Retarded Formation of the Hippocampal Commissure in Embryos From Mouse Strains Lacking a Corpus Callosum

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Abstract:
A precise description of the timing and route traveled by axons traversing the telencephalic midline through the ventral hippocampal commissure (HC) is essential for understanding the role it plays in the formation of the corpus callosum (CC). A normal baseline of HC development was described in B6D2F2 hybrid mice and then compared with two inbred strains of mice displaying callosal agenesis, BALB/cWah1 (50% CC defect) and 129/J (70% CC defect), their F2 hybrid (C129F2—33% CC defect), and a recombinant inbred strain (RI-1—100% CC defect) derived from pairs of C129F2 mice. Embryos weighing from 0.25 g to 0.70 g (E14.5–E17) were collected and fixed by perfusion. Axon tracts were labeled using crystals of the lipophilic dyes DiI and DiA inserted into the hippocampal fimbria and cerebral cortex. HC axons in B6D2F2 mice first cross the midline at about 0.350 g body weight (E14.8) by traveling over the dorsal septum and along the pia membrane lining the longitudinal fissure. Earlier crossing was prevented by the presence of a deep cleft formed by the longitudinal fissure extending down into the septal region. Subsequent axons fasciculated along existing axons, gradually building the dorsoventral height of the HC to about 200 μm by 0.600 g. The earliest callosal axons from frontal cortex crossed the midline at 0.620 g and were clearly seen fasciculating along and between existing hippocampal axons at the dorsal surface of the HC as they crossed. In the acallosal strains, HC formation was delayed by the continued presence of the cleft deep in the septal region. This delay in time of crossing was correlated with later CC defect expression. Initial HC crossing occurred at about 0.470 g (E1 6.25) in BALB mice and about 0.520 g (E1 6.5) in 129 mice. In the RI-1 embryos, first HC crossing was estimated at about 0.750 g (E1 7.5), although several older embryos showed no crossing. These results show the importance of the HC for successful CC formation and suggest that absent CC arises as a consequence of a developmental defect which affects the formation of the hippocampal commissure prior to arrival of CC axons at midplane.

Key Words: neurogenetics; fornix; axon guidance; inbred strain; BALB/c

Article:
INTRODUCTION
An accurate description of the route traveled by commissural axons is important for understanding the structural defects that arise during commissure formation. The mouse forebrain provides an excellent model for studying the traverse of the telencephalic midline by developing commissural axons. Inherent defects in the structure of the ventral hippocampal commissure (HC) and corpus callosum (CC) have been described in several strains of mice (Livy and Wahlsten, 1991; Ozaki and Wahlsten, 1992; Wahlsten and Bulman-Fleming, 1994), but the route taken by axons traveling through the hippocampal commissure has received little attention. These axons can first cross the midline by passing through the medial septum, over the top of the medial septum at the base of the longitudinal fissure, and have also been suggested to travel extracerebrally through the pia membrane lining the base of the longitudinal fissure (Glaser, 1975). Axons of the corpus callosum cross the midline between the HC and the pia membrane at about E16 in normal hybrid mice (Wahlsten, 1981; Ozaki and Wahlsten, 1992), which is about 1 day after the hippocampal axons across (Glaser, 1975; Wahlsten, 1981). Despite intensive study, the precise route traveled by callosal axons remains elusive. This is due in part to the difficulty in
differentiating between callosal and hippocampal axons at the time of crossing. In some cases the two structures have been considered as one owing to the indistinct border between CC and HC (Glas, 1975; Wahlsten and Smith, 1989). Silver et al. (1982) suggested that CC axons cross the midline using the ‘sling,” a layer of subventricular cells which forms a bridge between the lateral ventricles just anterior to the HC, but it is not yet clear whether these cells guide the first callosal axons to cross midline. The close apposition between HC and CC axons has led to the suggestion that the HC plays a role in guiding early CC axons across the midplane in rodents (Valentino and Jones, 1982). HC formation is retarded in some mice with callosal agenesis, suggesting a causal relationship between HC formation and CC agenesis (Wahlsten, 1987; Livy and Wahlsten, 1991).

Imprecise staining methods may not reveal axon routing. Staining axons with silver and/or hematoxylin/eosin (Glas, 1975; Silver et al., 1982; Wahlsten, 1987), or neurofilament antibody (Silver et al., 1993), does not distinguish between axons of differing origins, nor does this indicate positions of individual axons relative to cellular structure. Lipophilic dyes (Honig and Hume, 1989) diffuse within the membrane of the axons, providing a precise labeling of axon position, allowing the route traveled by the axons to be seen. The use of two dyes with different spectral properties permits differential labeling between sides of the brain or different structures within the brain. The fine labeling provided by lipophilic dyes permits a detailed view of growth cone morphology (Godement et al., 1987; Ghosh and Shatz, 1992) and the change in complexity of hippocampal axon growth cones as they approach and cross the midline. A similar description of growth cone morphology in developing callosal axons has revealed a decrease in structural complexity soon after the growth cones cross the midline region (Ozaki and Wahlsten, 1992).

In this work, we examine the formation of the hippocampal commissure in normal hybrid mice to provide a more precise description of the timing and location of first crossing of the HC axons and to show the relationship between the HC and CC during early development. This description is then used as a baseline for comparison with HC development in strains of acallosal mice. We show that early HC axons cross the midline in close association with the pia membrane and that early callosal axons cross the midline while contacting existing hippocampal axons. In the acallosal strains, hippocampal axons are unable to cross owing to the presence of a cleft extending deep between the hemispheres. Preliminary results of this work have been published previously in abstract form (Livy, 1992, 1994).

MATERIALS AND METHODS

Animals
Normal development was described using the F2 offspring (B6D2F2) from hybrid B6D2F1/J parents (C57BL/6J females × DBA/2J males) obtained from the Jackson Laboratories, Bar Harbor, Maine, at 7–8 weeks of age, then raised and bred at the University of Alberta. Acallosal development was described in two inbred strains, BALB/cWah1 (BALB), bred and maintained at the University of Alberta, and 129/J (129), obtained from the Jackson Laboratories, Bar Harbor, Maine, at 7–8 weeks of age, then raised and bred at the University of Alberta. Although the expression of CC deficiency is high in these two strains, the incidence of total CC absence is relatively low (see Table 1). The F1 offspring from a BALB × 129 (C129F1) cross show no CC defect, which supports a two-locus recessive model of inheritance (Livy and Wahlsten, 1991). Among the F2 offspring from crossing two C129F1 mice, there is about a 25% incidence of total CC absence (Wahlsten and Schalomon, 1994, plus unpublished data). Recombinant inbred strains are now being formed from pairs of 129 × BALB F2 mice (Wahlsten and Sparks, 1995), and in one of the RI lines (#1) every adult animal in recent litters has had complete absence of the CC and greatly reduced HC, as is often seen in the strain I/LnJ (Lipp and Waanders, 1990; Livy and Wahlsten, 1991). This study used both the C129F2 offspring and one line (RI-1) of recombinant inbred animals at the sixth generation of full-sib mating.

All mice were housed in 29 × 18 × 13-cm opaque plastic mouse cages with Aspen-Chip bedding (Northeastern Products Corp., Warrensburg, NY) with a few sheets of toilet tissue added. Pregnant females were provided with a Nestlet for improved nest building and free access to food (non-autoclaved Wayne Rodent Blox 8604) and tap water. Room temperature was maintained at approximately 23°C with a 12/12-hour light cycle (lights on at 6 am).
Embryo Collection

One male and one to three females between 80 and 150 days old were placed together in a cage for 4 hours or overnight, after which the females were checked for vaginal plugs. Plugged females were weighed and housed individually for the duration of gestation. Conception (0.0 days) was considered to be the midpoint between plug detection and the previous plug check. B6D2F2 embryos were extracted between gestational days 14 and 16.5 (E14–E16.5) to obtain body weights ranging between 0.250 g and 0.700 g to encompass initial midline crossing by both hippocampal and callosal axons. As proposed by Kaufman (1992), we refer to all mice studied prenatally as embryos, even though some have completed organogenesis and might be regarded as fetuses. Development is generally slower in inbred strains than in healthy hybrids (Wahlsten and Wainwright, 1977; Wahlsten, 1987), and therefore to observe early midline crossing by hippocampal axons in acallosal strains, embryos were removed between E15 and E17 to obtain body weights ranging between 0.300 g and 0.600 g. The numbers of litters and embryos collected from each of the strains and providing usable data are shown in Table 2.

To extract the embryos, pregnant females were euthanized with sodium pentobarbital (120 mg/kg IP), and their uteri were removed and rinsed in a solution of 0.9% saline in ice. The uteri were cut open, and each embryo was separated from its placenta by cautering the umbilical artery. Embryos were then rinsed in ice-cold 0.9% saline, carefully blotted to remove excess fluid, and weighed to the nearest mg (see Fig. 1). Immediately after weighing, each embryo was perfused intracardially with 3–5 mL of 10 mM phosphate-buffered saline (pH 7.6) followed by 10–15 mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.6) using a peristaltic perfusion pump, stereomicroscope, and micropipettes. After removing the scalp, the embryo head was placed in fresh fixative overnight. The following day the occipital bone was removed, and the head was placed in fresh fixative and stored until the dye crystals were inserted.

Dye Insertion

Labeling of axonal membranes was achieved using crystals of the fluorescent carbocyanine dye DiI (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) and the aminostyryl probe DiA (4-(4-dihexadecylaminostryryl)-N-methylpyridinium iodide) (Molecular Probes, Eugene, Oregon). The brain was extracted from the skull, and the caudal parts of the occipital and entorhinal cortices were removed to expose the hippocampal fimbria. A crystal of either DiI or DiA, between 30 and 50 mm in diameter, was placed in the area of the hippocampal fimbria using the tip of a fine dissecting pin. Most brains were double-labeled; either the right and left fimbria were labeled with contrasting dyes, or both fimbria were labeled with one dye, and the frontal cortex (if applicable) was labeled with several crystals of the contrasting dye (see Fig. 2) in slightly different locations to maximize the number of CC axons visualized. In a few cases these dye applications also involved the cingulate cortex. After insertion of dye crystals, the brains were placed in fresh fixative and stored

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>% CC abences</th>
<th>% CC defect</th>
<th>Initial HC crossing (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6D2F2</td>
<td>0*</td>
<td>0*</td>
<td>0.350</td>
</tr>
<tr>
<td>C129F2</td>
<td>24*</td>
<td>30*</td>
<td>0.440</td>
</tr>
<tr>
<td>BALB/ cWahl</td>
<td>20*</td>
<td>55*</td>
<td>0.470</td>
</tr>
<tr>
<td>129/ J</td>
<td>16.67*</td>
<td>70*</td>
<td>0.520</td>
</tr>
<tr>
<td>RI-1</td>
<td>100*</td>
<td>100*</td>
<td>0.750</td>
</tr>
</tbody>
</table>


### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of litters</th>
<th>Number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6D2F2</td>
<td>22</td>
<td>149</td>
</tr>
<tr>
<td>C129F2</td>
<td>10</td>
<td>77</td>
</tr>
<tr>
<td>BALB/ cWahl</td>
<td>7</td>
<td>30</td>
</tr>
<tr>
<td>129/ J</td>
<td>7</td>
<td>33</td>
</tr>
<tr>
<td>RI-1</td>
<td>6</td>
<td>40</td>
</tr>
</tbody>
</table>
in the dark at room temperature for at least 4 weeks, although some brains were stored up to 2 years with no apparent loss of labeling quality. Some brains were stored at 37°C to increase the rate of dye diffusion (Senft, 1990).

Sectioning and Photography
Sections were cut using a microslicer (DSK–DTK 1500E) and a sapphire knife (Pelco). Preliminary results indicated that tissue at these young ages suffered extensive damage because of shear stress during cutting, particularly at ages where midline fusion was limited and only one or two axons were crossing the midplane. Gelatin infiltration was found to be successful in providing the necessary structural support. The brains were first rinsed in 0.1 M phosphate buffer for 2 h at 37°C. They were then infiltrated with 2% gelatin at 37°C for 6 h, and then with 5% gelatin at 37°C for 12–24 h, after which the gelatin was fixed using 4% PFA for 48 h at 4°C. The brain was removed from the fixative and glued caudal surface down to the microslicer stage using a cyanoacrylate glue. Coronal sections cut at 30–60 μm were submerged in a 1:1 mixture of 8% PFA in 0.2 M phosphate buffer (pH 10.0) and glycerin at 4°C overnight and then mounted on glass slides using the same solution and coverslipped. To provide staining of cell nuclei, 0.02% bis-benzimide was added to the overnight solution of certain sections (Senft, 1990).

Most sections were immediately viewed and photographed with a Leitz epifluorescence microscope equipped with a rhodamine filter for DiI (N2) and a band-pass filter for DiA (L3). In some cases a fluorescein filter set was used (I2/3) to view DiA. Best results for photography were obtained using Kodak P800/1600 Ektachrome film at 1600 ASA, primarily due to the low intensity of the DiA viewed using the L3 filter. Sections not able to be immediately photographed were stored at 4°C until viewing was possible. Certain brains were viewed using a Leitz Confocal Laser Scanning Microscope (provided by the Dept. of Anatomy, Univ. of Alberta) and a Molecular Dynamics CLSM, to provide a more enhanced view of axon movement and location.

Results
In all brains, clear and complete growth cones could be observed, indicating complete anterograde transmission of the dyes. In older embryos retrograde labeling also provided information about commissural axon origins, although this labeling was not as consistent. Gelatin infiltration did not appear to interfere with the observation of either dye, nor did it appear to facilitate further diffusion of either dye. The bis-benzimide gave a clear view of the nuclei of the pia membrane and other cells which were present in the midline region. In most cases, labeled axons were present throughout the cross-sectional profile of the hippocampal commissure, although there were cases in which the dye did not include all HC fibers in older embryos. These unlabeled areas were quite apparent due to the absence of any fluorescence from the lipophilic dyes and low density of cells stained with bis-benzimide.

Body Weight vs. Chronological Age
Chronological age is not the best indicator of mouse embryo development (Wahlsten and Wainwright, 1977). A more accurate assessment of developmental stage in the brain is provided by the body weight of the embryo (Ozaki and Wahlsten, 1992; Wahlsten and Bulman-Fleming, 1994). Figure 1 shows the approximately linear relationship between body weight and chronological age of gestation between E1 4.5 and E1 6.5 in B6D2F2 embryos, which is similar to previous results (Ozaki and Wahlsten, 1992; Wahlsten and Bulman-Fleming, 1994). No significant difference was found in development rate between the B6D2F2, C129F2, and BALB embryos, although the C129F2 embryos appeared to lag about half a day and the BALB embryos lagged about 1 day behind the B6D2F2 embryos. The 129 embryos demonstrated a higher rate of development than all other strains but also displayed a more pronounced lag of about 1.5 days in their development.

HC Development in B6D2F2 Embryos
The columns of fornix were present in the septal midline region of the youngest embryo at 0.259 g (Fig. 3a). From the dye insertion site, the hippocampal axons moved rostrally and medially through the brain and then turned sharply ventromedially toward the midline, just rostral to the lamina terminalis, which is defined here as the most anterior border of the third ventricle. Upon reaching the midline area, the fornix columns turned
ventrally toward the anterior commissure. The columns of fornix were initially far apart, largely due to the presence of the longitudinal cerebral fissure extending deep between the hemispheres with its floor located just above the anterior commissure (Fig. 3b). As growth continued, fusion of the telencephalic vesicles progressed dorsally and rostrally, and the fornix columns rapidly thickened and moved closer to midplane. Axons appeared to grow along the dorsal surface of existing fornix fibers toward the midplane with extensive branching apparent. These axons were identified by their large and complex growth cones. Axons migrating within the fornix column could not be differentiated because of the fluorescence of the surrounding fibers.

As the columns of fornix made their ventromedial turn, some axons emerged and moved toward the midline, even in the youngest embryo (see Fig. 3a). These axons moved through the cells forming the medial border of the septal area and contacted the pia membrane lining the longitudinal fissure. In younger embryos, contact at the pia did not appear to be directed to any particular location along the depth of the fissure. Shortly before the first midplane crossing of the HC axons, most of the contact with the pia was very close to, but not necessarily directly at, the bottom of the fissure. After contacting the pia, many of the growth cones turned ventrally and migrated along the pia (Fig. 4). No growth cones were seen to turn dorsally. Axons emerging from the fornix fibers were seen all along the length of the ventromedial progression of the fornix column (Fig. 4). With increasing maturity, newly arriving axons continued to travel along the dorsomedial surface of existing fibers, providing increased contact between these fibers and the pia membrane. However, these axons were prevented from crossing by the continued presence of the pia-lined cleft at midline (Fig. 3b). Prior to the first crossing, these axons turned away from the midline and re-entered the fornix column, becoming indistinguishable from the surrounding fornix fibers.
As development proceeded, the floor of the longitudinal fissure continued to move dorsally as the midline fused. The first HC axons to cross the midline remained in contact with the pia membrane and followed the ventral contour of the fissure floor (Fig. 3c). The first few axons did not appear to precede a large bundle of axons in waiting. Instead, an increasing number of fibers gradually emerged from the fornix column to cross. Later-crossing axons were dorsal to those crossing previously but still followed along the fissure floor. The first two or three axons to cross did not necessarily fasciculate along each other. Some axons were separated by as much as 60 μm along the rostrocaudal axis and 40 μm along the dorsoventral axis. However, a small cylindrical bundle of axons was very quick to form at the very base of the fissure (Fig. 3d), and subsequent axons were seen to fasciculate along these existing axons.

The initial crossing of midline was made earlier by axons from the right hemisphere in six of eight brains labeled and viewed appropriately, whereas one brain showed a similar development between sides and one clearly had initial crossing from the left side. The earliest HC axon crossing was seen in the brain of a 0.328-g embryo which was surprisingly well formed and was the only one to appear in this manner. In most embryos of this age, axons had not yet crossed or were just about to cross. Most of the early crossings occurred between 0.340 g and 0.360 g body weight, but several larger embryos were found with no crossing. The largest embryo with no crossing was 0.391 g; the fornix columns were well formed, but an unusually deep fissure continued to separate the hemispheres.

New axons approaching midline were seen along the dorsal surface of the ipsilateral fornix and then across the midline on top of existing HC fibers. They continued up the dorsomedial surface of the contralateral fornix and fimbria up to their target areas in the hippocampal formation (Fig. 3e). Soon after initial crossing, individual axons were still apparent; however, by 0.400 g a larger bridge-like structure spanned midline (Fig. 3f).
Continued growth dorsally and rostrally resulted in a bridge that was roughly cylindrical in shape at midline with a dorsal-ventral height of about 200 μm at 0.620 g body weight. Figure 5 shows the rate of dorsal growth of the HC during this time of gestation. The initial formation of the HC bridge occurred at the bottom of the longitudinal cerebral fissure, and by 0.600 g the gradual accumulation of axons in the HC eventually reached or surpassed the dorsal limit of the primordium of the subfornical organ just caudal to the HC (Wahlsten and Bulman-Fleming, 1994). Fusion of the hemispheres in the regions ventral and posterior to the HC appeared to be complete, but the zone directly anterior to the HC was initially filled by a loose plexus of fibers in a region that later occupied the ventral portion of the cavum septi pellucidum (Hankin et al., 1988).

FIGURE 3. Development of the hippocampal commissure in mouse embryos. a: Well-formed columns of fornix can be seen in the midline region about 800 mm from the frontal pole at 0.25 9 g body weight in
normal B6D2F2 embryos. One DiA-labeled axon can be seen extending out of the column toward the midline. This particular axon has contacted the pia membrane lining the longitudinal fissure (arrows). b: The cleft formed by the longitudinal fissure (shown by arrows) extends deep between the hemispheres and prevents hippocampal axons from crossing the midline early in development as shown in this 0.345-g BALB embryo. c: A complex growth cone contacting the pia membrane in a 0.490-g BALB embryo. The lower process from this cone (arrow) is extending ventrally to cross the midline. Other growth cones may make contact farther up the fissure, but they typically proceed in a similar way. d: A typical early axon crossing shown here in a 0.595-g 129 embryo but which appears similar in all other strains. Note that the small bundle of crossing axons is at the immediate base of the longitudinal fissure (arrow). e: DiI-labeled axon (indicated with arrows) extending across midline and continuing up the dorsomedial surface of the contralateral fornix in a 0.413-g B6D2F2 embryo. Although some axons were present in the middle of the fornix column, newly arriving and migrating axons were only seen on the dorsal surfaces of existing axons. f: The bridge-like structure formed by the crossing HC fibers is shown in this 0.41 0-g B6D2F2 embryo. Some of the dye has diffused into the surrounding tissue, which in this section provides a descriptive view of the entire midline region including the future floor of the longitudinal fissure (solid arrows), and the cleft extending deep between the hemispheres to the point of fusion (open arrow). g: Midsagittal section of the same 0.620-g B6D2F2 embryo shown in Figure 6. Several early DiA-labeled (green) callosal axons (arrows) which have just crossed the midplane can be seen sitting directly on top of the HC (red). h: Confocal view of a midsagittal section during early callosal axon crossing in 0.692-g B6D2F2 embryo. Note that callosal axons appear as a bundle just rostral to the HC, but that individual callosal axons (arrows) have already extended across the midline along hippocampal axons at the dorsal edge of the HC. Scale bar 5 50 mm in a–f, 20 mm in g and h.

FIGURE 4. As development proceeds, the columns of fornix (F) move closer together in the septal region (S), and an increasing number of axons extend from the columns to contact the pia. In this 0.275-g B6D2F2 embryo, the growth cone of one of these axons (arrow) has just contacted the pia and has started to migrate ventrally along the pia. Note the complexity in many of the growth cones seen. Scale bar = 50 μm.

The visibility of individual HC fibers crossing midline was limited to those on the dorsal surface. These axons did not tightly fasciculate along other fibers but rather formed a loose association. By about 0.450 g, some axons had traveled far enough into the contralateral hemisphere to be labeled by the dye inserted into the

FIGURE 5. Growth of the HC in B6D2F2 embryos occurs in a fairly linear manner from the time of early crossing to about 0.7 g body weight. Thickness reaches about 200 μm by about 0.6 g body weight.
fimbria. In cases in which the dye was transported retrogradely, cell bodies that were labeled appeared primarily in the CA3 region of the hippocampus (Swanson and Cowan, 1977).

**Interaction Between HC and CC Fibers**

Callosal axons were seen crossing the midline in association with hippocampal axons as early as 0.485 g, but these callosal axons originated from the cingulate cortex and not the frontal cortex. Axons have previously been shown to emerge from the cingulate cortex and cross the midline this early in the development of rats (Koester and O’Leary, 1994). The first definitive crossing of CC axons from frontal cortex occurred in an embryo of 0.620 g. Figure 6 is a parasagittal view of this brain in which all of the labeled callosal fibers are emerging from the cortex and growing directly toward the HC. Figure 3g is a midsagittal view from this same embryo. A few callosal axons can be seen directly on top of the HC, while a few other fibers appear at the rostral edge. Confocal reconstruction showed that these axons reached midway through the adjacent section, after which there was a gap of about 30 μm before axons from the contralateral hemisphere could be seen. This is the first time the early crossing of callosal fibers from frontal cortex has been seen with such clarity and precision. The callosal axons did not follow a straight path but rather appeared to weave between and fasciculate along the loose association of hippocampal axons at the dorsal HC edge.

Figure 3h is a confocal image of the midsagittal plane from a 0.692-g embryo which shows several callosal axons intermixed among the hippocampal axons at the dorsal edge of the HC. Callosal fibers rapidly form a small bundle on top of the HC, and by 0.700 g the CC appeared relatively large, just dorsal and rostral to the HC in most brains. A layer of cells immediately ventral to the callosal fibers was quite thick in the area rostral to the HC, but this structure was almost nonexistent at the interface between the HC and CC. These cells were likely the sling cells described by Silver et al. (1993). Cell bodies were seen as a wedge-shaped mass along the medial edge of the lateral ventricle at the level of the floor of the longitudinal fissure as early as 0.586 g, but no cell bodies of this structure were seen close to the midsagittal plane until 0.682 g, when they were located rostral to the HC and ventral to a well-formed CC.

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**FIGURE 6.** Parasagittal section of a 0.620-g B6D2F1 embryo showing the callosal axons (CC) extending down from the cortex directly in line with the top of the hippocampal commissure (HC). At the midsagittal plane, the callosal axons were seen crossing directly on top of the HC in association with hippocampal axons (see Fig. 3g). Scale bar = 50 μm.

**FIGURE 7.** Coronal section of a 0.503-g BALB embryo stained with bis-benzimide. The nonstained areas show the location of hippocampal axons (HC) which are definitely in the correct location for crossing. These axons are not able to cross because of the small cleft (arrows) which can be seen still extending down directly in the middle of the septal region (S) where crossing would have occurred. Scale bar = 50 μm.
**HC Development in Acallosal Mice**

The pattern of development in the acallosal strains was remarkably similar to that in the B6D2F₂ embryos up until the time of first crossing. Fornix columns were present early, and axons emerged from the columns to extend to midline, but the cleft formed by the longitudinal fissure remained deep, long past the time of first crossing in B6D2F₂ mice (Fig. 7). As indicated in Figure 8, HC axons first crossed the midline at about 0.470 g in BALB embryos and at about 0.520 g in 129 embryos. The time of first crossing for the C129F₂ embryos was at 0.440 g, earlier than either of the parent strains, but in the RI-1 embryos it was necessary to extend the body weight range of collected embryos in order to see any evidence of hippocampal axon crossing, which eventually occurred much later than any of the other strains, at about 0.750 g. Despite the obvious delay in crossing, the first HC axons in RI-1 embryos crossed the midline at the ventral tip of the longitudinal fissure and appeared to remain in contact with the pia membrane as they crossed, similar to the B6D2F₂ embryos. In the RI-1 embryos, callosal axons were seen in the midline region after about 0.600 g and had begun to form Probst bundles in the larger embryos. The differences in crossing times between the acallosal strains are correlated with their adult expression of the CC defect (see Table 1).

![Diagram of HC axons crossing midline](image)

**FIGURE 8.** Crossing of the telencephalic midline by hippocampal axons in all strains. Inverted triangles indicate embryos in which crossing has not yet occurred, while those pointing up indicate that crossing has occurred. Arrows indicate the approximate median weight of first crossing for each strain which occurs at about 0.350 g in B6D2F₂ embryos, about 0.470 g in BALBs, 0.520 g in 129s, 0.440 g in C129F₂ embryos, and approximately 0.750 g in RI-1 embryos. Crossing appears to be increasingly delayed in strains with a higher frequency of CC defect.

**Growth Cone Structure**

Growth cone size and complexity changed during the growth of the hippocampal axons through the midline region in mice from the B6D2F₂ and acallosal strains. Prior to the first crossing, almost all axons emerging from or along the dorsomedial surface of the fornix columns were large and complex with extensive branching, particularly in those axons closest to the base of the longitudinal fissure (see Fig. 3b). Growth cones which had contacted and were migrating ventrally along the pia remained larger but no longer displayed the same degree of extensive branching (see Fig. 4). An abrupt change in structure was usually noted in axons which had just crossed the midline. A complex structure was maintained right up to the midline, from which a single fiber emerged and continued up into the contralateral hemisphere (Fig. 9a). Growth cones of those axons which had crossed the midline were all smaller with a very simple, at morphology. Early after the initial crossing, individual growth cones could still be seen as axons approached and crossed the midline. However, once the commissure had formed into a small bundle, only those axons immediately on the dorsal surface could be seen. Of these, most were small and simple in structure.

The variability in the sizes and shapes of growth cones prior to midline crossing is shown in Figure 9b and is contrasted with growth cones of axons after crossing midline. This structural variability has also been found in the first callosal axons to approach midline (Ozaki and Wahlsten, 1993). Growth cones continually change their...
shape during axonal growth (Godement et al., 1994; Halloran and Kalil, 1994), and the variability seen must result at least partly from the snapshot view of growth cone structure at the time of fixation.

**DISCUSSION**

The results clearly indicate that the first hippocampal axons to cross the midline travel over the dorsal septum and along the pia membrane lining the longitudinal fissure. These axons appear to remain in contact with the pia membrane as they cross, but no axons penetrated the membrane. These axons did not appear to precede a larger "main bundle" of axons as is seen in callosal axon outgrowth (Ozaki and Wahlsten, 1992); instead axon emergence was continual and gradual from the fornix columns. Earlier axons emerging from the fornix columns migrated toward midline but were unable to cross owing to the presence of a deep cleft formed by the longitudinal fissure. Dorsal fusion of the fissure eventually enabled the crossing of these axons at about 0.350 g or E14.8 in B6D2F<sub>2</sub> embryos. Axons arriving earlier migrated ventrally along the pia to the approximate location of crossing and then re-entered the fornix columns.

This is in contrast to callosal axons which will wait a few hours for midline development to support their crossing (Ozaki and Wahlsten, 1993). Delayed development results in callosal axons forming a large Probst bundle (Probst, 1901) from which axons will either cross the midline if development allows (Ozaki and Wahlsten, 1993) or will emerge to make ipsilateral connections (Ozaki and Shimada, 1988). Axons within Probst bundles formed by surgical transection of the midline region during the time of callosal development, retain their electrical function, and in the neonate are able to emerge from the bundle and cross the midline after the insertion of a nitrocellulose bridge (Lefkowitz et al., 1991; Silver and Ogawa, 1983). On the other hand, hippocampal axons that fail to pass through the HC in the most severely affected acallosal embryos rejoin the columns of the fornix rather than form a local whorl, although their eventual fates are unknown.
The results also provide clear support for the use of the HC by some early callosal axons to cross midline. These CC axons fasciculated along and between the hippocampal axons at the dorsal edge of the HC. This is the first time that CC axons have been clearly seen to associate directly with hippocampal axons during their traverse of the midline this early in the development of a normal mouse. Wahlsten (1987) has observed CC axons crossing on the dorsal surface of the HC in an acallosal mouse strain, but this was much later in development and the resulting CC was often abnormal in size and shape. The role of these early callosal axons remains unclear. Although they may provide structural support for subsequent callosal axons during midline crossing, early dye-labeled “main bundles” of callosal axons were usually seen at the dorsal-rostral edge of the HC. A more comprehensive distribution of cortical dye placements would demonstrate whether a main bundle of callosal axons was also present immediately dorsal to the HC. The early callosal axons may conceivably act as pioneers for the main bundles, establishing the existence of an intact substrate for crossing and perhaps effecting a signal change for main bundle crossing. On the other hand, there may be several possible locations of crossing for the very first CC axons, and it may not be absolutely necessary that the pioneering CC axons contact the HC bridge. A large sample of embryos clustered in a very narrow range of sizes would be needed to assess this. We examined dozens of normal hybrid embryos and detected only one in which the most advanced CC axons from frontal cortex were precisely at midplane.

The earliest callosal axons seen here to interact with the HC axons originated from the cingulate cortex. Koester and O’Leary (1994) reported an early emergence of callosal axons from the ventromedial cingulate cortex in the rat and have suggested that these axons act as pioneers, defined as the first axons to cross the midline, for the corpus callosum. Although these cingulate axons may be the first to cross the midline, their role in the guidance of subsequent callosal axons is not clear. Axons that use an existing axon pathway for directional guidance often display a simplified growth cone morphology (Dodd and Jessell, 1988; Harrelson and Goodman, 1988), yet growth cone morphology is complex in early cortical callosal axons first approaching the midline and then less complex after crossing (Ozaki and Wahlsten, 1992).

Growth cone complexity is thought to be related to environmental assessment occurring within the cone (Bovolenta and Mason, 1987; Norris and Kalil, 1991; Tessier-Lavigne and Placzek, 1991); however, it may also be a characteristic of neuronal origin (Nordlander, 1987). Callosal axons continue to display some degree of complexity during their growth through the contralateral hemisphere until they migrate up into their cortical target sites (Norris and Kalil, 1990; Halloran and Kalil, 1994). Growth cone complexity was also demonstrated by the hippocampal axons approaching the midline, although less so in those migrating along the pia, and for a short distance into the contralateral hemisphere as these axons made critical decisions about direction of travel. Once crossing was complete, growth cone complexity appeared to simplify markedly, corresponding to the appearance of axons fasciculating along existing axons.

The cell bodies which formed a wedge extending from the lateral ventricles toward the midline were likely the sling cells. In our tissue, nuclei of these cells did not reach the vicinity of the midline until after early callosal axon crossing, when these cells were clearly seen in a position rostral to the HC. It is possible that fine processes emanating from these cells did reach the midline earlier and play an important role in guiding CC axons to the opposite hemisphere. The presence of the sling lateral to the midline at the time of callosal axon crossing may provide a barrier to prevent callosal axon entry into the septal region (Hankin and Silver, 1986, 1988) and may guide the CC fibers in the direction of midplane (Wahlsten, 1987). A similar sling structure has been identified in the cat (Silver et al., 1986) and rat (Katz et al., 1983). Cells covering the surface of the sling have been identified as primitive astrocytes and radial glial cells (Hankin and Silver, 1986). Silver et al. (1993) identified primitive astroglial and radial glial cells at the midline prior to the arrival of callosal fibers.

The importance of mechanisms involved in HC formation is clearly demonstrated in the acallosal strains. HC development in these strains was disrupted by the continued presence of the longitudinal fissure extending deep between the hemispheres. The eventual time of initial crossing by the HC axons was later in strains with a more severe incidence of adult CC defect. Initial crossing occurred at about 0.470 g or E1 6.25 in BALB embryos and about 0.520 g or E1 6.5 in 129 embryos. Because most adults of these strains have a normal HC structure (Livy
and Wahlsten, 1991), it is likely that HC development continues normally once the axons do cross the midline (see Wahlsten, 1987, Fig. 12). However in the strains I/LnJ and RI-1 with 100% total CC absence, adult HC structure is often abnormal (Livy and Wahlsten, 1991). Initial crossing by hippocampal axons may be too late to permit the normal growth of the HC. In the RI-1 embryos, first crossing was estimated as about 0.750 g or E1 7.5, but there were several older embryos which had not displayed crossing. Total HC absence in adult mice has never been reported, and therefore it can be presumed that the HC axons do eventually cross in all animals. This suggests that there is a relatively long period of time when these axons can cross midline. Callosal axons in RI-1 mice arrive at midline prior to first crossing by the HC axons and must wait for these axons to cross and then for the HC to grow to the proper position to support callosal axon crossing. The callosal axons eventually grow back into the ipsilateral hemisphere to form Probst bundles before this can occur. Therefore, the time of initial crossing by the hippocampal axons would appear to be an important factor for the traverse of the interhemispheric fissure by callosal axons. It is significant that in every case in which the HC is markedly reduced in size in the adult mouse, there is no CC at all (Wahlsten and Sparks, 1995), whereas the presence of any amount of CC axons crossing midplane is always accompanied by a normal adult HC.

The substrate used by commissural axons crossing midline is often specific for that event. Commissural axons display an affinity for specific glial cells in the grasshopper (Bastiani and Goodman, 1986) and in Drosophila (Jacobs and Goodman, 1989). In the mammalian forebrain, neurons and radial glia have been identified at the site of the prospective optic chiasm and are thought to be required for the successful formation of the chiasm by retinal ganglion cell axons (Marcus and Mason, 1995; Marcus et al., 1995; Sretavan et al., 1995). Axons of the anterior commissure cross the midline through the medial septum dorsal to the preoptic recess using a tunnel-shaped formation of glial cell processes (Silver et al., 1982). In vertebrates, floor plate cells have been identified in the ventral midline of the spinal cord and brainstem which release a diffusible chemoattractant that orients spinal commissural axon growth in vitro (Tessier-Lavigne et al., 1988; Placzek et al., 1990) and may provide a physical substrate for these axons to cross the midline (Bovolenta and Dodd, 1990; Kuwada et al., 1990; Yaginuma et al., 1991). The lack of these cells disrupts the normal pattern of axon crossing (Bovolenta and Dodd, 1991). It is possible that similar events occur during hippocampal axon crossing; early axons that emerge from the fornix columns and grow toward midline may be orienting in response to a chemoattractant. Such chemical signals may emanate from the pial cells lining the longitudinal fissure, which would explain the early axon emergence toward the pia from the fornix column, but the greater growth cone complexity and extensive branching seen in axons approaching the area immediately ventral to the fissure suggests that chemical signals are released from the area of hemispheric fusion, perhaps owing to the degradation of the trapped pia membrane. Once these axons contact the pia membrane, they grow along the pia across midline. In some embryos, axons reached the midline but did not cross despite the lack of an obvious obstruction. This may indicate the necessity of a second event to complete the crossing event, which is likely to be specific for the substrate that provides physical guidance to the crossing axons. The proximity between axon and pia suggests that the pia provides this contact guidance, although it is known that later in development the pia does not act as a substrate for callosal axons, even when their usual midline substrate is missing in BALB mice (Wahlsten, 1987).

Differential staining with lipophilic dyes has demonstrated the relative locations of callosal and hippocampal axons when early callosal axons cross midline. Combining carbocyanine dye labeling with immunohistochemical techniques (Elberger and Honig, 1990) should enable the observation of axon interaction with specific substrate antigens. Similarly, the use of photo-oxidized Dil with regular and/or immuno-electron microscopy (von Bartheld et al., 1990) should provide a very detailed view of hippocampal axons as they first cross midline to determine if they remain in contact with the pia or whether there is another substrate present. Increased precision in dye labeling should determine whether later axons also travel within the columns of fornix and the HC as well as on their dorsal surface. It should also permit the identification of developing projection patterns of the hippocampal axons. In adult rat HC, axons from more septal regions in the hippocampal formation are found in the more rostral areas of the HC, whereas those axons from the more temporal areas are found in the more rostral area; fibers arising from areas near the ependyma of the lateral ventricles cross in more dorsal regions of the HC, whereas those arising along the pial border cross through the
ventral region (Wyss et al., 1980). Of particular interest is whether any particular axon can indeed cross within the commissure or whether only certain axons from particular origins cross the midline first.

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