Defects of the Fetal Forebrain in Mice With Hereditary Agenesis of the Corpus Callosum

By: Douglas Wahlsten


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Abstract:
Inbred BALB/c mice are genetically the same, yet less than half of adults show absent or small corpus callosum. Is this because only a minority has prenatal defects of the sling at the telencephalic midline, or do most fetuses have a defective sling but some are able to form a corpus callosum via some other substrate pathway? This question was addressed by comparing large samples of BALB/c fetuses at 17, 18, and 19 days after conception with a series of normal C57BL/6 and hybrid fetuses matched for body size.

At 17 days postconception almost all BALB/c fetuses show an unusual widening or bulge in the interhemispheric fissure anterior to the hippocampal commissure. Furthermore, formation of the hippocampal commissure is greatly retarded, although it eventually attains a normal size in adult mice. At 17 days, when mice of normal strains all have a corpus callosum at midplane, almost every BALB/c fetus lacks the structure, but 1 day later 67% of fetuses show delayed formation of this structure and by 19 days all but 7% of fetuses have some callosal axons crossing the midsagittal plane. Many BALB/c fetuses are able to form a corpus callosum without the benefit of a normal sling. The degree of delay of axon crossing is strongly correlated with the severity of sling defects. An unusually small adult corpus callosum occurs because fetal axons are able to follow unusual pathways and actively compensate for absence of the sling, not because of arrested midline development.

Key words: strain difference, hippocampal commissure, anterior commissure, cavum septi, axon guidance

Article:
Hereditary agenesis of the corpus callosum is a useful tool for exploring the processes that allow axons to traverse the region between the cerebral hemispheres. Fetuses of the BALB/c mouse strain that fail to form a corpus callosum also suffer from a malformation or absence of a band of cells termed the "sling" (Silver et al., '82). This evidence, together with study of normal anatomical development and effects of surgical interference with cells near the telencephalic midline prior to arrival of callosal axons (Silver et al., '82; Silver and Ogawa, '83; Hankin and Silver, '86), suggests that a normal sling is necessary for normal development of the corpus callosum.

There is a problem with the interpretation of development of the BALB brain, however. Even though mice of that strain are genetically the same, only a minority show absent or small corpus callosum as adults (Wahlsten, '74, '82a). The occurrence of "incomplete penetrance" of this defect casts doubt on explanations that focus on a single aspect of prenatal development. Because inheritance of the defect is clearly multifactorial rather than a single gene effect (Wahlsten, '82b), several developmental processes are probably abnormal.

The present study sought to discover differences among fetal BALB brains that might account for later differences in adult brain structure. Because the corpus callosum could not be assessed at one prenatal stage and then hours or months later in the same mouse, large samples of mice from closely matched litters were studied at successive ages. Mice from other strains at the same developmental ages served as a normal comparison
group. This approach made it possible to correlate anatomical features seen at one age with abnormalities present later in development and to make a strong case for a causal connection between them. In this respect, the study regarded incomplete penetrance of the hereditary defect as a help rather than a hindrance. The inherent variability in BALB brain development served as a noninvasive experiment of nature with a superb control condition: genetically identical littermates conceived and nurtured in the same mother at the same time.

Two contrasting questions were asked. (1) If a large sample of BALB mice is assessed at a particular fetal age, will the sling and associated structures be reasonably normal in many of these animals, and will the frequency of sling defects approximate the frequency of overt corpus callosum defects in adults? (2) Do most BALB fetuses suffer serious sling defects, but in many of them CC axons manage to cross between the hemispheres anyway? If the former, then sling defects must be a major cause or even the sole cause of aberrant corpus callosum. If the latter, other processes must be important.

The study was not designed to clarify the exact histological nature of the "sling" cells. These cells have been variously described as radial glia and primitive astrocytes forming a dense mat under the corpus callosum (Rankin and Silver, '86), as a "region of loosely organized glia-like tissue" made up of "cells from subventricular zone" and containing macrophages and astrocyte processes (Valentino and Jones, '82), and as an extension of the subependymal layer containing astrocytes, many of which are degenerating (Zaki, '85). Rather, the main objective was to observe the consequences for axon growth when the normal zone of these cells failed to extend to the telencephalic midline.

**MATERIALS AND METHODS**

**Animals**
Mice of the strains BALB/cCF and C57BL/6J, each inbred for over 100 generations, were bred and maintained in the author's colony as described in detail elsewhere (Wahlsten, '82b). Matings to produce these animals for the present study were between brother and sister, except for a few between cousins within the same family. B6D2F1/J mice, the offspring of a C57BL/6J female mated with a DBA/2J male, were obtained from the Jackson Laboratory and mated to produce B6D2F2/J hybrid animals. The inbred C57 mice and the F2 hybrids have no major brain defects. The F2 hybrids were from the same cross used for the normal comparison group in previous studies of prenatal development (Wahlsten, '81, '84).

**Design**
At least two litters of F2 mice and four litters of C57 mice were obtained at 16.0, 17.0, and 18.0 days from conception and 100 days after birth. Because the BALB mice were known to lag behind the F2 hybrids by about 1 day of developmental age in fetuses (Wahlsten and Wainwright, '77), five litters of BALB mice were obtained at 17.0, 18.0, and 19.0 days from conception and four litters at 100 days after birth. One or two litters of F2 or C57 mice were also obtained at the half-day intervals E16.5 and E17.5 to fill in gaps in the distribution of fetal sizes. This was not necessary for BALB because of great individual variability. For the C57 and especially the BALB mice, an effort was made to ensure that litters from mice in the same family were distributed equally across the four principal age groups. All of the female parents in this part of the study were 100- to 120-day-old virgins at the time of mating.

A larger sample of BALB mice was obtained in a separate series that used the same methodology as the first series, except that the mother mice were somewhat older. Thirteen litters were obtained at 18.0 days from conception and 14 at 19.0 days, with equal contributions of each family of BALB mice to each age. In this second series, litter sizes were slightly smaller and average body sizes were slightly larger than for BALB mice in the first series, but relations between body size and brain development were very similar.

A few BALB litters at various prenatal ages were obtained for plastic embedding and sectioning, and a few adult mice were also used for silver staining of axons. This tissue was used for detailed histological study and photographs but was not used in the statistical analysis.
Collection of fetal and adult brains

The time of conception was determined within 2 hours by checking for vaginal plugs at 4-hour intervals. Conception (E0.0 days) was defined as the time midway between detection of a plug and the previous plug check. After mating, the female was isolated until testing. At the designated time, the pregnant female was anesthetized with a pentobarbital sodium overdose, and the uterus was removed and immersed in cold saline. Each fetus was dissected from the surrounding membranes, rinsed in saline, blotted gently to remove excess fluid, and then weighed to the nearest milligram. Every third fetus in most litters was then marked in the midbrain for determination of shrinkage during histology, as described below. The fetus was then fixed by immersion in Bouin-Duboscq fixative for 48 hours. After 2-5 minutes in fixative, the scalp of the fetus was removed and small slits were made in the skull about 2 mm lateral and parallel to midline in order to aid fixation of the brain. Adult mice at P100 days were anesthetized with pentobarbital sodium and perfused intracardially with saline followed by 10% neutral-buffered formalin. The fixed brains were removed from the skull, trimmed to a standard configuration (Wahlsten, '83), blotted, and weighed to the nearest milligram.

<table>
<thead>
<tr>
<th></th>
<th>B6D2F2/J</th>
<th>C57BL/6J</th>
<th>BALB/cCF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Females</td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Sample size</td>
<td>11</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Body wt. (g)</td>
<td>21.3 ± 0.4</td>
<td>26.6 ± 0.4</td>
<td>20.5 ± 0.5</td>
</tr>
<tr>
<td>Brain wt. (g)</td>
<td>0.454 ± 0.006</td>
<td>0.464 ± 0.004</td>
<td>0.465 ± 0.004</td>
</tr>
<tr>
<td>CC area (mm²)</td>
<td>1.004 ± 0.020</td>
<td>1.082 ± 0.036</td>
<td>1.168 ± 0.027</td>
</tr>
<tr>
<td>HC area (mm²)</td>
<td>0.201 ± 0.006</td>
<td>0.211 ± 0.005</td>
<td>0.233 ± 0.006</td>
</tr>
<tr>
<td>AC area (mm²)</td>
<td>0.117 ± 0.003</td>
<td>0.126 ± 0.004</td>
<td>0.129 ± 0.003</td>
</tr>
</tbody>
</table>

CC, corpus callosum; HC, hippocampal commissure; AC, anterior commissure.

Fig. 1. Outline of the corpus callosum (CC) at the midsagittal plane for 100-day-old (P100) mice of three strains. The vertical brackets indicate littermates. Vertical dashed line passes through the centre of the hippocampal commissure (HC) such that the anterior-posterior location of CC is standardized for all mice. Anterior is to the right in this and all other figures showing CC in the sagittal plane.
**Histology**

Prior to embedding, the head was removed from each fetus and the skull lateral to the longitudinal slits was peeled away to allow rapid penetration of solutions. It was important to leave most of the skull in place because blood vessels and other tissues in the interhemispheric fissure are usually torn if the brain is removed from the skull. The brain with the dorsal skull intact was then embedded in paraffin wax; serial sections were cut sagittally at 10 μm; and tissue was stained with hematoxylin and eosin. Adult brains were cut frozen at 33 μm in the sagittal plane and stained with metachromatic thionin. For several BALB fetuses, brains were embedded in glycol methacrylate (Sorvall), sliced at 2μm in various planes, and stained with methylene blue and basic fuchsin. Several adult C57 and BALB mice were perfused with ethanol/trichloroacetic acid/ammonium hydroxide, and the whole brains were then impregnated with silver nitrate following the Ranson method (Foerster, ’82). After staining they were embedded in paraffin and sectioned at 10 μm; sections were then mounted, cleared of paraffin, and coverslipped.

**Correction for shrinkage**

The degree of shrinkage of the brain tissue during fixation and embedding was determined by piercing the midbrain region of a fetus with a cluster of four fine needles arranged in a square with tips 1.0 mm apart. The needle cluster was clamped in a holder fastened to a small stereotaxic tower, and it was lowered into the brain of a fetus placed on its left side so that the needles penetrated the skull just dorsal to the right ear. This resulted in a pattern of four small holes in the midbrain region that could be seen in a stained sagittal section. Because the measures of interest in this study were cross-sectional areas, the correction for area was obtained by comparing the area of the rectangle formed by the four, pins in the stained tissue to the original area of 1.00 mm². This method also corrected for compression of the tissue by the knife during sectioning. For brains that were not marked with the pins, the average shrinkage factor of all marked brains was used, because the degree of shrinkage did not vary significantly with strain or fetal size.

**Correction for angle of cutting**

Because of the opacity of paraffin, the fetal brains often were not sliced exactly in the sagittal plane. The deviation of the actual plane of cutting from true sagittal was determined from four landmarks: a) the anterior cerebral artery dorsal to the corpus callosum, b) the third ventricle just ventral to the anterior commissure, c) the cerebral aqueduct just ventral to the poster commissure, and d) the infundibulum. For each landmark, the section was found where it was at the midsagittal plane, and its distances from standard X and Y axes were measured. The four points measured in three dimensions were then analysed with vector geometry to determine the angle (0) of deviation from the true sagittal plane. For a fibre tract with measured cross-sectional area of A, the corrected area was A’ = Acos0. This correction was only appreciable when the angle of deviation was more than 10°, which was uncommon, and in any event it was much less than the correction for shrinkage.

**TABLE 2. Mean ± SD (n) for Body Weights of Fetuses at Several Ages**

<table>
<thead>
<tr>
<th>Age</th>
<th>B6D2F2/J</th>
<th>C57BL/6J</th>
<th>BALB/cCr</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.0</td>
<td>0.557 ± 0.028 (19)</td>
<td>0.537 ± 0.071 (14)</td>
<td>0.552 ± 0.060 (32)</td>
</tr>
<tr>
<td>17.0</td>
<td>0.782 ± 0.044 (27)</td>
<td>0.714 ± 0.098 (32)</td>
<td>0.788 ± 0.083 (37)</td>
</tr>
<tr>
<td>18.0</td>
<td>1.045 ± 0.088 (20)</td>
<td>0.928 ± 0.104 (20)</td>
<td>1.353 ± 0.120 (20)</td>
</tr>
<tr>
<td>19.0</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

**TABLE 3. Results of Multiple Linear Regression Analysis for Predicting Areas of CC and HC From Six, Three, or One Independent Variables for Combined Data of B6D2F2/J and C57Bl/6J Fetuses: Each Value is an F Ratio**

<table>
<thead>
<tr>
<th>Predictors</th>
<th>CC area 6 3 1</th>
<th>HC area 6 3 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>3.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Age</td>
<td>0.3</td>
<td>1.2</td>
</tr>
<tr>
<td>No. of sire</td>
<td>3.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Body weight</td>
<td>235.3*</td>
<td>36.4*</td>
</tr>
<tr>
<td>Quad. area</td>
<td>0.3</td>
<td>16.3*</td>
</tr>
<tr>
<td>AC area</td>
<td>24.1*</td>
<td>10.6*</td>
</tr>
<tr>
<td>Multiple R²</td>
<td>0.991</td>
<td>0.889</td>
</tr>
</tbody>
</table>

*F < .05
Measures of structure sizes
Outlines of the corpus callosum (CC), the hippocampal commissure (HC), and the anterior commissure (AC) were traced at the midsagittal plane with a Leitz tracing device on a Dialux microscope, and then cross-sectional areas of the structures were determined and corrected for shrinkage and angle of cutting where appropriate. The area at mid-plane of the quadrangle formed by the four landmarks listed in the above paragraph was determined by using vector geometry.

RESULTS
Adult brains
Measures of the 100-day-old animals are summarized in Table 1. The BALB mice had larger body and brain sizes than the other two groups, which demonstrates they did not suffer from poor nutrition or any other pervasive deficit in growth. BALB mice also had larger cross-sectional areas of the hippocampal and anterior commissures, and the larger sizes of these commissures were approximately proportional to the larger brains of BALB. These data are important for the interpretation of commissure size in fetuses. For example, if a BALB fetus of a particular body weight has an HC that is much smaller than the HC of an F2 or C57 fetus with the same body weight, this indicates retardation of HC development relative to growth of the whole fetus, because the HC of adult BALB mice actually tends to be larger than the HC of other strains.

The only striking abnormality of the BALB adult brain at the midplane was deficiency of the CC, as depicted in Figure 1. The cross-sectional area of CC was sometimes quite small, and in other instances the area was within normal limits (Wahlsten, '82b) but the shape was unusual, being thicker in the region of the truncus of CC than was ever seen in the F2 and C57 brains. In the present sample of 25 BALB brains, only ten had both size and shape similar to F2 and C57 mice. The extreme variability in CC anatomy is not a consequence of genetic variation in this BALB strain (Wahlsten, '78), and it has persisted despite many generations of inbreeding. Furthermore, the deficient size of the CC of some mice within the BALB strain is not associated with lower brain weight (Wahlsten, '82a) and is not accompanied by even a minor increase or decrease in the adult size of the anterior or hippocampal commissure (Wahlsten and Jones, '83).

Fetal development
Morphological age. A summary of body weights for fetuses at the principal ages is given in Table 2. As expected from previous research (Wahlsten and Wainwright, '77), body weights of BALB mice were close to those of F2 mice that were 1 day younger, whereas C57 mice grew only a little less rapidly than F2 mice of the same age. However, F2 mice at one age and BALB mice 1 day older were not equivalent developmentally, because BALB mice were about four times more variable than F2 mice in body weight, a difference that was highly significant at all three ages \((P < .005)\). A similar pattern occurred with C57 mice. Hence, the genetically uniform BALB and C57 mice were more variable in body size and growth rate than the genetically variable F2
hybrids, a clear example of developmental homeostasis or canalization (Waddington, '59). Because of this, the overall degree of fetal development of different strains could not be equated simply by comparing fetuses at specific ages.

A previous study had equated the degree of maturity by using aspects of external morphology such as eyes, ears, skin, and paws (Wahlsten and Wainwright, '77), but the time scale based on these measures was sensitive only to about E16.5 for F2 hybrids. Two observations showed that body weight was a good indicator of overall fetal maturity in the present study. First, comparisons of body weight and morphological age in BALB fetuses collected for an earlier study (Silver et al., '82) revealed a close relationship (see Fig. 2). Second, multiple linear regression analysis (Nie et al., '75) revealed that a large fraction of the variance in CC or HC area in normal F2 and C57 mice was associated with variation in fetal body weight. The analysis was done separately with either one or three or six independent variables in the equation, as shown in Table 3. With all variables considered simultaneously, there were no significant contributions of strain difference, fetal age, or litter size to the equation. This by no means implies that CC area does not vary with strain, age, or litter size; on the contrary, it grows rapidly with age and tends to be smaller in C57 mice of a given age and in larger litters. However, there
is no significant association between CC size and strain, age, or litter size when body weight is also taken into account. The area of AC is significantly related to CC area, even when body weight is also taken into account, but the increase in predictability, as measured by the multiple $R^2$ that occurs when AC area is included as a predictor is very small. Likewise, area at midplane of the quadrangle formed by the four landmarks used to measure the cutting angle enhanced predictability of HC by only a small amount. Consequently, the overall degree of morphological maturity of fetuses from different strains can be equated accurately and conveniently by comparing fetuses with the same body weight, and F2 and C57 data can be pooled to form a normal comparison group.

**Fibre tract growth.** Cross-sectional areas of three forebrain fibre tracts in relation to fetal body weight are portrayed in Figure 3 for individual BALB mice and for the pooled sample of F2 and C57 mice, which was used as the normal comparison group. The F2 and C57 data are summarized by a regression equation of best fit as well as confidence limits obtained by drawing lines that included 98% of normal mice. Any BALB fetus occurring below the lower limit is regarded as having a fibre tract that is unusually small for its body size. Figure 3a reveals that the anterior commissure grew at a reasonably normal rate for BALB fetuses less than 1.0 g, whereas a small degree of retardation was evident in larger BALB fetuses. The hippocampal commissure (Fig. 3b), on the other hand, was greatly retarded in the smaller BALB fetuses and was frequently below normal limits in larger fetuses. This clearly was a case of temporary delay of development, because the structure was not unusually small in adult BALB mice, and hence it must have recovered from the prenatal growth deficit. The CC of BALB fetuses (Fig. 3c) was greatly retarded in forming at midplane. Every F2 and C57 fetus heavier than 0.45 g had some CC axons crossing midplane, but no BALB fetus lighter than 0.60 g had any discernible CC at midplane. The smallest BALB fetus having a CC area within the normal limits weighed 0.75 g. Many BALB fetuses between 0.75 and 1.00 g had no CC at midplane, but total absence of CC was as rare in those above 1.00 g as it is in adult BALB mice.

Statistical comparison of a large sample of matched BALB fetuses at E18.0 and E19.0 (Fig. 4), consisting of over 18 litters at each age, revealed that the frequency of total CC absence was much higher at E18 than E19 ($z = 4.6$, $P < .00001$). However, litter sizes were not significantly different at the two ages ($t = 0.43$, $P > .05$).
Hence the dramatic change in frequency of absent CC was not caused by differential mortality. These data show unequivocally that many animals that lacked a CC at midplane on E18 did form one belatedly by E19.

Normal anatomy. In order to judge abnormalities of forebrain structure in BALB fetuses, the appearances of the CC and related structures were first determined for the F2 and C57 fetuses at various ages and body weights. Because there was relatively little variation in forebrain structures in these fetuses when equated for body weight, 11 individuals having the best histological quality of the stained tissue were used to establish a sequence of normal development for body weights from 0.49 to 1.16 g. Sections for three F2 fetuses near the mean body sizes for E16, 17, and 18 are shown in Figure 5. One section is at midplane, another is at the point where the CC passes ventral to the indusium griseum, and the third shows the CC just medial to the first occurrence of the cingulum. Small black arrows indicate the borderline between HC and CC. Large black arrows indicate the slit. Bar: 0.2 mm.

One feature of the normal brains was obvious: The cavum septi pellucudum (CSP) was absent or extremely small in fetuses of less than 0.60 g. The CSP of mice near 0.75 g was evident as a large gap ventral to the CC at midplane (Fig. 5d) and was often littered with cells having darkly staining nuclei, but it did not extend more than 50 μm on either side of midplane and was not evident ventral to the indusium griseum (Fig. 5e). When fetuses had reached 1.00 g, the CSP was very large at midplane (Fig. 5g) and extended quite far laterally (Fig. 5i). The very rapid expansion of this fluid-filled cavity may be partly a result of tissue shrinkage during dehydration and embedding, but the consistent location and age-related appearance of the structure demonstrate its reality in the fetal brain prior to fixation. The cavity did not appear to be open into the subarachnoid space in any normal fetus.
In E16 hybrid mice near 0.60 g, the structure identified as the "sling" by Silver et al. ('82) was evident laterally (Fig. 5c) as an agglomeration of small, darkly stained cells. The putative CC axons passed over these cells and through the larger spaces between them. In more mature fetuses the lateral sling was clearly visible as a dense clustering of cells ventral to CC (see Fig. 5f) with bands of cells penetrating almost the entire thickness of the CC. Beneath the indusium griseum the sling appeared as a loose clustering of darkly staining cells (Fig. 5e). In E18 hybrid fetuses the sling was usually absent from the more caudal portion of the CC (Fig. 5h,i).

![Image](image_url)

**Fig. 6.** Midsagittal sections of brains of a normal B6D2F1/J fetus (a) at E16 weighing 0.64 g and two BALB fetuses at E17 weighing 0.68 g and 0.66 g (b,c); a: The large white arrow indicates the CC and the smaller white arrow indicates the cavum septi. b: The small white arrow indicates a narrow gap dorsal to the HC and ventral to a branch of the anterior cerebral artery (black arrow). c: The white arrows indicate erythrocytes filling the gap around the HC. Bar: 0.2 mm.

**TABLE 4. Frequency of Six Types of Defect of the Gial Sling in BALB Mice**

<table>
<thead>
<tr>
<th>Age</th>
<th>None</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.0</td>
<td>0</td>
<td>12</td>
<td>20</td>
<td>13</td>
<td>17</td>
<td>6</td>
<td>88</td>
</tr>
<tr>
<td>19.0</td>
<td>5</td>
<td>2</td>
<td>36</td>
<td>18</td>
<td>17</td>
<td>11</td>
<td>99</td>
</tr>
</tbody>
</table>

Includes only fetuses 0.75 g or larger.

**BALB brains at E17.** Of the 42 BALB fetuses examined at E17, only two had the beginnings of a CC at midplane and almost every one had an obvious defect dorsal and anterior to the HC where a distinct gap or bulge occurred (see Fig. 6b). It varied in size from a narrow fissure next to an HC of nearly normal size and extending laterally about 30 μm to a large cavity surrounding a diminutive HC (Fig. 6b). In seven brains with very small HC the cavity had many erythrocytes floating in it (Fig. 6c), apparently the result of hemorrhage, but these blood cells did not spill into the subarachnoid space. In brains where the cavity extended laterally more than 50 μm there were axons of the CC approaching midplane but they stopped short, usually a few microns medial to the most lateral extent of the cavity, and they had no or very few cells of the sling in contact with them. Further laterally, equivalent to the region shown in Figure 5c, the sling of these fetuses generally did not have any evident abnormalities.

**BALB brains at E18.** The nature of this gap or bulge was most clearly revealed in coronal plastic sections of a 0.76-g BALB fetus having no CC axons crossing midplane. At a region about 0.5 mm anterior to the HC, the gap was evident in two respects (Fig. 7a). Dorsally, there was an obvious bulge in the longitudinal cerebral fissure at the zone where CC axons were near midplane. In this region the meninges were attached to cortical tissue by fine processes. Ventrally, the walls of the septal region were separated by a narrow groove from which
the meninges had detached. This groove closely resembled the sulcus medianus telencephali medii identified in human fetuses by Rakic and Yakovlev (‘68). The unusual bulge in the longitudinal fissure in the septal region was apparent as far as 0.75 mm anterior to the HC. At the level of the AC, about 0.15 mm anterior to the HC, the bulge in the longitudinal cerebral fissure was very large, especially where many CC axons were approaching midplane (Fig. 7b). At the dorsal portion of the gap, many CC axons made contact with the meninges but had not grown medially along this tissue. The fibres at the ventral portion of the large bundle of CC axons were in direct contact with axons of the fornix, because of the absence of the usual subcallosal sling cells, but they did not fasciculate along them or enter the septum.

Comparison with another BALB fetus of 0.38 g at E16 revealed an interesting fact. In a coronal section through the AC (Fig. 8), at the same level as Figure 7b at E18, a bulge was present between the columns of the fornix, which had not yet reached the AC. Although the cortical plate and the entire medial cortex were much thicker at E18 than E16, the width of the zone extending from the most medial angle of the lateral ventricle (star) to the longitudinal cerebral fissure, the zone of the sling in normal fetuses, changed little in this period. While nearby tissues were growing rapidly, this zone was not, and these differential growth rates, combined with lack of fusion of hemispheres, created a large bulge in the longitudinal cerebral fissure at the very location where CC axons arrived by the thousands.

![Fig. 7. Coronal plastic sections of a BALB fetus at E18, 0.76 g. Section in a is about 250 μm anterior to the one in b, which passes through the anterior commissure (AC) and is about 150 μm anterior to the HC. In a the small arrow indicates a groove where the hemispheres are not fused and meninges are not attached. Large arrow indicates a bulge in the longitudinal cerebral fissure at the region where axons of the CC approach midplane. Meninges are still attached by fine processes here and more dorsally. In b the CC axons form a large bundle that terminates in a very large bulge in the longitudinal cerebral fissure. There is a wide gap between the two columns of the fornix (F). CC axons at the ventral portion of the bundle (small arrows) contact axons of the fornix but do not fasciculate along them into the septum (S). Star indicates medial aspect of the lateral ventricle (LV) corresponding to the same region in Figure 8. Methylene blue–basic fuchsin. Bar: 0.4 mm.](image)

Among the 102 brains examined at E18, many had formed a CC at midplane, and several of these showed no signs of the gap dorsal to HC that was so prevalent and distinctive at E17. However, when the CC was present, the cavity ventral to CC and anterior to HC was also present and was extraordinarily large compared to the CSP of F2 and C57 brains for fetuses weighing less than 0.80 g. Of particular interest at E18 were the many brains in
which the CC axons had not yet reached midplane. In virtually every case, the CC axons were clearly present laterally but had not yet made the traverse (Fig. 7b).

**BALB brains at E19.** Most fetuses at this age had formed a CC at midplane, but in certain cases this clearly occurred late in development. Figure 9 shows the location of crossing in two large fetuses with a very small CC but a large CSP. Neither brain had any sling apparent within 300 μm of midplane. The CSP of all BALB brains at this age was very large but was within the range of values for F2 and C57 fetuses. Among BALB fetuses with a substantial CC, it was difficult to perceive any defect at midplane that might give some sign of the gap above HC seen at E17; the gap had been filled by axons. In those seven of 103 fetuses at E19 that still had no CC at midplane, the putative CC axons were present laterally in a tangled, disorderly array from which, in several cases, emerged a large bundle of CC axons growing anteriorly and close to the longitudinal cerebral fissure. In three of the seven brains the closest CC axons were still more than 100 μm from midplane. It is highly unlikely that any of these CC axons would ever have reached midplane, had the mice lived longer. They showed clear signs of formation of a longitudinal Probst bundle quite far from midplane and ventral to the cingulum, which is characteristic of adult BALB mice with total absence of the corpus callosum (see Lent and Schmidt, '86: Fig. 3c).

![Image](image.png)

**Defects of the sling.** Many of the E18 and E19 BALB fetuses with a substantial CC nonetheless showed abnormalities of the sling. These defects were especially clear in fetuses weighing at least 0.75 g when the cells were examined where the CC passed ventral to the medial portion of the cingulum, because in normal brains there were usually remnants of the sling even in the most mature fetuses at this region after the cells had largely disappeared near midplane. The degree of defect was judged against brains of a standard series of F2 and C57
fetuses weighing from 0.76 to 1.16 g. The appearance of a typical F2 brain is shown in Figure 10a. There was a large number of small cells with dark nuclei ventral to the CC. Emanating dorsally from this group of cells were numerous other small cells arranged in radial interdigitating rows between large bundles of CC axons. The CC axons themselves were grouped in rows with a clear radial orientation, and each axon bundle was nearly perpendicular to the midsagittal plane.

Abnormalities in BALB mice were categorized as six different types for ease of description, although in reality there was a continuous gradation of defects.

Type I: Rows of cells and bundles of CC axons were present in radiating rows, but the layer of cells in the sling below the CC was markedly thinner than in F2 and C57 fetuses.

Type II: This was like type I, except that rows of cells and axons were quite curved or wavelike (Fig. 10b).

Type III: A thin layer of sling cells was present under all or most of the CC anterior to HC. Fewer cells were present within the CC than type I and only a small fraction of these were organized in radiating rows. A few bundles of CC axons travelled longitudinally.

Type IV: Sling cells were sparse and lined only a portion of the CC. Cells within the CC were present in moderate numbers but not in radiating rows. Many axons were grouped in longitudinal bundles (Fig. 10c).

Type V: Very few sling cells were present, and only a few cells were scattered in the CC. Axon bundles were scrambled, coursing in many directions.

Type VI: No sling was present at all and there was a distinct gap under the CC. There were very few cells within the CC, especially near its ventral edge. Axons were scrambled (Fig. 10d).

The frequency of each type of defect for BALB fetuses at two ages is given in Table 4. There were only five among the total sample of 187 fetuses weighing 0.75 g or more that had no perceptible defect. Another 70 had the very mild type I or II defects. Thus, about 37% of fetuses showed either no or mild defects, a proportion that is similar to the frequency of adult BALB mice with a CC having normal cross-sectional area and shape. The larger fetuses with type III or IV defects usually had a substantial CC present at midplane, despite the clear abnormality of the sling.

The similarity of the distributions of type of sling defect at E18 and E19 suggests that these morphological abnormalities were not transient. In fact, closer study of adult BALB brains with a Ranson silver stain revealed many distinctly longitudinal bundles of axons in the genu of the CC in a location never observed in normal strains (see Fig. 11). These unusual bundles must be the enduring consequences of a prenatal sling defect that modified CC morphology but not its eventual size. Some of them were probably perforating fibres that leave the cingulum and enter the ipsilateral septum by passing through the CC (Rankin and Silver, '86).

The type of sling defect showed an interesting relationship with growth of the CC and HC. This was revealed by linear regression of commissure cross-sectional area on body size separately for fetuses with each type of sling defect. As shown in Figure 12, the HC was smaller in animals with more severe midline anomalies, but rate of HC growth was similar in all categories. This meant that midplane defects had a retarding influence on early formation of HC but did not impair its later growth. Because of this, the HC was able to reach normal size in all adult BALB mice (Table 1). The situation was different for the CC. Fetuses with type IV defects showed delayed crossing of CC axons but normal growth rates, whereas those with type V defects had delayed crossing and slower growth rates. Most of the fetuses with a type VI sling defect had no CC at midplane at all, but a few of them had acquired a very small CC by E19 (see Fig. 9).
As the BALB fetuses grew, anomalies became evident further and further from midplane. By E19 several brains showed large gaps in the subventricular layer near the lateral ventricle (Fig. 13a) where CC axons from many regions of the cerebral cortex converged and formed a disordered neuroma (Fig. 13b). Presumably this secondary anomaly resulted when rapidly growing CC axons were unable to traverse the hemispheres because of the midplane bulge, much as ganglion cell axons in the ocular retardation mutant mouse form large whorls when they are unable to leave the eyeball (Silver and Robb, '79). The convergence of axons from anterior and posterior regions of the cerebral cortex into a zone slightly dorsal and anterior to the HC makes it highly likely that in mice that belatedly form a very small CC at midplane, the CC contains axons from many regions of the cortex. This is confirmed by tracing of CC axons in adult BALB mice with very small CC, which shows a normal topographic distribution but greatly reduced numbers of cells of origin (Serra-011er et al., '86).

DISCUSSION
The principal anomaly of CC formation in the BALB mouse appears to occur at or near the telencephalic midline. The distinct layers of the cerebral cortex in the normal hybrid mouse (Crandall and Caviness, '84) are present in BALB/c, and CC axons approach the midsagittal plane at an appropriate time. The region near the most medial portion of the lateral ventricle is very similar to that of normal C57 and hybrid mice at earlier developmental stages. However, as the CC axons approach midplane, they encounter a large fluid-filled gap or bulge that is sometimes engorged with blood and that often separates the HC from the pial membrane. The width of the gap increases rapidly with age and eventually resembles the septal cavity of normal hybrid fetuses. In BALB mice with a large gap the sling identified by Glas ('75), Silver et al. ('82), and Zaki ('85) does not form or is extremely fragmentary.

The gap is of special interest because it is present in almost every BALB fetus at E17 days, whereas only a small minority of adult animals in this inbred strain lacks a corpus callosum. The abnormal gap shows nearly complete "penetrance" of the BALB heredity, and hence it is a good candidate for single gene inheritance. Inheritance of absence or deficiency of CC in adult BALB mice is recessive and multifactorial (Wahlsten, '82b), so it is likely that several distinct developmental defects must occur jointly in order to produce a severe deficit in the adult.
The midline gap in the E17 BALB fetus may impair formation of the CC, but it is not clear what produces the gap itself. It is always difficult to localize the time and place of first action of a genetic defect, and this difficulty is compounded when the defect is genetically complex. From the present data it is not known whether something peculiar about the surface features of cells impairs migration from the ventricular layer of the lateral ventricles or whether the proliferation of putative sling cells from the lateral ventricles is itself deficient.

Despite the presence of the gap, a CC forms in most BALB fetuses between E18 and E19, which means there is recovery from or compensation for a severe deficit in the sling and associated structures. In some fetuses it is obvious that the CC axons cross over the anterior portion of the dorsal surface of the hippocampal commissure (Fig. 9) without any assistance of a sling. Apparently, the later the CC axons cross midplane, the smaller will be the CC in the adult. However, if the axons do not cross by E19, they never will. The frequency of E19 fetuses with no CC at midplane is about the same as in adult BALB mice (Wahlsten, '82a, '84). This threshold effect

Fig. 10. Sagittal sections of CC ventral to the most medial location of the ingulum. White arrows indicate remnants of the sling: a: A normal Balb/De2F2 hybrid brain at E18, 1.18 g. The black arrow shows radial rows of interdigitating cells. b: A BALB brain at E19, 1.12 g, with a type II sling defect. The black arrow shows wavy rows of cells and axon bundles. c: A BALB brain at E19, 1.30 g, with a type IV sling defect. The black arrow indicates ependymal axon bundles. d: A BALB brain at E19, 1.47 g, with a type VI sling defect. This is the same brain shown in Figure 9b. Bar: 0.2 mm.

Fig. 11. Sagittal sections of the genu of CC lateral to midplane in adult mice. The black arrows indicate the stria longitudinale: a: A normal C57 mouse with axon bundles in the CC perpendicular to the sagittal plane. b: A BALB mouse with longitudinal axon bundles (white arrows) that are presumably a sign of an earlier type IV sling defect. Ranson silver stain. Bar: 0.2 mm.
must be the consequence of an interaction of two separate processes—the formation of the sling and growth of the septal cavity. In normal mouse strains the sling forms and CC axons cross before the cavity appears. The cavity appears later and expands rapidly to form a large volume ventral to the CC (Fig. 5), but it does not interfere with subsequent CC growth because this occurs largely by fasciculation along axons that have already crossed. In BALB fetuses, on the other hand, the gap and deficient sling retard crossing of the CC axons, and then the gap enlarges rapidly to form a septal cavity. If incoming CC axons are not able to traverse the gap when it is relatively small, they cannot do so later when it has become an immense cavity (Fig. 12).

These observations require a revision of previous notions about the developmental events producing callosal agenesis. Loeser and Alvord ('68) reviewed the anatomy of adult human brains with varying degrees of CC agenesis and concluded that partial agenesis was produced by "a midline lesion occurring quite late" in development. Likewise, Stefanko and Schenk ('79) attributed partial CC agenesis to "arrested normal development," an opinion also adopted by Wahlsten ('81), Milner ('83), and Jeeves ('84). This opinion, derived indirectly from the study of abnormal adult brains, is not supported by data from prenatal development of abnormal BALB mice. In these animals the primary lesion occurs relatively early, even in those that later acquire a small CC. Compensatory processes enable some fetuses to overcome the early defect and grow to be seemingly normal adults, although a closer look at the large CC of some adults (Fig. 11) reveals signs of an earlier prenatal malformation. Partial CC occurs when the axons cross midplane relatively late in development, but this does not indicate any kind of "arrest" or cessation of development. On the contrary, it is the continuing growth of axons and development of midline structures that enables so many fetuses to form a CC despite an early midline defect. This sequence of events provides further evidence that plasticity of early development is an important source of a normal adult pattern of brain structure and that comparable patterns can often be established via quite different sequences of events.

Fig. 12. Lines of best fit for predicting CC and HC areas from body weight for BALB fetuses in the given weight range. Separate lines are shown for fetuses with different types of sling defect.
The wide range of midline defects seen in BALB fetuses may help to clarify certain questions about the formation of the CC. There is some disagreement about where the first axons cross midplane. Some investigators maintain that the first wave of CC axons crosses over the hippocampal commissure (Valentino and Jones, '82). The present findings (e.g., Fig. 9) prove unequivocally that this can happen in BALB mice with no other neural tissue anterior to the hippocampal commissure, but this in no way proves that the first axons normally do cross in this region. Evidence suggests otherwise, because crossing exclusively via the HC occurs quite late in development and results in a very unusual adult CC, one that is diminutive and contiguous with the HC over most of its length at midplane. Earlier researchers (e.g., Kallen, '54; Rakic and Yakovlev, '68) argued that axons of both HC and CC cross in a commissural plate, a thickening of the "lamina reuniens." More recent studies of mice using improved histological methods and closely spaced series of fetuses have modified this view somewhat. Glas ('75) concluded that axons cross not in but over a midline structure termed the area septalis and under the meninx primitiva. He found that the first crossing of the hippocampal commissure is "situated in the dorsocaudal edge of the area septalis" and that the early corpus callosum "borders dorsally the area septalis without belonging to it" (p. 74). He also noted that there is no clear borderline between HC and CC at midplane and that the two form one dorsal commissure at E16. Silver et al. ('82) observed that CC axons enter the septal region and cross via a pathway called the "sling" that extends over the septum anterior to the hippocampal commissure. They noted that a few early CC axons cross caudal to the sling and "blend imperceptibly" with the HC. The sling has been observed by Silver and colleagues in fetal rats (Katz et al., '83) and cats (Silver et al., '85). Finally, Zaki ('85) also observed that "the subependymal layer between the medial angles of the ventricles forms a sling on top of which the corpus callosum fibres cross" (p. 137). The present findings with BALB mice confirm the importance of a complete slinglike structure dorsal to the septum, because the absence or fragmentary nature of that structure at the very least leads to a corpus callosum of highly unusual morphology characterized by many axons parallel rather than perpendicular to midline. They provide further evidence of the importance of substrate pathways, especially subventricular cells (Schneider and Silver, '86), for guidance of axons over relatively long distances (Katz et al., '80, '83), especially in regions where axons with an intrinsic tendency to grow straight (Katz, '85) must make an abrupt change in direction of growth.

This study has shown how the phenomenon of incomplete penetrance of a hereditary anomaly can be helpful in dissecting complex processes of brain development. In most previous studies of genetic defects research has concentrated on instances where the effects of a particular genotype are relatively consistent across individuals. This approach raises serious problems of interpretation when consequences secondary to a primary lesion are of interest, because every cell in the brain carries the aberrant genetic material and may therefore develop anomalously because of an intrinsic defect. Studies of chimeras have helped to separate the effects of defects intrinsic to a cell from those produced by interactions with other defective cells. Incomplete penetrance is especially promising as a means of further examining interactions at the cellular level, because the brains of normal and anomalous littersmates have the same genetic constitution but different epigenetic outcomes. This approach has certain advantages over surgical or pharmacological interventions in that defects may sometimes be better confined in time and space. Destruction of the sling by surgery (Silver et al., '82; Silver and Ogawa, '83) or gamma radiation (Lent and Schmidt, '86) and sectioning of the corpus callosum at a very early age (Lent, '83, '84) produce anomalies of the adult CC that closely resemble the most severe defects in BALB mice, but it is difficult to interpret these results because surgery and radiation inevitably impinge upon many other cells and membranes other than those of primary concern. Incomplete penetrance cannot supplant more invasive procedures; rather it can supplement them and help to interpret results obtained with various methods.

Besides being a useful analytic device, incomplete penetrance can tell us many things about the role of heredity in brain development (e.g., Stent, '81; Wahlsten, '82c). In particular, it reveals how concepts such as a genetic "blueprint" for brain structure (e.g., Lorenz, '65; Singer et al., '79) are teleological, inflexible, and incapable of explaining the multitude of differences between individual brains. The phenomenon further demonstrates the important role of epigenetic interactions between cells during development (e.g., Rakic, '84; Edelman, '85; Mikami and Onishi, '85).
Fig. 13. Sagittal plastic sections of a BALB fetus at E19, 1.33 g, at the most medial location of the lateral ventricle (LV), about 1.0 mm from midplane. The fetus has a type V sling defect and a small CC crossing midplane. In axons from both anterior (Ant.) and posterior (Post.) cerebral cortex converge into a whorl of axons forming the CC, which is located in a large gap in the subventricular zone (SVZ). Enlarged picture of inset is shown in b. H, hippocampus; HC, hippocampal commissure; IZ, intermediate zone; S, septum; VZ, ventricular zone. Methylene blue-basic fuchsin. Bars; 0.4 mm.

LITERATURE CITED


