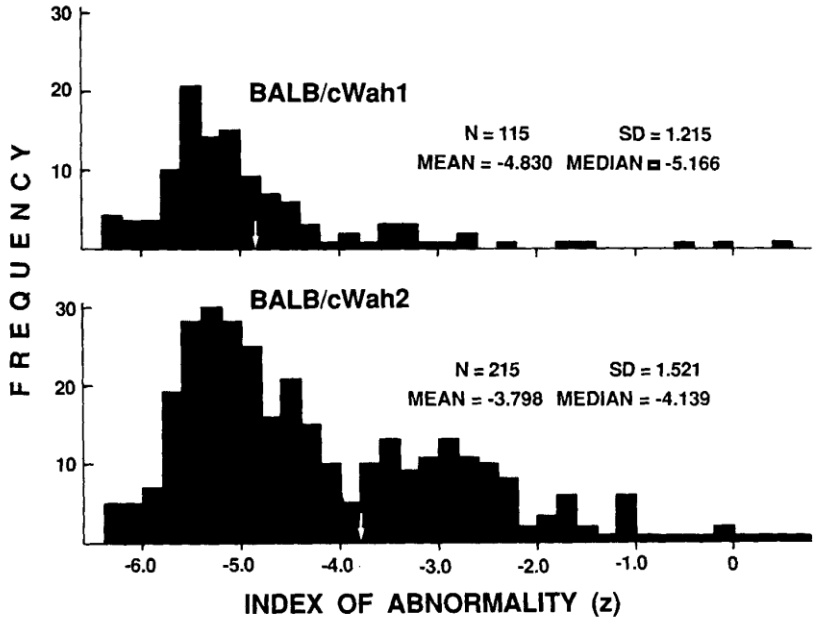


Fig. 3. Frequency distributions of the z score index of abnormality for BALB/cWah1 and BALB/cWah2 fetuses. White arrows indicate means.

Because results from these tests indicated that strain 1 and strain 2 fetuses were two distinct subgroups, most subsequent analyses were carried out separately for each of the two lines.

Table 1
Litter Mean Values (± SD) of z Score, Body and Placental Weights, Number of Resorptions, and Litter Size for BALB/cWah1 and BALB/cWah2 Fetuses and Results of Independent Groups t tests.

Variable	BALB/cWah1	BALB/cWah2	t^a
No. of Litters	23	29	
z Score Index of Abnormality	−4.89 ± 0.69	−3.90 ± 0.80	4.71[b]
Body Weight (g)	0.7149 ± 0.0434	0.7620 ± 0.0397	4.08[c]
Placental Weight (g)	0.1073 ± 0.0239	0.1042 ± 0.0100	0.629
Litter Size	5.04 ± 1.61	7.59 ± 2.18	4.67[b]
No. of Resorptions	2.39 ± 1.56	1.90 ± 1.14	1.32

[a] $df = 50$
[b] $p < 0.0001$
[c] $p < 0.001$, two-tailed

Effects of Sex

Results of *t* tests revealed no significant differences between z scores for males and females in strain 1 or strain 2 (strain 1 males z = — 4.77 ± 1.00; females z = — 4.86 ± 1.37; strain 2 males z = — 3.73 ± 0.97; females z = — 3.80 ± 0.91). Body weights also were not different for males and females in strain 1 (all ps > 0.05); in strain 2, however, the males were heavier *(t* ---- 2.01, *df*25, $p < 0.05$). Male placentas were heavier than those of females for both lines (strain 1: $t = 2.22$, $df = 19$, $p < 0.05$; strain 2: $t = 2.9$, $df = 25$, $p < 0.01$).

Effects of Litter Size

The correlation between litter size and litter mean z score for strain 1 was not significant ($r = 0.26$, $p > 0.05$) but was highly significant for strain 2 ($r = 0.52$, $p < 0.01$). Examination of scatterplots (not shown) of litter mean z scores as a function of litter size for strain 2 revealed that this was the result of two litters of two, both of which had very negative (about — 5.6) z scores. Elimination of these two data points resulted in a correlation of 0.22 *(p* > 0.05). Possibly, systemic factors which caused some females to have abnormally small litters also retarded brain development in the fetuses so that the reasons for the very low z scores in these small litters could be fundamentally different from whatever environmental factor(s) or other influences may be acting to cause retarded development in larger litters. Because very small litters are not informative concerning the effect of uterine position variables, the two litters of two fetuses ($n = 4$) from strain 2 as well as one litter of one in strain 1 were excluded from further analyses.

Litter mean body weights were not significantly correlated with size of the litter for either strain (both p 's > 0.05) whereas a strong inverse relationship between litter mean placental weight and litter size for both lines was evident (strain 1: r = — 0.68; strain 2: r = — 0.64, both $ps < 0.001$). These results are in agreement with those of McLaren (1965) working with C57BL/McL mice of the same chronological age. Between E 17.5 and birth at E 19 or 19.5, fetuses roughly double their weight; the placental weight-litter size correlation most likely is translated into a body weight-litter size correlation during that period.

Uterine Position Variables

Horn Membership

Matched-pairs t tests were conducted on mean values of the right and the left uterine horns for z score, body and placental weights, number of resorptions, and number of fetuses. For strain 1, no differences between the horns for z score, body weight, or number of resorptions were found. The mean number of fetuses in the right horn was significantly larger than that in the left (3.1 vs. 2.2, respectively) and right horn placentas were, on average, heavier than those on the left side (0.1052 g vs. 0.0983 g, respectively). On the other hand, right horn z scores for strain 2 were significantly higher (less negative) than those on the left side (— 3.47 vs. — 4.12, respectively) but none of the other variables which could possibly account for this z score difference for strain 2 (e.g., crowding, number of resorptions) differed between the horns.

Table 2
Results of Multiple Regressions of z Score Index of Abnormality on Measures of Uterine Position for E 17.5 Fetuses of BALB/c Wah1 and BALB/c Wah2 (Bulman-Fleming, 1988)

	t values	
Measure*	BALB/cWah1	BALB/cWah2
Dir	0.61	−0.73
Sex	−1.24	0.05
Body	−1.10	−0.58
Plac	−1.31	−2.95[a]
Side	−0.18	−2.89[a]
Hornno	−0.30	0.55
Pos	0.22	0.65
Rfetus	−0.67	0.23
Mprox	2.75[a]	0.23
End	0.28	0.74
Multiple R^2 (adjusted)	0.03	0.05
Sample Size (fetuses)	101	208

* Abbreviations: Dir—direction of head: 1—toward ovary, 2—toward cervix; Sex—1—male, 2—female; Body—body weight (g); Plac—placental weight (g); Side—uterine horn side: 1—right, 2—left; Hornno—number of fetuses in horn; Pos—position in horn (Endo et al., 1987): 1—ovarian, 2—middle, 3—cervical; Rfetus—number of resorptions next to fetus; Mprox—proximity to males: 0—no male neighbors, 1—one male neighbour, 2—two male neighbors; End—placement with respect to ends of uterine horn: 0—having two neighbors, 1—having one neighbor.

[a] $0.01 > p > 0.001$

Relative Position in the Horn

Fetuses were classified by position as being in either the cervical (C), middle (M), or ovarian (O) portion of a horn according to the scheme of Endo, Goto, and Sakai (1987). Horns containing fewer than three implantations were not included in the analysis. The following orthogonal contrasts were performed: (O + C)/2 vs. M; 0 vs. C. For z scores in both lines, none of the t values reached significance (all $ps > 0.05$). For strain 2, the average of the fetal weights at the ends of the uterine horns was significantly larger than the mean weight of the middle fetuses (t = 3.49, df = 25, $p < 0.01$) and fetuses near the ovary were significantly heavier than those placed near the cervix (t = 3.00, df = 25, $p < 0.01$). Placental weights near the ends of the horn were heavier than those in the middle for strain 2 (t = 2.05, df = 25, $p < 0.05$) but those near the ovary and near the cervix did not differ. These results are consistent with those of other investigators (McLaren & Michie, 1959; Healy et al., 1960;

McLaren, 1965; Kalter, 1975). Conversely, strain 1 bodies and placentas exhibited none of these position effects.

Within-Horn Variables

Correlations between z scores and other uterine position variables (direction of head, number of fetuses in horn, resorptions in horn and next to the fetus, & proximity to males) were nonsignificant (all *ps* > 0.01) in spite of the large sample sizes, and were often inconsistent between the lines. Because of the number of correlations performed and the consequent probability of obtaining a spurious significant relationship, the alpha level was set at 0.01. Possibly, it is not one or two factors, but rather a combination of many factors which makes the difference between a high and a low z score. To test this possibility, multiple regressions of z scores on several measures of uterine position were performed for each of the two lines. Because some measures (e.g., number of resorptions next to the fetus & number of resorptions in the horn) were highly correlated and would have introduced problems of multicolinearity (Pedhazur, 1982), not all were used in the regressions. As above, the alpha level was set at 0.01. Table 2 shows the *t* values obtained for the multiple regressions of z score on the uterine position variables for each of the two strains. Although placental weight and horn side for strain 2 as well as proximity to males for line 1 reached statistical significance, there was no meaningful pattern overall. Furthermore, the adjusted multiple R^2 for the regression for strain 1 was less than 0.03 and for strain 2, less than 0.05. It appears that, either there exists an important factor or factors not measured or that placement in the uterine horn of fetuses having severe retardation of CC + HC development is not related to variations in the uterine environment.

Table 3
*Results of the Runs Test for Randomness on Sex and z Score Index of Abnormality for BALB/cWah1 and BALB/cWah2 Fetuses**

	BALB/cWah1				BALB/cWah2			
Two Element Set	r	E(r)	SD(r)	z[a]	r	E(r)	SD(r)	z[a]
SEX: male/female	81	79.40	2.55	0.63	128	126.22	4.22	0.42
z SCORE INDEX[b] ≤ median of strain/> median of strain	72	70.98	2.09	0.49	130	126.27	4.43	0.84

* Abbreviations: r—actual # of runs; E(r)—expected # of runs given a random distribution of the two-element set; SD(r)—standard deviation of E(r).
[a] Test statistic for the runs test for randomness.
[b] Index of abnormality for the combined area of corpus callosum and hippocampal commissure (CC + HC).

Tests for Randomness

The Runs Test

Although it cannot be demonstrated by the results of a single test that the distribution of a given element is random, it is possible to demonstrate statistically that the distribution in a series of two types of elements does not differ significantly from what would be expected assuming a random distribution of the two elements. If each fetus is classified on the basis of its z score as being in 1 of 2 groups (elements)—severely affected and not-so-severely affected—a runs test for randomness can be performed. The number of runs in a sequence of elements of two kinds can be defined as "a subsequence of elements of the same kind which is both preceded and followed by either an element of the opposite kind or by no element" (Kalbfleisch, 1979). For a given number *n* of elements ($n = a + b$ where *a* and *b* are the two types of elements) and a given frequency of *as* and *bs* in a series, the expected value of the number of runs as well as the variance can be determined (Brunk, 1965). Assuming the right and left horns are independent, the expected values and variances for the number of runs can be determined separately for each horn, and (making use of the equality $E(\Sigma) = \Sigma(E)$, where E = expected value), summed over all horns to produce a test of randomness for placement of 2-element sets. The distribution of the standard score, produced by subtracting the sum of the expected number of runs from the sum of the actual number of runs and then dividing this quantity by the square root of the sum of the variances of the number of runs, approaches a normal distribution as n approaches infinity (Brunk, 1965). If, for example, factors were clustering severely retarded fetuses within horns, relatively fewer runs would result thus leading to a negative standard score; conversely, a systematic alternation of severely affected and other fetuses would result in a greater number of runs than would be expected on the basis of randomness, producing a positive test statistic. Standard scores were obtained for the following two 2-element sets: 1) males and females and 2) z scores ≤ median z score for that strain and z score > median z score for that strain. The results of the runs test

(Table 3) show no evidence for a nonrandom distribution of sex or of high and low z score indices of callosal abnormality in either of the BALB sub-strains. This result is expected for the distribution of sex because other investigators have obtained similar results (vom Saal, 1981; Endo, Goto, & Sakai, 1987; but see Clark & Galef, 1990).

z Score Differences Between Neighbors

A method using the continuous z index may provide a more sensitive test. One useful statistic is the difference between z scores of adjacent fetuses within a uterine horn, symbolized $d_{ij} = z_i = z_{j=i+1}$. If the more severely affected animals tend to be clustered together, the Σd^2 should be relatively small. On the other hand, if very small patches of the uterine environment confer severe abnormality, the Σd^2 should be rather large.

When *n* fetuses are arranged in a sequence, the variance of the difference *d* is estimated by $s_d^2 = \Sigma d^2/(n - 1)$, because there are *n* — 1 differences among *n* fetuses and the expected value of the sum of the mean differences is zero. If the variance of the *n* scores without regard to order is simply σ^2, then it can be shown2 that the expected value of the variance of the differences, when ordering of z scores is random, is $E(s_d^2) = 2\sigma^2$. The observed s_d^2 can then be compared to $2\sigma^2$ with a chi-squared test. The number of fetuses in any one horn is far too low for a reasonable test. There may be systematic differences in mean z score between substrains or even left and right uterine horns, but there is no reason to believe any position effect within a horn should differ markedly for substrains or sides. Therefore, it should be possible to combine data across the 63 horns having three or more fetuses. The value of s^2 = est σ^2 obtained this way is simply the error variance from a one-way ANOVA. For the z index of abnormality, this s^2 is 1.941 with 197 degrees of freedom, and twice this is 3.882. For the 260 fetuses, 197 of which are adjacent, s_d^2 is 4.198. The two values are close indeed, but are not really independent. If $\sigma^2 = 3.882$ is taken as the population value, $\chi^2 = 213.04$, and the critical value of χ^2 is 237.8 when $\alpha = 0.05$, using a normal approximation for large degrees of freedom. This indicates no significant departure from a random distribution, but the test is somewhat dubious because the distribution of z scores is obviously skewed and 3.882 is an estimate of the population parameter.

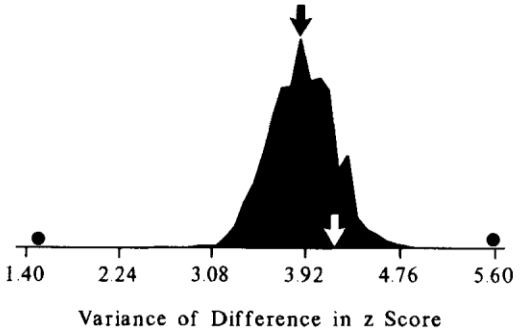


Fig. 4. Frequency distribution of the variance of the differences of z score indices of abnormality between nearest neighbors when order of z scores was randomized within horns 1000 times. The black arrow indicates mean variance of the differences for 1000 randomizations and the white arrow indicates actual variance of the differences for our data. Also shown are the values when the data in each horn are rank ordered (1.492) and when they alternate high and low ranks (5.532).

In this situation a randomization test is appropriate (e.g., Tarone, 1989). The order of z scores within each horn was randomized using the ran0() function from Press, Flannery, Teukolsky, and Vetterling (1988) to minimize sequential dependencies, and s_d^2 was recomputed across the 63 horns. One thousand repetitions of this procedure yielded the frequency distribution in Figure 4. The mean of this sample (3.883) was very close to the expected value (3.882). The observed value of 4.198 was at the 87th percentile, which amounted to a two-tailed probability of 0.26. Also shown in Figure 4 are the values of s_d^2 that occur when the data in each horn are rank ordered, which yields the smallest possible value (1.492), and when they alternate high and low ranks, which gives the largest possible value (5.532). All 1000 of the random values as well as the obtained $s_d^2 = 4.198$ are far from these nonrandom outcomes. Thus, the uterine location of fetal z scores does not depart significantly from a random distribution.

Discussion

The two BALB substrains exhibit differences in mean litter size, body weight, and z score index of abnormality. Both the litter sizes and body weights of strain 1 animals were smaller than those of strain 2 and the development of CC and HC in strain 1, as measured by the z score index, was more severely retarded compared with the Wah2 substrain. In addition, though the difference did not reach significance, strain 1 litters contained more resorptions than strain 2. In the eighth generation of a breeding program in Wahlsten's laboratory involving full-sibling matings for 13 BALB substrains, the proportion of Wahl animals having a defective callosum rose dramatically (from about 14% to about 50%), and has stayed at that level to the present time. This was probably a result of a mutation, and if so, may well pertain to basic events very early in development. It has been suggested that rate of cleavage (for which a gene or group of genes has been identified in mice) could influence reproductive performance as well as postimplantation loss in mouse embryos (Wilmut, Sales, & Ashworth, 1986). The effects of this putative single gene difference between our two BALB substrains certainly are not unique to retardation of CC development, as evidenced by the litter size and body weight differences .

Virtually 100% of BALB fetuses exhibit an unusual bulge or gap at the telencephalic midline and the scaffold for pioneering callosal axons, which is present in strains showing normal CC development, is conspicuously absent (Wahlsten, 1987a). This means that for BALB, vanguard fibers must find innovative pathways by which to cross to the contralateral hemisphere (Wahlsten, 1987a). Fibers which never find their way to the opposite side course through the ipsilateral hemisphere to form a Probst bundle. The great variation in CC development within a genetically uniform inbred strain argues strongly against any notion of a "blueprint" for brain development. Yet, plasticity obviously has its limits. Inspection of Figure 3 suggests a possible threshold for the degree of CC and HC retardation which allows eventual recovery from the developmental anomaly. Fewer Wahl fetuses at E 17.5 have attained that crucial level of development so that more animals have defective callosa as adults. The threshold corresponds to an index of about z = — 5.5.

This is the first experiment from our laboratory in which fetal sex has been determined. The lack of observed differences between male and female z scores in these data is in agreement with previous work on adult BALB. The observed difference between male and female body weights in strain 2 (male > female) and the lack of such a difference in strain 1 is most likely a manifestation of the relative retardation of strain 1. Both lines exhibited significant differences with respect to placental weights (males > females), a result which has been reported previously (Ward, Karp, & Aceto, 1977).

The finding of the extremely low z scores for fetuses in very small litters (n = 1 or 2) merits further study. Of course, quite possibly the result could be accounted for by sampling error since only five fetuses are involved. More data from very small litters will be needed (which may prove difficult for strain 2) in order to resolve this question.

The higher z scores for fetuses occupying the right uterine horns compared with those in the left horns in strain 2 is especially puzzling in the absence of other evidence of asymmetry. An experiment which investigated left/right asymmetry in ovarian and uterine function in random-bred Swiss-Webster mice and a hybrid strain found a right side advantage for ovulation rate, number of fetuses, and survival of transferred embryos, as well as differences between the two groups in the extent of the right side advantage (Wiebold & Becker, 1987). Conceivably, the effect on z scores seen in strain 2 was not merely a result of sampling error, although the fact that no such z score effect was seen in strain 1 casts doubt on this interpretation and brings into question the generalizability of the effect to other substrains of BALB.

Data from the present study concerning fetal weights and uterine position support the haemodynamic theory which was first proposed by Eckstein, McKeown, and Record (1955) to account for local as opposed to systemic effects on guinea pig weight. In our data, the end positions (ovarian & cervical) contained fetuses and placentas which were heavier than those occupying middle positions, at least in strain 2. That neither fetuses nor placentas in strain I showed these effects may be a result of that line's general retardation developmentally or of

strain l's smaller litter sizes, which would not be expected to result in large within-horn differences in blood pressure (McLaren, 1965).

Apart from the relatively higher z scores observed for fetuses in the right horns as opposed to the left in strain 2, no intrauterine position effects on z score were detected for either strain. Neither was a combination of intrauterine factors responsible for the z score variability, as evidenced by the extremely low adjusted squared multiple *R* in each of the multiple regressions (both <0.05). The question remains then: What are the factors acting to predispose only certain fetuses within a litter to exhibit the ability to recover from the prenatal defect with which they are virtually all afflicted?

Because the animals used in this study can be considered to be, at least within each strain, genetically homogenous (Wahlsten, 1989), genotype cannot contribute to this variability. Possibly, either some crucial aspect of the intrauterine environment exists which was not measured in this study, or there is no such aspect, in which case a third source of variability must be hypothesized. In order to provide evidence for the existence of such a third factor, it is necessary to show that the z scores of fetuses are distributed randomly within the uterine horns. In this study, the number of sequential runs of more severely affected fetuses and the variance of differences in z score between nearest neighbors do not depart significantly from randomness. While remaining open to the possibilities that even larger samples or measures of complex interactions among features of the uterine environment will reveal nonrandomness, we believe there are sufficient reasons to hypothesize that variance within these BALB/c substrains is neither genetic nor environmental.

This putative third source of variation, if not environmental, must be inherent to the fetus but not genetic (Lewontin, 1982). The existence of nongenetic individuality in bacterial cells has been claimed and can be explained by stochastic (chance) differences in small numbers of generator molecules which can ultimately give rise to large differences in enzyme concentrations (Spudich & Koshland, 1976). Similar differences in the end result, with initial starting conditions which only differ to a very small degree, can also occur as a result of chance in multicell systems as a result of cell-cell interactions (Wahlsten, 1987b). Kurnit, Layton, and Matthysse (1987) reported that computer simulations modeling cardiac malformations can produce quite different end results (i.e., defect or no defect). The parameters of these simulations were identical but the model included a stochastic component. The authors state that "the final outgrowth of large numbers of cells depends sensitively on probabilistic interactions among small numbers of cells and growth clusters, thereby amplifying the effects of initial stochastic variations and introducing a significant role for chance" (p. 983). They point out that an 8-day mouse embryo comprises a sufficiently small number of cells (about 104) so as to be subject to variations, similar to the ones observed for cardiac septal defects, in the fate of large groups of cells (Kurnit et al., 1987). For BALB, stochastic variations in small numbers of cells at the telencephalic midline during a critical period in development could conceivably result in the seemingly random occurrence of absent or extremely small CC in adult animals.

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