

## Recombinant inbreeding in mice reveals thresholds in embryonic corpus callosum development

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Wahlsten, D., Bishop, K.M., and Ozaki, H. (2006) Recombinant inbreeding in mice reveals thresholds in embryonic corpus callosum development. *Genes, Brain and Behavior*, 5: 170-188.

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### **Abstract:**

The inbred strains BALB/cWah1 and 129P1/ReJ both show incomplete penetrance for absent corpus callosum (CC); about 14% of adult mice have no CC at all. Their F<sub>1</sub> hybrid offspring are normal, which proves that the strains differ at two or more loci pertinent to absent CC. Twenty-three recombinant inbred lines were bred from the F<sub>2</sub> cross of BALB/c and 129, and several of these expressed a novel and severe phenotype after only three or four generations of inbreeding – total absence of the CC and severe reduction of the hippocampal commissure (HC) in every adult animal. As inbreeding progressed, intermediate sizes of the CC and the HC remained quite rare. This striking phenotypic distribution in adults arose from developmental thresholds in the embryo. CC axons normally cross to the opposite hemisphere via a tissue bridge in the septal region at midline, where the HC forms before CC axons arrive. The primary defect in callosal agenesis in the BALB/c and 129 strains is severe retardation of fusion of the hemispheres in the septal region, and failure to form a CC is secondary to this defect. The putative CC axons arrive at midline at the correct time and place in all groups, but in certain genotypes, the bridge is not yet present. The relative timing of axon growth and delay of the septal bridge create a narrow critical period for forming a normal brain.

**Keywords:** Complementation test, corpus callosum, critical period, hippocampal commissure, incomplete penetrance, recombinant inbred strains

### **Article:**

The corpus callosum (CC) is a large bundle of axons that conveys information between the two cerebral hemispheres of placental mammals. Many molecular genetic mechanisms involved in guiding axons toward and across the cerebral midline are known (Kaprielian et al. 2001; Nieto 1996; Stein & Tessier-Lavigne 2001), and the major anatomical steps in the normal ontogeny of the CC have been outlined (Rakic & Yakovlev 1968; Richards 2002). Several components of the midline developmental system have been explored with the aid of more than 30 targeted mutations of mouse genes that impair formation of the CC (Campos et al. 2004; Ha et al. 2005; Richards et al. 2004; Steele-Perkins et al. 2005), and more than 35 genes have been implicated in human syndromes that often involve callosal agenesis (Richards et al. 2004).

Several inbred mouse strains express CC absence, including BALB/c, 129, I/LnJ (Wimer 1965; Lipp & Wahlsten 1992; Magara et al. 1999; R.E. Wimer personal communication) and BTBR T/+ tf/tf (Wahlsten et al. 2003b). BTBR was formed by Lyon (1956) and then maintained by outcrossing to a 129 strain by Dunn (Flaherty, personal communication) followed by inbreeding, and microsatellite markers indicate that BTBR is most closely related to the 129 group of strains (Wahlsten et al. 2003b). Recent evidence suggests the role of a major X-linked gene in the CC defect of BTBR (Kusek et al. 2003).

One of the most remarkable features of CC defects in the mouse is the extraordinary developmental variability seen in the strains BALB/c and 129. Despite the identical genotypes of mice within a highly inbred strain, the developmental outcomes range from animals with no callosal axons crossing midline at all to mice that appear to be anatomically normal, and the degree of incomplete penetrance in a strain remains relatively stable over

many generations (Wahlsten 1974, 1982a, 1989). This non-genetic variation might reflect some inherent instability in the gene-related developmental processes of these strains, but it could also arise from complex interactions of embryonic axons with their surroundings. We applied recombinant inbreeding to demonstrate that incomplete penetrance arises from a developmental threshold in the embryo that magnifies the ubiquitous, microscopic individual variation seen in all embryos into dramatic, macroscopic abnormalities in some adult mice that have anatomically normal littermates.

Recombinant inbred (RI) strains are widely used for mapping genes and demonstrating genetic correlations involving neural and behavioral phenotypes (Broman 2005; Crabbe et al. 1994; Crusio 2004; Williams et al. 2001). When the difference between the two progenitors of a set of RI strains involves more than one major gene, recombination of multiple genes may create novel phenotypes not present in either progenitor (Seyfried et al. 1980), and, as we show here, can create abnormalities more severe than those seen in either progenitor. Rather than starting the experiment by crossing progenitors differing greatly in their phenotypes, as is the common practice when doing linkage analysis, we began with two inbred strains, BALB/cWah1 and 129P1/ReJ, that suffer from almost identical, relatively mild forebrain commissure defects and then generated both normal and extremely abnormal phenotypes. Genetic mapping studies are done after more than 20 generations of full sib mating of an RI line, whereas the focus in this study was on the rapid changes in brain development over the first few generations of inbreeding.

The BALB/cWah1 and 129P1/ReJ strains have similar degrees of incomplete penetrance of absent CC, but they differ at two or more autosomal loci that are important for abnormal CC development (Livy & Wahlsten 1991), and their alleles exhibit a recessive inheritance in crosses with C57BL/6J (Lipp & Wahlsten 1992; Wahlsten 1982b). RI strains derived from progenitor strains that differ at two major loci will end up with one of four genotypes with continued inbreeding, and there could be  $2^L$  different homozygous genotypes if they differ at  $L$  loci. Considering the simplest situation of two major loci where BALB is  $a/a+/+$  and 129 is  $+/+b/b$ , recombinant mice with the hypothetical  $a/a b/b$  genotype should be more severely defective than either of the progenitor strains, and about 25% of RI lines should show this novel phenotype. Both predictions were supported by our set of RI lines.

The events leading to the traverse of the interhemispheric fissure by callosal axons in normal mouse and rat embryos have been documented in considerable detail. Because there are substantial differences among inbred strains as well as inbred vs. hybrid genotypes in the overall rate of embryonic development, equivalent to more than 24 h of development at 16 days of chronological age (Wahlsten & Wainwright 1977), we find it more accurate to express the timing of important events in the mouse embryo with respect to body weight rather than chronological age (e.g. Livy & Wahlsten 1997). The hemispheres begin to fuse at the anterior edge of the third ventricle (lamina terminalis), leading to a gradual thickening of the septal region at the mid-sagittal plane (midplane) that is apparent in normal mouse embryos of 0.25-g body weight or more (Glas 1975; Silver et al. 1982; Wahlsten & Bulman-Fleming 1994). The first population of axons to cross in the dorsal septal region is the hippocampal commissure (HC) (Livy & Wahlsten 1997), which first appears at midplane in normal mouse embryos of 0.35–0.4-g body weight. Meanwhile, putative callosal axons emerge from their cells of origin in several regions of cerebral cortex in embryos of about 0.45 g of body weight, and they grow at a rate of 1–4 mm/day toward the midplane crossing point (Ozaki & Wahlsten 1992). Prior to arrival of CC axons near midplane, a wedge-shaped population of glial cells forms, extending from the medial edge of the lateral ventricles toward midplane (Shu & Richards 2001). These cells are anatomically distinct from the later-forming glial sling (Silver et al. 1982); they express glial fibrillary acidic protein and the Slit-2 protein, and the glial wedge repels cortical axons, thereby directing the axons toward midplane. The genetic mechanisms involved in forming the glial wedge and the sling are distinct (Ha et al. 2005). The first cortical axons to cross to the opposite hemisphere arise from the cingulate cortex (Koester & O’Leary 1994; Ozaki & Wahlsten 1998; Richards et al. 2004), and they cross at about 0.47 g in C57BL/6 embryos (Rash & Richards 2001). As shown clearly in Fig. 2(a) of Rash and Richards (2001), many cingulate axons cross just above the HC. The first axons from neocortex to cross midplane arise from frontal cortex, and there is a general rostral-caudal gradient of axonal crossing (Deng & Elberger 2001; Ozaki & Wahlsten 1992, 1998; Richards et al. 2004), such that axons

from frontal cortex cross in embryos of about 0.65-g body weight, whereas those from occipital cortex first cross at about 0.9 g. It is generally believed that a thin layer of glial processes separates the CC axons from the HC in normal embryos (Shu & Richards 2001; Silver et al. 1982; Valentino & Jones 1982), although a few CC axons have been observed to intermingle with HC axons at midplane (Livy & Wahlsten 1997).

The situation is somewhat different in the BALB/c and 129 strains of mice. The axons of the nascent CC emerge from cortical neurons and grow toward the midline of the brain at the right time, and they reach the interhemispheric fissure at the same stage of development as normal hybrid mice (Ozaki & Wahlsten 1993), but formation of a bridge of tissue at the dorsal septal region is markedly delayed in many individuals of these strains (Livy & Wahlsten 1997; Wahlsten 1987). In moderately afflicted mice, the glial sling is malformed, and CC axons that manage to cross midplane belatedly do so directly atop the HC (Wahlsten 1987). If the septal bridge is not present when the CC axons arrive, those axons continue to grow; many of them enter a whorl of axons growing longitudinally, referred to here as the Probst bundle (Loeser & Alvord 1968; Probst 1901; Rakic & Yakovlev 1968; Vogt 1905), and then re-enter the ipsilateral cerebral cortex (Ozaki et al. 1987; Ozaki & Wahlsten 1993), where they eventually form synaptic connections, become myelinated and survive into old age (Lefkowitz et al. 1991). The anomalous Probst bundle only appears when the CC is absent or markedly reduced in size. It is distinct from the commissure of Probst that contains axons from the dorsal nucleus of the lateral lemniscus (Gabriele et al. 2000) and the Probst tract that contains axons joining the mesencephalic trigeminal nucleus with the hypoglossal nucleus (Zhang et al. 2001).

The formation of the HC is substantially delayed in BALB/c and 129 strains and grossly delayed in one of the RI lines present here (Livy & Wahlsten 1997), and it seems highly likely that it will be similarly delayed in other severely abnormal RI lines. The situation in embryos of the F<sub>1</sub> hybrids involving BALB/c and 129 and RI lines that are normal as adults is more difficult to anticipate. One possibility is that genotypes associated with normal adult brains will also result in completely normal embryos if the early neural defect is discrete and permanent. On the other hand, it is feasible that the embryos of RI lines and the F<sub>1</sub> hybrids will show substantial retardation of HC formation but not severe enough to prevent CC formation, and retardation that does not extend to or below a threshold will allow complete recovery when CC axons are able to cross over a late-forming septal bridge.

## Materials and methods

### *Animals*

The progenitor strain BALB/cWah1 was derived by Wahlsten from BALB/cCF mice obtained from Carworth Farms in 1976. Carworth Farms obtained their mice from the Laboratory Animals Centre in the United Kingdom in 1968, who in turn derived their strain from BALB/cJ mice obtained from The Jackson Laboratory in 1955 at F61 (see Wahlsten 1989). The strain had passed through about 48 generations of full sib mating in this laboratory at the time of the experiments described here. The progenitor 129P1/ReJ was purchased from The Jackson Laboratory, Bar Harbor, ME. It is one of the genuine 129 substrains (see <http://jaxmice.jax.org/html/nomenclature/129nomenclature.pdf>) that has not been out-crossed (Simpson et al. 1997; Threadgill et al. 1997) and it retains the original coat color genes of the ancestral 129 strain (Beck et al. 2000; Festing et al. 1999). A few 129P3/J mice were also obtained from the Jackson Laboratories. Samples of the strain I/LnJ were generously donated by Dr Robert L. Collins of The Jackson Laboratory. The F<sub>1</sub> and F<sub>2</sub> hybrid crosses of the C57BL/6J and DBA/2J strains from the Jackson Laboratories, termed B6D2F1/J and B6D2F2/J, were used to establish standards for normal adult forebrain anatomy and prenatal forebrain development.

All adult mice analyzed in this study were bred in the laboratory at the University of Alberta. The BALB/cWah1 and 129P1/ReJ strains were mated to obtain reciprocal F<sub>1</sub> hybrid and reciprocal F<sub>2</sub> hybrid generations. The RI lines described in this report were all descended from a F<sub>1</sub> hybrid cross of a 129P1/ReJ female by a BALB/cWah1 male that was used to produce an F<sub>2</sub> hybrid cross. Beginning with the F<sub>2</sub> hybrid litters, 23 independent RI lines were established with randomly chosen littermate breeding pairs. Because most recombinant mice were examined before the line had undergone 20 generations of full-sib mating, the criterion

for designation as an inbred strain, the lines are referred to in this report simply as lines 1–23. After at least six generations of full-sib mating, the most severely afflicted lines were crossed with each other, and certain of them were also crossed with males of the strain I/LnJ.

Matings to obtain hybrid crosses and recombinant lines were done with one female and one male sib per cage when they had reached  $56 \pm 2$  days of age, and their offspring were weaned into same-sex groups at  $21 \pm 1$  day from birth. The mice were chosen as parents on the day of mating using random numbers from an electronic calculator. In the early phases of the study, two breeding pairs were formed per RI line, and the mice were kept together until two female–male littermate pairs had been weaned to propagate the next generation. In nearly all cases, this quota of four mice was obtained from the first litter. When both pairs produced large and healthy litters, the first born was used to propagate the line. If neither mated pair produced a healthy litter within 2 months, the line was considered extinct, unless a female was visibly pregnant or had recently given birth to a litter that perished. Careful records were made of the breeding performance of all mice in this study in order to assess possible correlates with absent CC.

Embryos were studied from the inbred strains BALB/ cWahl and 129P1/ReJ, their F<sub>1</sub> and F<sub>2</sub> hybrids and seven of the 23 RI lines. The RI lines were chosen to span the range from consistently and severely abnormal (lines 1, 3, 4), intermediate and highly variable (lines 14, 15), to almost always normal (lines 21, 23) as adults. The parents of the RI lines examined as embryos were from generations 11 to 16 of inbreeding. Embryos were also obtained from the 129P3/J strain and the B6D2F1/J and B6D2F2/J hybrid crosses.

Methods of animal husbandry and euthanasia were conducted according to the guidelines of the Canadian Council on Animal Care, and protocols were approved by the Biological Sciences Committee on Animal Care at the University of Alberta. All mice were maintained at about 22 °C with room lights on from 0600 to 1800 h. They were housed in plastic cages (29 × 18 × 13 cm) filled with Aspen Chip bedding (North-eastern Products, Warrensburg, NY) that were changed once per week. They had free access to Edmonton tap water and solid food (PMI Laboratory Rodent Diet 5001 for weaned mice and PMI 11 % fat breeder diet 5015 for mated mice).

### **Adult brains**

After enough offspring had been weaned, the mated pairs were processed for histology, yielding brain data on at least four adult mice per line per generation for the RI lines. Almost all were 100 days of age or older, and some were considerably older. Mice were deeply anesthetized with an overdose of sodium pentobarbital (120 mg/kg) and then per-fused intracardially with physiological saline followed by 4% neutral buffered paraformaldehyde. The brain was removed from the skull within 24 h of perfusion and stored in fresh fixative for at least 1 week, whereupon it was trimmed to a standard configuration and weighed (Wahlsten et al. 1975). Commissures were visualized by cutting the fixed brain in half at the mid-sagittal plane, immersing one entire half in gold chloride solution (Schmued 1990; Wahlsten et al. 2003a) for one-half to 3 h until myelinated structures appeared distinctly reddish or brown and then fixing the stain with 2% sodium thiosulphate. Cross-sectional areas of the CC, HC and anterior commissure (AC) were measured as described previously (Livy et al. 1997). The AC was almost invariably normal and is not discussed further. The HC, termed the ventral commissure of the fornix in older studies, was carefully distinguished from the dorsal commissure of the fornix (DCF) that is located just posterior to the HC at midplane and makes contact with the HC when the CC is very small. The area of the DCF at midplane was not included in the HC area. The DCF connects neurons in the two halves of the entorhinal cortex and is part of the limbic system. Its development origins were not investigated in this study.

### **Adjustment of commissures for adult brain size**

Not only is the genetics of commissure defects complex, but the phenotype itself is complex, being continuous rather than dichotomous and substantially correlated with other features of the brain. Forebrain commissure size in normal animals has an allometric relation with whole brain size (Gould 1966). Consequently, it is important in genetic analysis that a distinction be made between a CC that is unusually small, because the whole brain is small vs. a CC that is small in relation to brain size (Wahlsten 1984). When no adjustment for brain size is made, commissure size will tend to be markedly multifactorial, because so many genetic and environmental

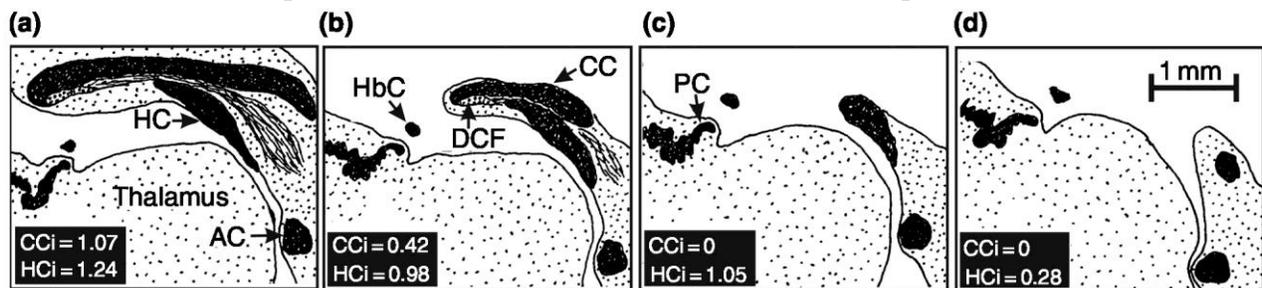
factors influence brain size (Williams 2000). The pattern of inheritance should be substantially simplified by taking brain size into account.

In normal adult mice, the relation between commissure size and brain size is nearly linear (Bishop & Wahlsten 1999). Adjustment of CC size for brain size can be done effectively with regression methods in the normal range of variation, but this approach can yield counterintuitive negative CC values when the CC is very small or absent. Consequently, we find it helpful to express commissure size as an index of abnormality formed by the ratio of the actual commissure size to the size expected from the mouse's brain weight using a linear regression equation fit to the data of a large sample of mice with no commissure defects (Livy et al. 1997). This can be done separately for cross-sectional areas of the CC and the HC in a mid-sagittal section to obtain the CC and HC indices. An index of 0 indicates that the structure is totally absent, whereas 1.0 denotes a structure equal to the value expected for its brain size. Instances of brains with different CC and HC indices are shown in Fig. 1.

### Collection of embryos

One adult male and one or more females were mated for 4 h during the day or overnight, after which females were checked for the presence of a vaginal plug. When a plug was detected, the female was weighed and then housed singly until testing. Conception (0.0 days) was considered to be the midpoint between plug detection and the previous plug check. It is important to examine embryos that are at the proper degree of maturity or morphological development, during the period when both the HC and the CC should have formed in normal animals. Embryos were extracted at ages ranging from gestation days 16–18 (E16 to E18), depending upon genotype, in order to obtain samples within the appropriate weight ranges. As proposed by Kaufman (1992), all mice studied prenatally are referred to as embryos, even though some had completed organogenesis and might be termed fetuses. Pregnant females were given an overdose (120 mg/kg) of pentobarbital sodium and then embryos were removed. The umbilical cord of each embryo was cauterized, the embryo was rinsed in ice-cold 0.9% physiological saline, and it was then blotted and weighed to the nearest mg.

Embryos chosen for study with lipophilic dyes were per-fused intracardially with saline followed by 6% neutral buffered paraformaldehyde, and then heads were removed and stored in fresh fixative for 3–5 days. Those chosen for general anatomical study were fixed by immersion in Bouin-Duboscq solution for 48 h after the scalp was removed and slits placed in the skull lateral to midline to facilitate penetration of fixative.

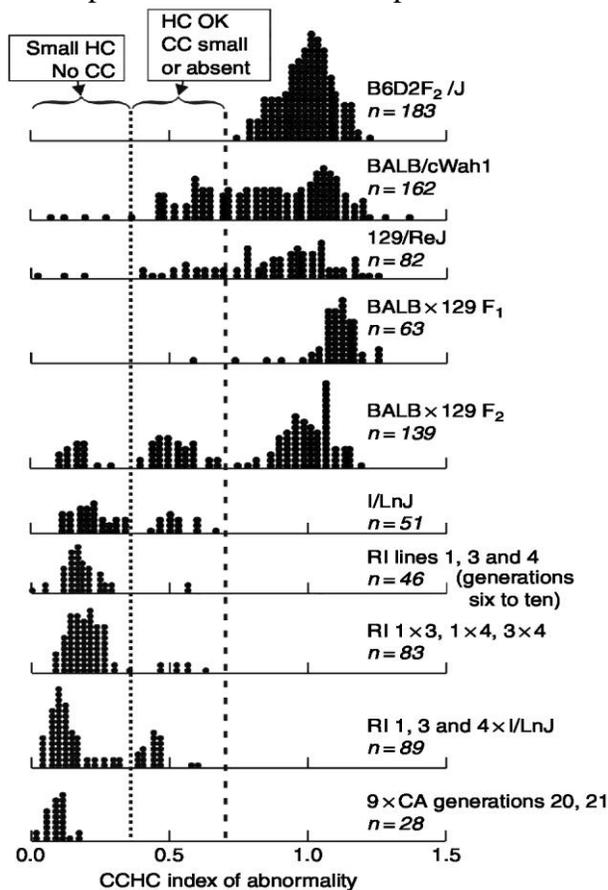


**Figure 1: Mid-sagittal diagrams of four adult mice, showing commissures ranging from normal to absent.** The index of abnormality for the corpus callosum (CC) is the ratio of actual CC cross-sectional area ( $\text{mm}^2$ ) to the area expected from the linear equation  $E(\text{CC}) = -0.1 + 2.2X$ , where  $X$  is brain weight in g. Likewise, the index for the hippocampal commissure (Hci) is  $(\text{HC area}) / (0.1 + 0.40X)$ . Linear equations were estimated in a previous study for normal hybrid mice (Livy *et al.* 1997). (a) Normal brain with ratios near 1.0. (b) Brain with abnormally small CC but normal HC. (c) Brain with no CC or dorsal commissure of the fornix (DCF) but HC of normal size. (d) Brain lacking CC and DCF and having an exceptionally small HC. AC, anterior commissure; HbC, habenular commissure; PC, posterior commissure.

### Tract tracing and axon growth in embryo brains

After fixation, the brain was removed from the skull and the caudal portions of the entorhinal and occipital cortices were removed to expose the hippocampal fimbria. With the aid of a dissecting microscope and eyepiece graticule, a 30–50- $\mu\text{m}$  crystal of Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) was inserted into both the left and right fimbria with a fine dissecting pin (Livy & Wahlsten 1997). Frontal cortex was labeled with a crystal of DiA [4-(4-dihexadecylaminostryryl)-N-methylpyridinium iodide] inserted into the center of the anterior third of each hemisphere. Dyes were obtained from Molecular Probes, Eugene, OR. Brains

were then placed in fresh fixative and stored in the dark at 37 °C for 4–6 weeks. Serial slices were cut at 50 μm in the coronal plane with a DSK-DTK microslicer and then incubated overnight at 4 °C in 0.02% bis-benzimide to label cell nuclei. Sections were coverslipped and viewed with a Leitz epifluorescence microscope using rhodamine (B2) and fluorescein (I3) filter sets, and photographs of selected slices were made with Ektachrome ASA 400 film. The progress of axon growth was determined by identifying growth cones, and measures of CC growth were taken for (a) the distance along the main axon bundle from the center of the crystal placement site to the leading edge of the main bundle and (b) the distance from the leading edge of the main CC bundle to the midplane crossing point at the surface of the interhemispheric fissure just anterior to the velum transversum and the primordium of the subfornical organ (Ozaki & Wahlsten 1992; Wahlsten & Bulman-Fleming 1994). The latter structures were always present, even when the HC was grossly retarded. Both measures (a) and (b) were deemed necessary, because the precise location of the crystal in frontal cortex was not identical in all embryos; measure (a) would not be affected substantially by crystal location, whereas measure (b) would be greater when the crystal was placed further from midplane.



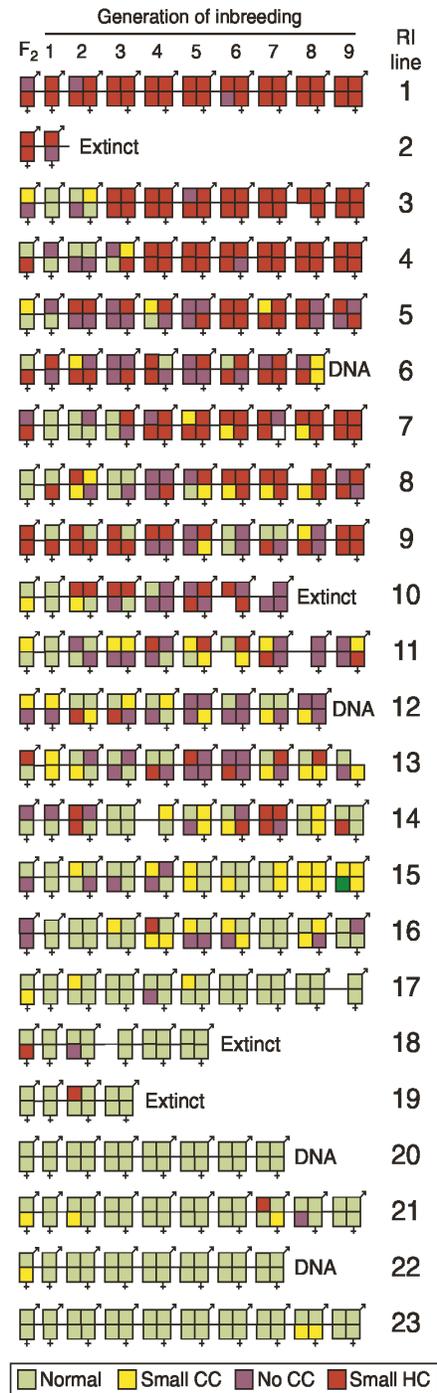
**Figure 2: Frequency distributions of the corpus callosum hippocampal commissure (CCHC) index of abnormality in adult mice of several inbred strains, classical hybrid crosses and recombinant inbred (RI) lines.** The CCHC index is the average of the CC and HC indexes described in Fig. 1. For B6D2F<sub>2</sub> hybrid mice, no animal ever had a ratio less than 0.7, and this value (dashed line) was used as the lower limit for normal commissure size. Mice with a ratio near 0.5 had no CC but normal HC size, whereas those below 0.35 (dotted line) had no CC and an unusually small HC. The inbred progenitor strains BALB/c and 129 showed very similar distributions with extreme variability, whereas their F<sub>1</sub> was almost always normal. In their F<sub>2</sub> hybrid, the distribution was distinctly trimodal, whereas in the I/LnJ strain, it was bimodal and no mouse ever had even a small CC. The three RI lines (RI lines 1, 3 and 4; see Fig. 3) with the most severe abnormalities never had any CC and almost always had a small HC, and the crosses among the three lines showed the same kind of distribution with no reduction of severity compared with their parent lines. Crosses of RI lines 1, 3 and 4 with I/LnJ yielded a distribution of CCHC<sub>i</sub> scores similar to I/LnJ, and no animal had even a small CC. After 20 generations of inbreeding RI line 4, a new inbred strain 9XCA/Wah was created that consistently had a severe reduction in size of the HC.

### Morphometry of embryo brains

Brains used for analysis of anatomical regions were embedded in paraffin, and 10-μm serial slices were taken in either the sagittal or coronal plane, then mounted on slides and stained with hematoxylin and eosin. The sagittal slice closest to midplane in the vicinity of the most dorsal portion of the HC was chosen for measurement. Tracings of commissures were made with a Leitz tracing device at ×40, and then areas and thicknesses of various structures were determined. The cross-sectional area of the CC and HC at mid-plane was estimated by summing dorsal–ventral thicknesses of these structures in serial slices. All measures were corrected for shrinkage and compression of the tissue during embedding and slicing (Wahlsten & Bulman-Fleming 1994).

Whether or not the cingulate axons are considered part of the CC proper is immaterial for our morphometric analysis, because tissue processed in a manner that allows measurement of the entirety of a commissure does not even allow a clear demarcation between the HC and the CC at the midsagittal plane in the mouse embryo. As noted several years ago by Glas (1975) and confirmed by us (Wahlsten 1987; Wahlsten & Bulman-Fleming

1994), in the embryo, the HC and CC form one continuous commissure precisely at mid-plane. Although the axons arising from different regions can be distinguished by labeling with different kinds of dyes, this tract-tracing approach does not allow us to measure the overall size of the commissure. Instead, the combined CC plus HC, including cingulate axons, can be measured morphometrically, and a regression equation allows us to find the size of the CCHC expected from the embryo body weight (Wahlsten & Smith 1989). An index of commissure abnormality can then be formed as the ratio of actual CCHC size to expected size in a normal embryo.



**Figure 3: Pedigree of 23 recombinant inbred (RI) lines derived from the F<sub>2</sub> hybrid generation of a cross between BALB/cWah1 and 129P1/ReJ. Each square represents one individual. A male has an arrow pointed upwards and a female has a + below. Each line was started with a single male–female pair, and in the first generation of inbreeding, there was also one pair. For the next eight generations, each line was bred to obtain at least two full-sib pairs. In instances where fewer than four mice are shown after the first generation, either one of the four mice died or one sex was absent because of a small litter size. Lines where reproduction ceased within seven generations are shown as ‘Extinct’ and had no DNA collected, whereas those ceasing reproduction in generations eight or nine had DNA sam-**

*ples collected from the parents. Color of a square indicates brain status of that individual. Mice with small corpus callosum (CC) (yellow) or no CC (purple) always had normal hippocampal commissure (HC) size, whereas mice with small HC (red) never had any CC present. The lines are ranked and numbered according to the average severity of brain defects over the first few generations. Lines with consistently total absence of the CC and reduced HC emerged by generation six.*

## **Results**

### ***Adult mice: classical crosses***

As observed previously (Livy & Wahlsten 1991; Wahlsten & Schalomon 1994), an unusually small HC occurred in the inbred strains and crosses only when the CC was totally absent. Consequently, it was possible to combine the CC and HC indices into a single index of commissure abnormality (the CCHC index) by averaging them. When both commissures were normal, the CCHC index was close to 1.0, whereas the index was close to 0.5 when the CC was completely absent but the HC was normal. Distributions of the CCHC index for the inbred mice and crosses are shown in Fig. 2. Data for normal hybrid B6D2F<sub>1</sub> and F<sub>2</sub> mice indicated that a CC index less than or equal to 0.7 or 0.65 was below the normal range, and a criterion ratio of 0.65 or 0.7 was also applicable to the HC index. Thus, a mouse with CCHC index less than 0.35 had an abnormally small HC and no CC. Because indices for the CC and HC were averaged, the striking bimodality of CC size seen in BALB/cWah1 (Wahlsten 1989) is not so obvious in Fig. 2.

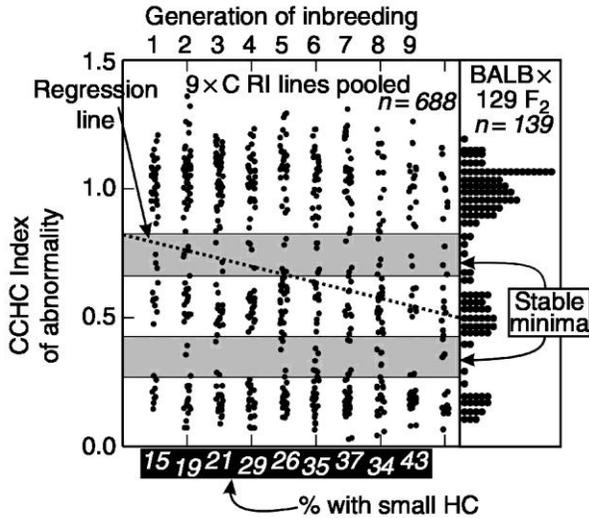
The distributions for the BALB/cWah1 and 129P1/ReJ progenitor strains were very similar; they had almost identical frequencies of small or absent CC (43% for BALB, 44% for 129), totally absent CC (12% for BALB, 16% for 129) and small HC (4% for both BALB and 129). I/LnJ, on the other hand, never showed any CC and commonly (62%) had a small HC. As shown previously (Livy & Wahlsten 1991), the F<sub>1</sub> hybrid between BALB and 129 was almost entirely normal; one mouse had a small CC but none had small HC. Thus, the BALB and 129 strains must differ at two or more loci relevant to commissure development. In their F<sub>2</sub> cross, however, severe commissure defects appeared with a fairly high frequency (30% total CC absence, 14% with small HC). Of special importance was the distinctly trimodal distribution of the CCHC index in the F<sub>2</sub> hybrids.

### ***Adult mice: recombinant lines***

The evolution of commissure abnormalities in the RI lines is shown in Fig. 3 for the first nine generations of inbreeding. After generations eight and nine, many of the intermediate and highly variable lines were eliminated from the colony after DNA samples were preserved. Three lines (1, 3 and 4) quickly stabilized on an extreme phenotype in which every mouse showed absent CC and a very small HC. Not only were these lines far below the level of their BALB and 129 progenitors, but they were more severely afflicted than even the I/LnJ strain. At the other end of the spectrum, several lines (e.g. 21, 23) showed almost entirely normal brains. When the most severely abnormal lines were crossed among themselves in a complementation test (Fig. 2), there was no reduction of the severity of the defect, unlike the situation when the BALB and 129 progenitors were crossed. Crossing lines 1, 3 and 4 with an I/LnJ male invariably yielded absent CC, but the frequency of small HC was intermediate between lines 1, 3 and 4 (100%) and I/LnJ (62%). These data are consistent with a model involving two major loci plus an unknown number of lesser modifier genes. With continued inbreeding for 20 generations until line 4 achieved formal inbred strain status (9XCA/Wah; see Schimanski et al. 2002), the size of the HC gradually declined until some mice had almost no HC axons crossing midplane (Fig. 2).

While the severity of forebrain commissure defects increased gradually and substantially with continued inbreeding for the group average (Fig. 4), the minima in the frequency distribution of the index of abnormality showed no change from those observed in the F<sub>2</sub> hybrid sample. The frequency of totally absent CC doubled from 25% in generation 1 to about 51% in each of generations five through nine, and the frequency of deficient HC more than doubled from 15% in generation 1 to 35% by generations 7, 8 and 9. Nevertheless, throughout the first nine generations of inbreeding, there were very few mice with intermediate sizes of either the CC or the HC. As inbreeding progressed, individual abnormality scores jumped abruptly from one category of scores to another in most cases.

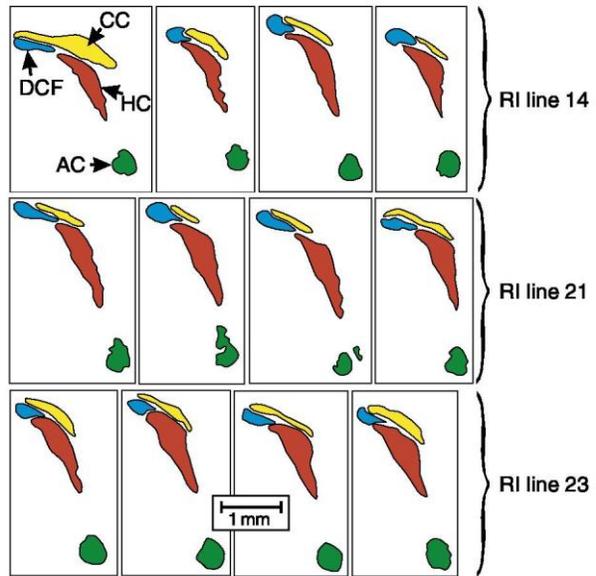
The stable minima in Fig. 4 lend strong support to the reality of a developmental threshold, but the threshold is not a matter of all or nothing. Instead, the threshold is a narrow region with fuzzy boundaries in developmental time and space. When large samples are observed, a few mice always occur in the minima of the distributions; every value of adult CC and HC size is possible. When only a few CC axons traverse midplane, they invariably do this directly above what in the adult appears to be a normal HC and just anterior to the DCF that always makes contact with the HC in such a case (Fig. 5). If the CC is small enough, a Probst bundle can usually be identified in coronal sections (Fig. 6A), especially in the 129 strain.



**Figure 4: Distributions of the corpus callosum hippocampal commissure (CCHC) index of abnormality over the first 10 generations of inbreeding.** Data shown as a jitter plot (Systat, Richmond, CA) that imposes a slight random displacement of each dot to reveal overlapping data points. Sample sizes per line were limited to four animals in a single generation, so that weightings of normal and severely abnormal lines (Fig. 3) were equal in each generation. Although the average CCHC index gradually declined with continued inbreeding, two minima in the distribution remained in the same locations, and these minima were the same as minima in the large F<sub>2</sub> hybrid sample. The larger minimum near CCHC<sub>i</sub> = 0.75 represented mice with normal HC and small CC, whereas the smaller minimum near CCHC<sub>i</sub> = 0.35 were mice with no CC and moderately reduced HC size. The dashed line shows the best fit for the regression of CCHC index on generation of inbreeding. Percentage of mice in each generation with abnormally small HC gradually increased with continued inbreeding.

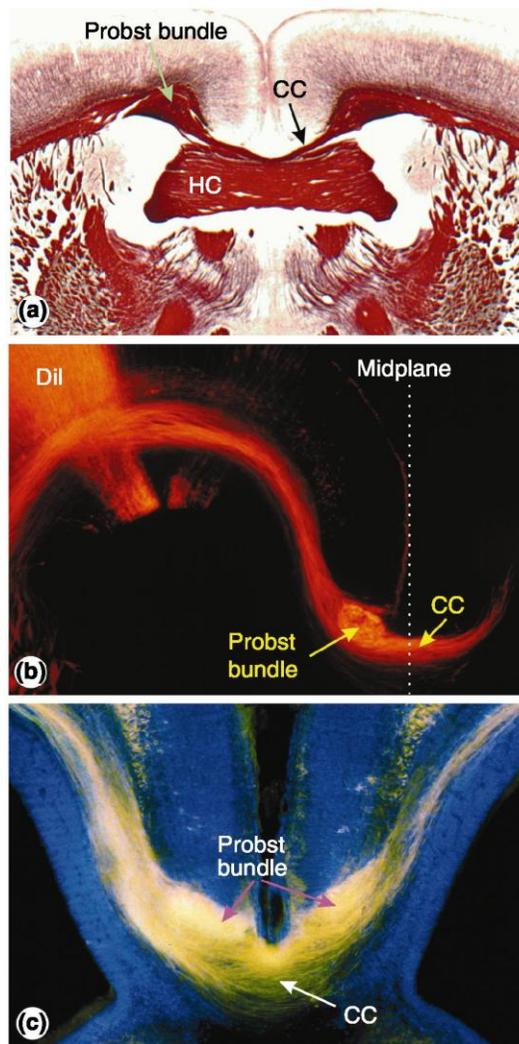
## Reproductive performance

Table 1 summarizes several measures of reproduction in the inbred progenitors as well as their pooled reciprocal F<sub>1</sub> and F<sub>2</sub> hybrids. As expected, F<sub>2</sub> hybrid litters were born sooner, contained more pups and showed much better survival than those with an inbred mother. An index of reproductive success was constructed on the basis of the data for the F<sub>2</sub> hybrid litters in a manner that placed it on the same scale as the commissure index of abnormality. Any female whose litter was similar to the F<sub>2</sub> hybrid litters would have an index near 1.0, whereas one who failed to become pregnant would score 0. It was evident that reproductive success had no relation with forebrain commissure defects for three reasons. (a) The F<sub>2</sub> hybrid litters were larger and healthier than the F<sub>1</sub> hybrids but they had many more brain defects (Fig. 2). (b) The F<sub>2</sub> hybrid parents of the 23 RI lines had average parental CCHC index of abnormality scores ranging from 0.13 to 1.10, and the reproduction index also ranged widely from 0.17 to 0.98, but the two measures were not significantly correlated ( $r = 0.10$ ,  $P > 0.05$ ). Neither was the maternal CCHC index related to her reproduction index ( $r = 0.07$ ); in fact, among the seven F<sub>2</sub> hybrid females with no CC and small HC, three bred very well ( $r > 0.9$ ). (c) Both the reproduction index and the commissure index of abnormality declined as inbreeding progressed and both averaged a little below 0.7 by generations five through seven. The CCHC index differed greatly among the 23 lines ( $R^2 = 0.72$ ), whereas the reproduction index did not differ significantly among lines ( $P = 0.16$ ), and the correlation between mean



**Figure 5: Locations of the corpus callosum (CC), hippocampal commissure (HC) and the dorsal commissure of the fornix (DCF) at the mid-sagittal plane.** Shown are four adult mice from each of three RI lines (see Fig. 3) when the CC was very small. The diminutive population of CC axons invariably crossed dorsally over the HC. No exception to this rule was observed in any mouse of any generation. Diagrams were drawn from gold chloride-stained half brains. The identity of tissue between the CC and HC could not be ascertained with this method. AC, anterior commissure.

reproduction index and mean CCHC index across the 21 lines that survived until generation six was not significant ( $P = 0.2$ ). Two of the RI lines with the most severe brain abnormalities (lines 1 and 3) proved to be among the best breeders.



**Figure 6: Coronal sections of three mice showing both a small Probst bundle and a small corpus callosum (CC).** (a) An adult mouse of strain 129P1/ReJ where a very small CC crossed directly over an hippocampal commissure (HC) of normal size. Gold chloride stain. (b) An embryo of strain 129P1/ReJ with callosal axons labeled by a crystal of the lipophilic dye Dil placed in parietal cortex, where some axons entered the Probst bundle and others traversed midplane. The location of the traverse (not shown) was just dorsal to the HC. (c) An embryo of recombinant inbred line 14 with callosal axons labeled by crystals of Dil placed symmetrically in the left and right frontal cortex. Some axons in both hemispheres formed a Probst bundle, and others traversed midplane dorsal to the HC (not shown). False color image with contrast enhancement.

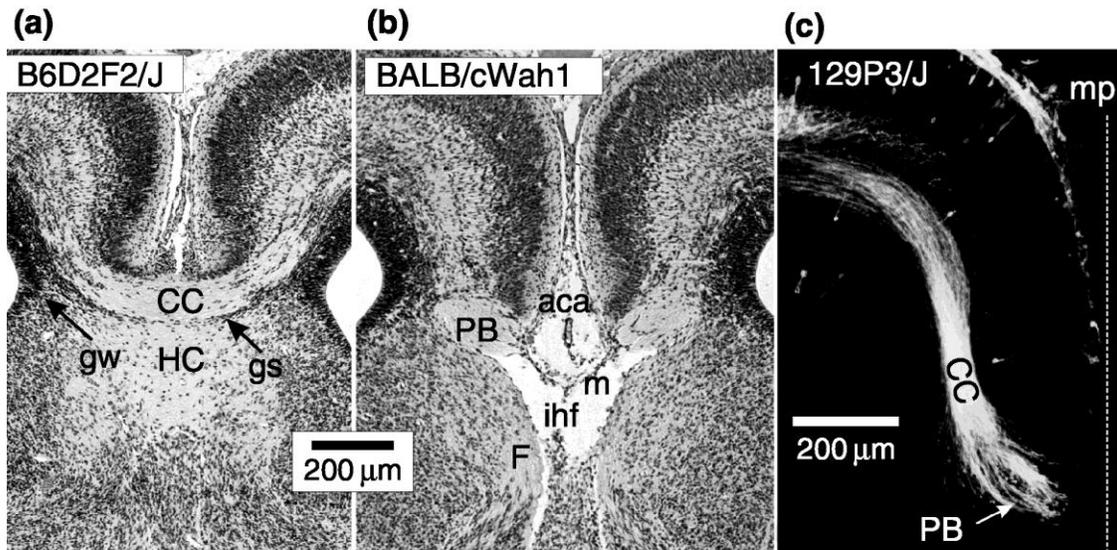
### ***Embryos: general appearance***

Coronal sections of normal hybrid mice (Fig. 7A) revealed the location of the CC dorsal to the HC with the glial wedge extending from the vertex of the lateral ventricles, as described by Shu and Richards (2001), and the glial sling lining the ventral surface of the CC near midplane, as described by Silver et al. (1982). In the genetically defective BALB/cWah1 strain at an equivalent degree of developmental maturity (Fig. 7B), on the other hand, the glial wedge was present but the glial sling was missing, as described previously by Silver et al. (1982) and Wahlsten (1987). Furthermore, the interhemispheric fissure had not yet fused at the medial septal region, and the HC was not yet present in many embryos. A clear Probst bundle was invariably present in progenitor and in RI mice of at least 0.75 g of body weight that lacked any visible CC or HC.

**Table 1:** Reproductive performance (mean  $\pm$  standard deviation of first litters) of 129P1/ReJ and BALB/cWah1 progenitors and their F<sub>1</sub> and F<sub>2</sub> hybrid crosses

Group	BALB, 129	BALB $\times$ 129	F <sub>2</sub> hybrids	
Parent genotype	Inbred	Inbred	BALB $\times$ 129	
Pup genotype	Inbred	BALB $\times$ 129	F <sub>2</sub> hybrid	R <sup>2</sup>
Number of litters	18	14	16	–
Days to first litter	24.7 $\pm$ 5.7	31.2 $\pm$ 17.9	20.9 $\pm$ 1.4	0.14
Number of pups born	4.3 $\pm$ 2.7	7.2 $\pm$ 2.8	8.4 $\pm$ 2.0	0.35
Mortality (%)	52.1 $\pm$ 50.1	49.8 $\pm$ 48.8	2.0 $\pm$ 4.4	0.27
Weaning weight (g)	10.2 $\pm$ 1.6	10.4 $\pm$ 0.8	11.4 $\pm$ 1.0	0.19
Reproduction index	0.60 $\pm$ 0.21	0.69 $\pm$ 0.26	0.96 $\pm$ 0.07	0.41

Data for reciprocal crosses were pooled. Data were analyzed with multiple regression using effect coding to evaluate pup and maternal effects. The squared multiple correlation (R<sup>2</sup>) estimates the proportion of total variation accounted for by differences among groups. Reproduction index is the average of three ratios: 20/(days to first litter); (pups born)/9 and (weaned pups)/(pups born).



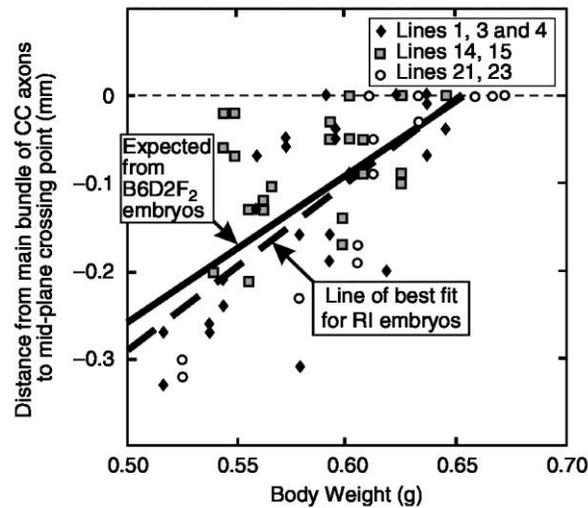
**Figure 7: Coronal sections of three mouse embryos.** (a) A normal B6D2F2/J embryo of 0.78 g of body weight showing the corpus callosum (CC) crossing above the hippocampal commissure (HC) with the glial sling (gs) lining the ventral surface of the CC and the glial wedge (gw) extending medially from the lateral ventricles. Hematoxylin and eosin stain of 10- $\mu$ m paraffin section. (b) BALB/cWah1 embryo of 0.80 g of body weight where putative callosal axons have entered the whorl of the Probst bundle (PB) and the halves of the dorsal septal region are still separated by the interhemispheric fissure (ihf). (c) 129P3/J embryo at 0.74 g of body weight that had a Dil crystal inserted into parietal cortex. CC axons have approached midplane (mp) and entered the PB but failed to cross to the opposite hemisphere. aca, anterior cerebral artery; m, meninges.

**Table 2:** Sample sizes and body weight ranges (g) for embryos studied with different techniques

Group	Paraffin/H&E – sagittal		Paraffin/H&E – coronal		DiA and Dil	
	n	Range	n	Range	n	Range
BALB/cWah1	27	0.51–0.80	–	–	–	–
129P1/ReJ	28	0.50–0.93	–	–	–	–
BALB $\times$ 129 F <sub>1</sub>	21	0.52–0.66	–	–	4	0.52–0.64
BALB $\times$ 129 F <sub>2</sub>	61	0.55–0.93	–	–	6	0.56–0.62
Rl line 1	11	0.54–0.88	9	0.62–0.78	6	0.54–0.65
Rl line 3	10	0.64–0.74	10	0.60–0.81	6	0.54–0.63
Rl line 4	10	0.62–0.77	10	0.55–0.80	6	0.54–0.65
Rl line 14	10	0.63–0.86	10	0.51–0.81	5	0.58–0.67
Rl line 15	10	0.63–0.86	9	0.59–0.75	5	0.52–0.67
Rl line 21	11	0.62–0.82	10	–	–	–
Rl line 23	11	0.62–0.78	8	–	–	–

### Embryos: axon growth

The present study examined embryo forebrain commissures in the same genotypes that were assessed as adults, with the exception of the I/LnJ strain. Whole commissure size was assessed in one series of embryos, whereas growth of axons from well-defined regions of forebrain was assessed with crystals of lipophilic dyes in separate embryos. Weight ranges and sample sizes of the various groups are summarized in Table 2.



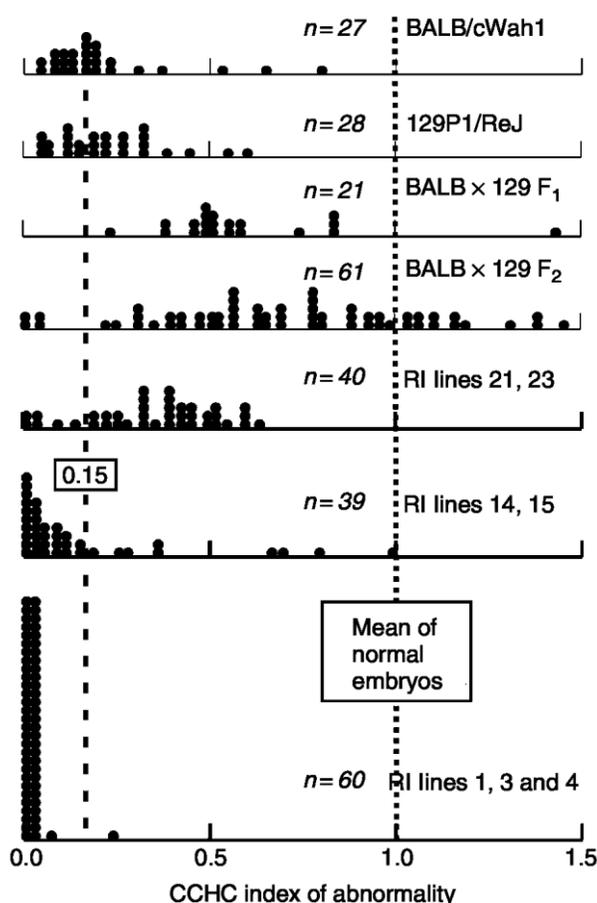
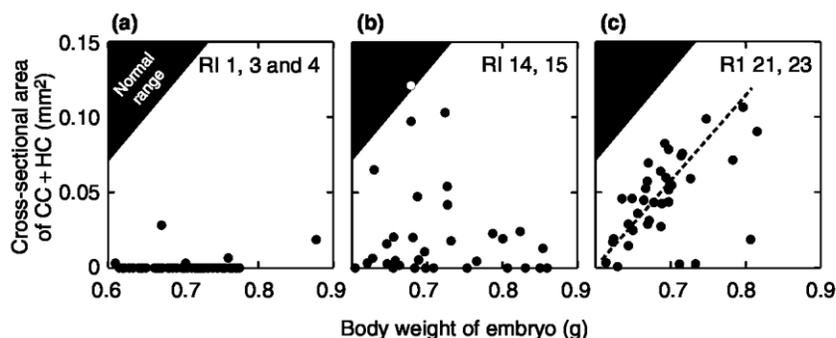
**Figure 8: Growth of corpus callosum (CC) axons toward midplane in recombinant inbred (RI) lines.** Axons were revealed by DiI or DiA labeling from a crystal placed into frontal cortex of paraformaldehyde-fixed embryos having different body weights. The line of best fit for the seven RI lines (dashed line) was almost identical to the comparable line from normal B6D2F<sub>2</sub> mice computed by Ozaki and Wahlsten (1992). Differences between the three categories of RI lines were not statistically significant at  $\alpha = 0.01$  (diamond, severely abnormal lines 1, 3 and 4; squares, moderately abnormal lines 14 and 15; circles, normal lines 21 and 23). Average time-course of growth of CC axons toward midplane was normal in all RI lines.

The growth cones of all RI embryos had normal appearance and were generally of the complex type as they approached midplane (Livy & Wahlsten 1997; Norris & Kalil 1990; Ozaki & Wahlsten 1992, 1993). For those embryos that were sufficiently advanced to have axon tips near the mid-plane crossing point, the CC axons appeared to be in the proper location, but in most instances, they encountered a wide interhemispheric fissure with no septal bridge present. Only in one RI line 21 embryo (0.67 g) had any CC axons crossed midplane in the size range studied here (Table 2). As shown in Fig. 8, the arrival of CC axons at midplane was entirely normal with respect to embryo body weight, as judged by data from a large sample of normal B6D2F<sub>2</sub> embryos studied previously with the same methods (Ozaki & Wahlsten 1992). It has previously been observed that CC axons from frontal cortex do not normally cross midplane until the embryo body weight is at least 0.65 g (Ozaki & Wahlsten 1992, 1998). Thus, the almost universal absence of labeled CC axons in the contralateral hemisphere in this study is not at all surprising. Obviously, from results for adult mice, we know that in many of the embryos from RI lines 14, 15 and especially 21 and 23, the CC axons would eventually cross midplane. The crucial finding from tract tracing with lipophilic dyes is that CC axons appeared to be quite normal in all respects until they arrived at midplane where the septal bridge normally is found. These results also imply that cross-sectional areas of the CCHC structure measured morphometrically were almost entirely comprised of HC axons in embryos less than 0.65 g, with some contribution likely from cingulate axons (Ozaki & Wahlsten 1998; Rash & Richards 2001). This conclusion was confirmed by our observations of abundant HC axons crossing midplane that were labeled by DiI crystals in the fimbria in RI lines 14, 15, 21 and 23.

### *Embryos: commissure size*

The cross-sectional area of the combined CC and HC at mid-plane differed greatly among the RI lines, as shown in Fig. 9. For lines 1, 3 and 4 that suffer severely reduced HC as adults, only two of 60 embryos showed a measurable commissure. Because most adults of these lines evidenced a small HC, the HC axons must have traversed midplane after embryonic day 18 (E18), the age of the oldest embryos studied here. For RI lines 14 and 15 having intermediate severity of defects as adults, the commissure sizes were highly variable, with a few mice being close to the normal range while others remained at zero. Lines 21 and 23 that were usually normal as adults showed significantly delayed formation of the CCHC but, for most individuals, normal growth of the commissures, once the first axons had crossed.

**Figure 9: Cross-sectional area of the corpus callosum (CC) plus hippocampal commissure (HC) in relation to embryo body size.** Shown for mice from severely abnormal lines (1, 3 and 4), moderately abnormal (14, 15) and mostly normal as adults (21, 23). Only lines 21 and 23 showed a reasonably normal rate of commissure growth, although the onset of growth was delayed by almost 1 day.

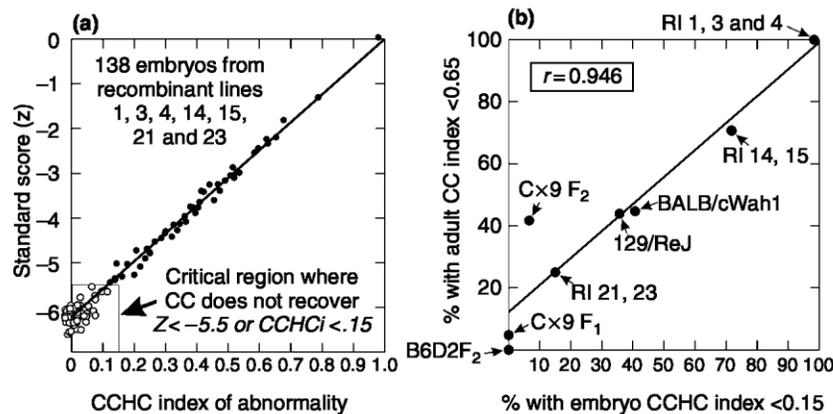


**Figure 10: Frequency distributions of the corpus callosum hippocampal commissure (CCHC) index of abnormality in embryos.** Shown are the inbred progenitors, their F<sub>1</sub> and F<sub>2</sub> hybrids, and three categories of RI lines (lines 21, 23 usually normal as adults; lines 14, 15 moderately abnormal but highly variable as adults; lines 1, 3 and 4 consistently and severely abnormal as adults; see Figs 2 and 3). Although the F<sub>1</sub> mice and RI lines 21, 23 were almost always normal as adults, the growth of the CC and HC in their embryos clearly was retarded, and most mice of these groups must have recovered from the embryonic delay. The proportion of embryos below the threshold value of CCHCi = 0.15 (heavy dashed line) was close to the proportion of adults with very small or absent CC (see Fig. 11).

The raw area of the CCHC at midplane was converted to a CCHC index of abnormality in two steps. First, the quadratic regression equation derived by Wahlsten and Smith (1989) for normal B6D2F<sub>2</sub> embryos studied with the same methods was used to obtain the value of the CCHC that was expected on the basis of the embryo body weight. Then the index of abnormality was calculated as the ratio (actual CCHC)/(expected CCHC). Because of the very rapid growth of both the HC and the CC during this period, with the HC clearly predated the CC at

midplane, a ratio of 1.0 certainly denotes normal morphometric development, but an index of 0.5 does not necessarily indicate absent CC with normal HC, as was the case with the CCHC index in the adults (Fig. 1). For an animal weighing 0.65 g, for example, the CCHC is expected to be almost entirely comprised of HC plus cingulate axons, and an index of 0.5 would point to seriously delayed growth of the HC. The distributions of CCHC index of abnormality scores are shown in Fig. 10 for the inbred progenitors, hybrid crosses and RI lines.

Several interesting findings are apparent. First, both the F<sub>1</sub> hybrids and RI lines 21 and 23 that are almost entirely normal as adults showed substantial retardation of HC formation in the embryo. Second, the distributions for both BALB/cWah1 and 129P1/ReJ were far below normal for almost all animals, even though most mice of these strains show normal adult CC and HC sizes. Third, the dispersion of scores in the F<sub>2</sub> hybrids was very wide indeed, as would be expected for a system of two or more segregating loci. Fourth, in the RI lines 1, 3 and 4 that never show any CC in the adult, all but two of 60 embryos had no sign of an HC present in the body weight range studied here. Finally, there were no clear signs of a bimodal or trimodal distribution of the embryonic CCHC index in any group in this weight range.



**Figure 11: Comparison of two measures of embryo commissure abnormality.** (a) Corpus callosum hippocampal commissure (CCHC) index of abnormality for 138 recombinant inbred (RI) mouse embryos from seven lines vs. a standard score ( $z$ ) that compares CCHC size with body weight based on the data of Wahlsten & Smith (1989). The data of Bulman-Fleming & Wahlsten (1991) indicated that an embryo with CCHC value more than 5.5 standard deviations below the value expected from its body size would never recover and would have very small or absent CC as an adult. That threshold value corresponds to a CCHC index score of about 0.15. Data are jittered to show overlapping points. (b) Percentage of embryos with CCHC index < 0.15 vs. percentage of adult mice with CC index < 0.65 (very small or absent CC) in eight genetic groups. Data from embryos and adults were from independent samples of each genotype. The correlation between the two quantities was very high. Only the C  $\times$  9 F<sub>2</sub> hybrid deviated substantially from the linear relation.

### Embryos: locating the thresholds

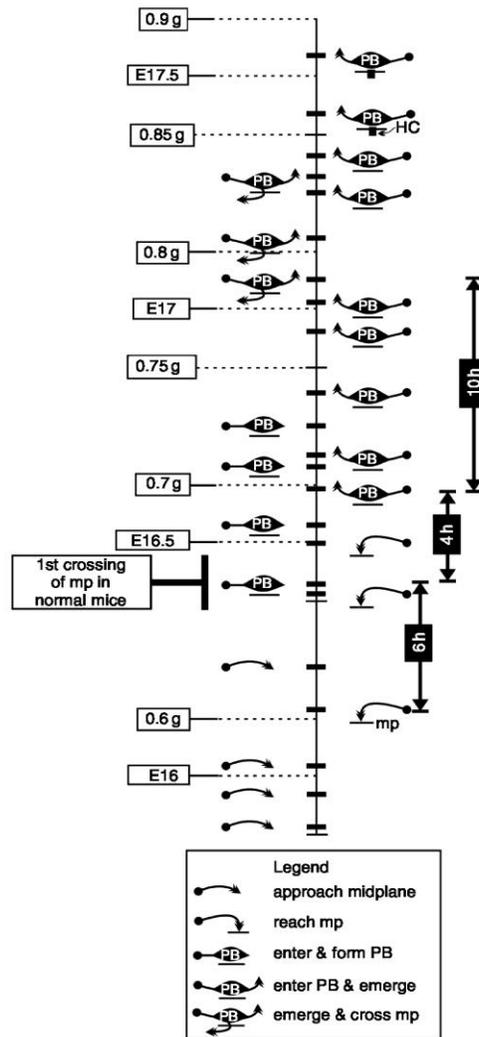
In the BALB/cWah1 strain, most embryos that lack a CC at day E18 show recovery 1 day later, just before birth at about 1 g of body weight (Wahlsten 1987). A previous study (Wahlsten & Smith 1989) quantified the degree of retarded CCHC growth as a standard score, the number of standard deviations below normal B6D2F<sub>1</sub> and F<sub>2</sub> mice for a given animal's CCHC area compared with the value expected from its body weight. Large samples of BALB/cWah1 and BALB/cWah2 embryos suggested that the threshold value for recovery from grossly delayed commissure formation is about 5.5 standard deviations below normal (Bulman-Fleming & Wahlsten 1991). The method of Wahlsten and Smith (1989) was used to determine the standard score ( $z$ ) for each recombinant embryo CCHC in the present study, and Fig. 11A shows that  $z = -5.5$  corresponds to a CCHC index of abnormality of about 0.15.

If an embryo with a CCHC index below 0.15 when it is between 0.5 and 0.8 g of body weight will never recover and achieve CC growth into the normal range, then the proportion of embryos below that threshold value should correspond closely with the proportion of adults having no or small CC. As shown in Fig. 11 B, these two values were almost perfectly correlated across genetic groups. Only two of the points in Fig. 11 B were established before the present study was done; the B6D2F<sub>1</sub> and F<sub>2</sub> mice were used to define criteria for normal development, and the BALB/cWah1 mice served to locate the hypothesized threshold in a previous study (Bulman-Fleming & Wahlsten 1991). Omitting those two points has almost no effect on the strain

correlation. Interpretation of these data is aided by an earlier observation that there is no differential mortality among mice with no CC after birth (Wahlsten 1984).

A wide range of CC plus HC size was observed in adults (Fig. 2) only in those groups where the CCHC index of 0.15 was in the middle of the frequency distribution for embryos. Of special importance for our argument was the fact that the spread of the frequency distributions for the CCHC index in embryos (Fig. 10) was similar for all genetically uniform groups except RI lines 1, 3 and 4 that had almost no variation and were far below threshold. It appeared that microscopic variation in growth processes in the embryo forebrain were magnified to create macroscopic variation in the adult.

The approximate location of the second threshold evident in adult mice in Fig. 4 could also be established with the correlational method. The proportion of adult mice with abnormally small HC was similar to the proportion of embryos in the weight range 0.5–0.8 g that had no measurable CCHC. A more precise definition of this threshold will require study of large samples of embryos in the 0.8–1.0 g range when the HC shows clear signs of recovery in many mice of genetically abnormal strains (Wahlsten 1987).



**Figure 12: Sequence of events in development of the corpus callosum (CC) in a series of 129P1/ReJ and 129P3/J embryos.** These animals were studied previously by Ozaki and Wahlsten (1993, 1998). The entire series from 0.58 to 0.9 g consisted of 34 embryos, but they were too closely spaced to show each individual. The earliest occurrence of each kind of pattern is always indicated. The scale is given in both body weight (g) and days of embryonic development (based on Livy & Wahlsten 1997; Wahlsten & Bulman-Fleming 1994). The time from the first appearance of a Probst bundle (PB) to axons clearly emerging from the PB and returning to ipsilateral cortex was about 4 h.

### *Embryos: width of the threshold region*

The source of the developmental threshold for CC formation is fairly well understood. The growth cones of the CC axons remain near midline for only a short time and then grow back into the Probst bundle and ipsilateral cortex (Ozaki & Wahlsten 1993), and axons arriving later tend to follow the earlier ones into the maelstrom of the Probst bundle rather than approaching and contacting the midline fissure. Hence, there is a critical period for CC formation in these strains, after which all putative CC axons will enter the Probst bundle and then return to ipsilateral cortex, despite the belated erection of the septal bridge (Wahlsten 1987). The threshold is not a matter of all or none; every size of the adult CC is possible (Fig. 2), but small sizes are relatively rare, especially in the F<sub>2</sub> hybrid cross of BALB/c and 129 and the RI lines formed from those hybrids.

The approximate duration of the critical period may be estimated from several facts. In normal hybrids as well as the BALB/c and 129 strains and their recombinant lines, CC axons from frontal cortex grow at an average rate of about 3–4 mm per day or 150–200  $\mu$ m per hour (Ozaki & Wahlsten 1992; present data). As shown in Fig. 7b,c, the Probst bundle is about 200  $\mu$ m wide in embryos of about 0.8 g of body weight, and it grows rapidly to a diameter of about 400  $\mu$ m in totally acallosal embryos (Ozaki & Wahlsten 1993). Thus, the time required to grow into the Probst bundle, reach mid-plane, then turn back into and finally exit the Probst bundle would be only 3 or 4 h if axons from frontal cortex continue to grow at a rate similar to their rate when approaching midplane. These time estimates agree reasonably well with timing of events observed in a closely spaced series of 129 and BALB/c embryos (Fig. 12) where axons from frontal cortex were labeled with lipophilic dyes. A Probst bundle was first identified in an embryo just a little larger than 0.65 g, and axons from frontal cortex were first seen exiting the Probst bundle and re-entering ipsilateral cortex at about 0.7 g, a body size that is usually reached about 4 h later (Livy & Wahlsten 1997; Wahlsten & Bulman-Fleming 1994).

On the other hand, the delay of formation of the septal bridge is about 1 full day in BALB/c mice and 2 days in the most severely retarded RI lines (Livy & Wahlsten 1997; present data). Thus, the time required for individual axons to explore the mid-plane tissue environment and then grow back into ipsilateral cortex is substantially less than the delay in formation of the septal bridge. Consequently, recombinant inbreeding that extends the delay in septal bridge formation by 2 days in RI lines 1, 3 and 4 moves the distribution beyond the region where many CC axons are still reaching midplane.

It is noteworthy that mice with an unusually small CC sometimes exhibited a small Probst bundle. The foundation for this rare structure was laid early in commissure formation, as shown in two embryos (Fig. 6b,c). Although our sample of these fascinating cases is small, in every instance, the CC axons that crossed midplane did so near the dorsal edge of the HC. There was a considerable span of time (about 10 h) from when the CC axons first emerged from the Probst bundle and entered ipsilateral cortex to when some CC axons entered the Probst bundle and then continued across to the opposite hemisphere (Fig. 12). We interpret these observations to mean that in embryos in the body weight range 0.65–0.78 g with a Probst bundle but no CC axons crossing midplane, an adequate septal bridge had not yet formed. Thus, a Probst bundle in the presence of a small adult CC must have arisen in an embryo at or in the threshold region.

The small Probst bundle may have contained axons that arrived earlier, when there was not yet an adequate septal bridge, whereas those that managed to cross midplane arrived a little later, just as the septal bridge reached a critical size. Because a small crystal of dye was inserted in lateral cortex of the case in Fig. 6(b), the axons in both the Probst bundle and the small CC must have emerged from a very similar region of cortex. It is possible that both kinds of axons may have arrived at midplane at about the same time, but that those located more ventrally in the loosely associated bundle of axons were more likely to contact crucial tissue that is part of or associated with the HC. Thus, the distinction between axons that are recruited into the whorl of the Probst bundle and those that traverse the interhemispheric fissure may be both temporal and spatial. Whatever the fine details of success and failure of the putative CC axons, the distinction must be quite small in mice that exhibit both a Probst bundle and a small CC. In cases of a small CC, the route of callosal axons is sometimes ‘tortuous and convoluted’ (Ozaki et al. 1987), but axons from many regions of dorsal cortex cross to topographically correct, homotopic regions despite the disorganization in the CC at midplane (Olavarria et al. 1988).

## Discussion

These results prove beyond doubt that recombination of strain BALB/cWah1 and 129P1/ReJ genes can yield a much more severe and at the same time much less variable forebrain commissure defect than occurs in either progenitor strain. Three of the recombinant lines rapidly evolved a pattern of total CC absence and greatly reduced size of the HC in every animal examined after only six generations of inbreeding. This novel result provides a useful source of material for anatomical and physiological study of mouse brain (e.g. Schimanski et al. 2002). It also reveals an important fact about the genes involved in the commissure abnormalities: there is nothing inherent in the particular BALB/c and 129 alleles that make development so highly variable. If this were so, then recombining them to obtain an even more devastating defect should have accentuated the already extreme variability within the genetically uniform progenitor strains. Instead, increasing the severity of the defect actually decreased the variability of its expression.

At least in principle, the decrease in variability could have proceeded gradually and continuously, involving every possible intermediate degree of defect, until it approached total absence of the HC and therefore could not get much worse. In fact, the state of uniformly poor commissure development was reached by abrupt transitions that resulted in very few mice with intermediate commissure sizes. This pattern of results is consistent with the existence of two developmental thresholds in forebrain commissure formation. Although the transition from normal brain to absent CC to deficient HC undoubtedly arose from specific combinations of abnormal genes, the sparse zones in the distribution of the index of abnormality cannot be attributed to a scarcity of specific gene combinations. On the contrary, gene combinations with intermediate degrees of severity must have been the most common situation in the F<sub>2</sub> hybrids and RI lines, given that the BALB/c and 129 progenitors themselves must have differed at two or more loci pertinent to the commissure defects. Furthermore, the most extraordinary degree of developmental variability was seen within the highly inbred, isogenic progenitors.

Many of the data in this study are consistent with a genetic difference between the progenitors involving two major loci. For the major genes, the genotypes of the progenitors would be reproduced in many F<sub>2</sub> animals as well as the early generations of severely affected RI lines. The phenotypic distribution in the F<sub>2</sub>, however, was remarkably less dispersed and more distinctly trimodal than in BALB/cWah1 and 129P1/ReJ (Fig. 2). Two phenomena could give rise to this pattern, both of which could be acting in the present study. First, there could be several modifier genes involved, and homozygosity at all of these loci might require many more generations of inbreeding. Although the probability of fixation at one locus beginning with two alleles is about 98% after 20 generations of full sib mating (Green 1981), genetic purity at multiple loci requires many more generations. Thus, continued inbreeding of the RI lines might lead to more severely abnormal lines. Second, there is evidence that developmental homeostasis or buffering is generally disrupted in highly inbred animals that are homozygous at many loci compared with animals that are heterozygous at many loci (Lerner 1970). This notion could also be addressed by continued inbreeding for many generations in order to determine whether the distinct modes and sparse zones seen in the F<sub>2</sub> distribution would gradually vanish in the intermediate severity RI lines. Cloning to create many genetic copies of a heterozygous animal might provide additional insight into the question of developmental variability and homozygosity, but cloning itself can augment phenotypic variation (Archer et al. 2003a, b; Humphreys et al. 2001).

Incomplete penetrance for CC defects is sometimes reported in studies involving transgenic mice (e.g. Hu et al. 2003), but it is difficult to adduce its origins, because small samples are often involved. Furthermore, targeted mutations are often created in embryonic stem cells from a 129 strain (Wynshaw-Boris et al. 1999) that has CC defects of its own (Magara et al. 1999) and then backcrossed onto another strain background, which creates a highly variable genetic background unless the study is done after many backcross generations.

Our data indicate that an embryo which fails to achieve an HC size that is at least 15% of the normal value before it has reached a body weight of 0.8 g will never form a normal CC, although the HC itself may recover fully. This was especially apparent in the F<sub>1</sub> hybrid cross of BALB and 129, in which almost every adult showed a normal CC, while almost every embryo showed substantially retarded HC formation. Because the lower tail of the distribution of HC size in the F<sub>1</sub> hybrid embryos was close to the threshold value of 15%, it is

not surprising that one adult hybrid was found to have a very small CC (Fig. 2). An occasional BALB/c × 129 F<sub>1</sub> hybrid with no CC at all would be supportive of the threshold model, and such an occurrence has been observed (F. Biddle, personal communication).

A threshold for CC formation occurs because of the relative timing of CC axon growth and erection of the septal bridge. HC axons normally cross midplane considerably before CC axons arrive there, and the region of septal fusion grows sufficiently large that early CC axons arrive near the dorsal surface of the HC. If the bridge is not present, however, the growth cones of CC axons turn away from mid-plane and enter the Probst bundle. Once an axon enters the Probst bundle, it generally does not grow back toward mid-plane and retest the conditions for crossing; instead, it re-enters ipsilateral cortex. Once enough CC axons are recruited into the massive whorl in the Probst bundle, it appears that all subsequent CC axons will fail to approach midplane, even though a septal bridge may have formed belatedly. These axons may be provoked into crossing by a surgical intervention that creates an artificial bridge, however (Silver & Ogawa 1983).

If the threshold or critical region in the embryo forebrain is fairly wide, adult mice with intermediate degrees of CC deficiency should comprise the most abundant phenotypic class in strains such as BALB/c and 129 with intermediate average severity of commissure defects (see Fig. 13). Only when the critical region in the embryo is narrow relative to the ubiquitous individual variation in timing of growth of the septal bridge will the striking bimodal distribution of CC size be seen in adult mice of an inbred strain. It is the narrow window of opportunity for successful traverse of the interhemispheric fissure by callosal axons that creates a developmental threshold and gives rise to incomplete penetrance of absent CC in isogenic BALB/c and 129 mice.

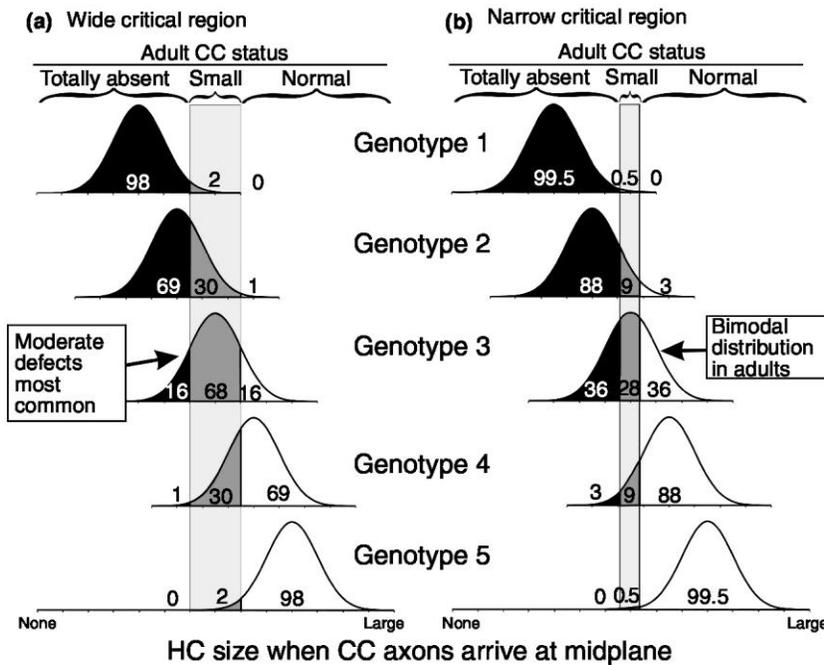
Some of the evidence for a threshold presented in this study is correlational (e.g. Figure 11), but the crucial methodology was experimental – the deliberate recombination of multiple genes to alter the relative timing of developmental processes. Surgical intervention in the embryo, a method used previously to disrupt the glial sling (Silver et al. 1982) and insert an artificial bridge between the hemispheres (Silver & Ogawa 1983), currently lacks sufficient precision in a 0.5-g embryo to damage selectively the HC at midplane without also disrupting the glial sling and causing massive bleeding into the third ventricle. Fine-scale tissue interactions in the mouse brain may be revealed by the inherent variability in neural development within a strain, for example the intra- and infra-pyramidal mossy fiber projections to the hippocampal CA3 region (Lipp et al. 1989). Thus, incomplete penetrance can be utilized as a non-invasive experiment to study subtle processes in the embryo brain with exquisite sensitivity.

The timing of events must also be inferred by comparing large samples of embryos at different maturities (Fig. 12), because we cannot follow the progress of axon growth in vivo in complex tissues the way it can be observed in vitro in simplified situations. Inferences based on correlation of events at different ages are strong, because mice of identical genotypes are being compared in the absence of differential mortality. It is reasonable to infer that the animals most severely abnormal at one age would generally be the most severely afflicted only a few hours later. There is no evidence that an entirely normal embryo at 0.7 g would suddenly retract all its CC axons from the opposite hemisphere and construct an elaborate Probst bundle in only a few hours. Our inability to know the exact history of individual embryos limits the precision of assertions about timing and location, although this precision may be enhanced by the study of very large samples. For our argument about developmental thresholds, the crucial observation is that the duration of a critical period is several hours, whereas the difference between genetic groups in the delay of forming the septal bridge is a day or more.

Our results attribute callosal agenesis in common inbred strains to the complete absence of a tissue bridge between the cerebral hemispheres when callosal axons arrive at mid-plane. The term bridge implies a mechanical structure, but this interpretation does not deny the importance of chemical guidance mechanisms. Unlike laboratory research on mechanical factors that employs non-biological devices to isolate the phenomenon (Rajnicek et al. 1997), the mouse forebrain is entirely biological. CC growth cones have receptors that can sense critical chemicals diffusing across midline (Richards et al. 2004; Stein & Tessier-Lavigne 2001), and disruption of these processes by targeted mutations can impair formation of the CC. Nevertheless, a major

problem in many mice is the lack of a suitable substrate at the mid-plane crossing point. It is not a situation where the usual cell bodies and processes are present, but some critical cell surface molecule is lacking; instead, the cells and their processes are not there at all.

The threshold model presented here is similar to the classical observations of Wright (1934a) on the genetics of extra toes in guinea pigs. Whereas his original model involved just one threshold separating three- and four-toed pigs, he later posited an additional threshold to distinguish between partial and complete development of the extra toe (Wright 1960, 1968). In Wright's models, the position of the thresholds was in arbitrary units that yielded the appropriate frequency of the various phenotypes in the adult animals, and he noted that 'no direct evidence has been obtained on the nature of the individual factors' distinguishing normal and abnormal litter-mates (Wright 1934a; p. 536). In the present study, on the other hand, we have identified the anatomical processes that create one of the thresholds and demonstrated why the threshold region is so narrow that a bimodal distribution is seen in isogenic adult mice.



**Figure 13: Models of width of threshold region in mouse embryos.** Each of five abnormal genotypes has a different, normally distributed size of the hippocampal commissure (HC) when the first corpus callosum (CC) axons arrive at the middle of the brain. Numbers give percentage of the area in the distribution in each of three phenotypic classes. If the tissue bridge that is associated with the HC is too small because it is so retarded, CC axons will return to the hemisphere from which they originated. If it is large enough, an anatomically normal adult CC will result. For a few embryos in the threshold region, a small CC will form, while most putative CC axons will enter the Probst bundle. Genetically normal mice would have a distribution shifted even further to the right, such that the HC forms a full day before CC axons arrive, and no animal would ever have even a small CC. (a) If the threshold region is wide, the most common phenotype in adults of strains with intermediate frequency of CC defects should be a small CC. This situation is never observed in the strains and crosses studied here. (b) A narrow threshold region will give rise to a bimodal distribution of CC size in adults, where very few mice exhibit a small CC.

Wright (1934a) demonstrated that the percentage of animals exceeding the thresholds for extra toes, while being a product of genotype, was also influenced by several environmental factors such as maternal age, season of birth and quality of the feed. In the case of absent CC, on the other hand, distribution of the defect among and between litters of BALB/cWah1 mice is effectively random (Bulman-Fleming & Wahlsten 1991; Wahlsten 1989), and the degree of penetrance is unaffected by a wide range of environmental treatments including prenatal ethanol (Wainwright & Fritz 1985), prenatal malnutrition (Wainwright & Gagnon 1984), early postnatal handling (Bulman-Fleming et al. 1992) and the inbred maternal environment (Bulman-Fleming & Wahlsten 1988). When a mother mouse is pregnant with a second litter while nursing the first litter, the frequency of absent CC almost doubles in the second litter (Wahlsten 1982c), but that maternal environmental factor was not germane to the present findings because almost all mice were studied in first litters.

In his classic study of otocephaly in the guinea pig, Wright (1934b) remarked about characteristics showing incomplete penetrance: 'being inconvenient for genetic work, they are largely discarded. The outsider is thus likely to obtain an exaggerated impression of the frequency with which genes determine clear-cut, absolute

effects'. The present study illustrates how extreme phenotypic variability among animals with the same genotype, while complicating genetic analysis, can help us perceive and understand developmental interactions more clearly.

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