Prenatal Formation of the Normal Mouse Corpus Callosum: A Quantitative Study With Carbocyanine Dyes

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Abstract:

Judgment of abnormalities in fetal cortical axon development is more sensitive when a good standard of normal ontogeny is established. The recent availability of postmortem tract tracing methods has greatly improved the observation of axon extension and growth cone morphology in mouse fetuses, which allows much stronger statements about the timing of crucial steps in the formation of the corpus callosum in particular. The first outgrowth and crossing of midplane by axons of the corpus callosum (CC) were examined in 153 normal mouse embryos and fetuses of the hybrid cross B6D2F_2/J with carbocyanine dyes applied to brains fixed by perfusion. In most brains a crystal of DiI was inserted into either frontal, parietal, temporal, or occipital cortex in one hemisphere, and a crystal of DiA was placed into a different site in the opposite hemisphere. Although dye diffusion obscured the emergence of axons, linear regression analysis revealed that the first callosal axons emerged from their cortical cells of origin at about 0.4 g body weight or 15.5 days after conception for all four sites. Subsequent axon growth rate was substantially faster for those from frontal cortex (3.2 mm / day) than occipital cortex (1.8 mm/day). Axons from frontal cortex crossed the cerebral midplane first (0.69 g, E16.3), followed by those from parietal (0.74 g), temporal (0.77 g) and occipital cortex (0.92 g, E16.9). Prior to crossing midplane, the pioneering CC axons were usually 200 μm or less in advance of the main bundle, but when they crossed midplane and encountered CC axons growing from homotopic sites in the opposite hemisphere, the pioneering axons were often 0.5 to 2.5 mm ahead of the main bundle. Growth cones were usually large and complex until they had crossed midplane and were thereafter smaller with simple and flat morphologies. The topography of axons in the CC at midplane was organized according to cortical region of origin from the very beginning, when the CC was only a small cap over the hippocampal commissure and dorsal septum. The quantitative results provide a convenient standard for normal callosal development in mice and should facilitate comparative studies.

Key words: axon guidance, growth cone, pioneer axon, morphometry, regression analysis, tract tracing

Article:

The traverse of the interhemispheric fissure by axons of the corpus callosum is an intriguing example of axon guidance over relatively long distances. The prenatal location and timing of arrival of the first callosal axons at the midsagittal plane are known very approximately in humans (Rakic and Yakovlev, '68), rats (Valentino and Jones, '83), hamsters (Lent et al., '90) and mice (Glas, '75; Silver et al., '82; Wahlsten, '81; Zaki, '85). In rodents the time of first crossing is especially difficult to judge because of the close apposition of the corpus callosum and the hippocampal commissure (Glas, '75). More precise experiments are now possible with the aid of carbocyanine dyes to trace axons and reveal growth cones in fixed fetal tissue (Godement et al., '87; Honig and Hume, '89; Senft, '90). Anatomical tract tracing with axonal transport of dyes or large proteins such as horseradish peroxidase (HRP) is readily done postnatally in placental mammals (Heimer and Zaborszky, '89). Although tracing pathways with HRP is possible prior to birth (Crandall and Caviness, '84; Dehay et al., '88; Floeter and Jones, '85; Lent et al., '90; Norris and Kalil, '90), it is inconvenient and relatively inaccurate. Carbocyanine dyes, on the other hand, are ideally suited to the study of cortical origins of early callosal axons.

Double labelling can be done with dyes having different emission spectra and these lipophilic dyes reveal axonal architecture at the moment of fixation rather than several hours after injection of a tracer, which aids interpretation. They may also be more sensitive than HRP, especially for staining one or a few pioneer axons (Hogan and Berman, '90).

Precise knowledge of normal callosal origins is especially important when making judgments about pathological development, such as hereditary absence of the corpus callosum (Lipp and Waanders, '90; Ozaki et al., '84, '89; Wahlsten, '87). Once the animal is sufficiently mature, it is usually obvious where the zone of abnormality occurs. To determine when and where the first deviation from the normal course of ontogeny occurs, however, requires sensitive quantitative indicators. It has been hypothesized that processes of axon guidance in acallosal mice are relatively normal until the fibres approach the midsagittal plane and find that the proper substrates for crossing midplane are missing (Silver et al., '82; Wahlsten, '87). To test this notion, one must know when these fibres normally emerge from their cortical cells of origin and reach midplane. Quantitative standards for normal development should also facilitate comparisons among mammalian species.

The present study establishes standards for normal callosal origins using B6D2F_2/J hybrid mice. Being derived from a commercially available F_1 hybrid cross with excellent breeding characteristics, the sample can be replicated in other laboratories and future studies. Because the two inbred parent strains (C57BL/6J and DBA/2J) are very different genetically (Atchley and Fitch, '91; Taylor, '72), the F_2 hybrids possess substantial genetic variability and hence generality. The same F_2 hybrid cross has been used to establish standards of normal development for several other aspects of the fetal brain (Wahlsten, '81; Wahlsten and Smith, '89).

MATERIALS AND METHODS

Mice and matings

Subjects were the F_2 hybrid offspring of B6D2F_1/J hybrid mice (cross of C57BL/6J female and DBA/2J male) obtained from the Jackson Laboratory, Bar Harbor, Maine. One male and one to three females were placed together in a cage and the females were checked for presence of a vaginal plug every 4 hours or, in a few cases, after being mated overnight. When a plug was detected, the female was weighed and then housed singly until testing. Conception (0.0 day) was defined as the time midway between detection of a plug and the previous plug check. All mice were housed in a common room with 12 hours light--12 hours dark illumination and maintained at about 23°C. They were allowed free access to Wayne Rodent Blox 8604 and Edmonton tap water. Aspen Chip® (Northeastern Products Corp., Warrensburg, NY) cage bedding was changed weekly, and pregnant females were given a Nestlet for nest construction.

Fetus extraction and fixation

At embryonic ages between E 15 and E 17, pregnant mice were deeply anaesthetized with sodium pentobarbital (120 mg/kg, intraperitoneal injections) and fetuses were retrieved from the uteri and placed in ice-cold 0.9% physiological saline. After the excess solution surrounding the fetus was carefully removed, the body weight was measured to the nearest mg (Fig. 1). Immediately afterwards, the fetus was perfused through the left ventricle with 3-5 ml of 10 mM phosphate-buffered saline followed by 10-15 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.6) by means of a tubing pump and micropipettes (ca. 400 μm internal tip diameter). Brains were left in situ and the whole heads were immersed in fresh fixative for 3-5 days. At this time, the cerebral cortex of both hemispheres was exposed by removing the top of the skull with toothed forceps.

Dye application

With the aid of a dissecting microscope and a graticule in the 10× eyepiece, 50-100 μm crystals of carbocyanine dyes (Molecular Probes Inc.), DiI (1,1' -dioctadecy1-3,3 ,3',3' - tetramethyl-indocarbocyanine perchlorate) and DiA (4-(4- dihexadecylaminostryry1)-N-methyl-pyridinium iodide), were inserted into the cerebral cortex with the tip of a fine pin. Preliminary experiments showed that uptake and transport of the dye DiO were not entirely reliable for quantitative work. In most brains (109 of 153), one hemisphere received DiI application in one of four cortical regions (frontal, parietal, temporal and occipital cortex) and the other hemisphere had DiA placement in one of the remaining regions. In 27 of the brains, there was only one injection site, and there were

three sites in 17 brains. Locations of injection sites were determined by dividing the cortex into thirds longitudinally. Frontal, parietal, and occipital sites were at the center of the anterior, middle, and posterior thirds, respectively. Lateral distances from the cerebral midline varied from 0.6 to 2.3 mm, depending on size of the head. The temporal site was in the middle third, 2.2 to 3.0 mm from midline. The heads were returned to fresh fixative and maintained in the dark either at room temperature or in a 37°C oven for 4-10 weeks (longer times for older fetuses). Incubation of some specimens at 37°C was employed because diffusion of carbocyanine dyes is much faster at higher temperatures (Senft, '90). These parameters yielded excellent images of callosal axons and growth cones via anterograde transport. However, retrograde transport to reveal the architecture of neurons of origin was not consistent with either DiA or DiI.

Sectioning and viewing

The brains were dissected from the heads and sectioned with a vibratome in the coronal plane at 60 μm thickness. Sections were collected in serial order in chilled distilled water, soaked in a 1:1 solution of 100% glycerol and 8% paraformaldehyde (pH. 10.0) overnight at 4°C, then mounted on slides in the same solution and coverslipped (Senft, '90). Specimens were stored at 4°C in order to keep the dye labelling crisp for longer periods. The sections were observed with a Leitz epifluorescence microscope equipped with a standard Rhodamine filter set (N2) for viewing the orange-red DiI fluorescence and a selective FITC filter set (I 2/3) for viewing the yellow DiA fluorescence. Presence of growth cones at the end of most axons was taken as evidence of complete staining. Photographs were made with Kodak Ektachrome ASA 400 slide film. Black and white prints were made from colour slides of DiA labeled axons.

Measurements

Several distances were estimated for each dye injection site with the aid of a graticule in a 10× eyepiece: (1) distance between the centre of the dye injection site and the front edge of the main bundle of growing CC axons; (2) distance between the injection site and the tip of pioneer CC axons; (3) distance of the front edge of the main bundle from the point at the midsagittal plane where the CC axons would eventually cross; and (4) distance of the tip of the most advanced pioneer axon from the midplane crossing point. The approximate location of the crossing point is known from previous studies (Glas, '75; Silver et al., '82; Wahlsten, '87). It could be visualized with the FITC filter set owing to moderate autofluorescence of the neural tissue. When a distance was large and the axons were found in several consecutive sections, the measurement took account of both the growth along the curved path evident in a single section and longitudinal growth across a series of sections.

Statistical analysis

A multiple regression equation provided the best prediction of a measure ($\hat{Y}$) from a sum of predictor variables (X_1, X_2, . . .), each weighted by a regression coefficient (B_1, B_2, . . .), plus a Y-intercept B_0 which is the expected value of Y when all X values are zero. For the Y values of N individuals, given no knowledge of their X values, the sum of squared deviations from the average value of Y is SS_y. When p predictors are used, the predicted value $\hat{Y}_i$ for an individual deviates from the actual value Y_i by an amount termed the residual. The *unexplained* portion of variation is $SSR_{Res} = \Sigma(\hat{Y}_i - Y_i)^2$ and the portion said to be *explained* by the regression equation is $SSR_{Reg} = SS_Y - SS_{Res}$. The fraction of explained variation is approximately $R^2 = SS_{Reg}/SS_Y$. Whenever a predictor variable is added to the equation, the SS_{Reg} increases. If q predictors are added, the increment in SS_{Reg} termed SS_{add}, is tested for statistical significance with an *F* ratio (Marascuilo and Serlin, '88).

$$F = \frac{SS_{add}/q}{SS_{Res}/(N - p - q - 1)}$$

The predictors can be "dummy" variables with values 0 or 1 to represent groups (e.g., site of injection) or continuous variables such as weight or age. Many hypotheses, be they very simple or complex, can be evaluated within this framework. Because several tests of significance were done in the course of this study, the criterion for a Type I error was set at $\alpha = .01$.

Where group differences among sites of dye injection were clearly significant, differences between specific sites were tested using planned multiple pairwise *t* tests and Dunn's critical *t* values with a set at .01 for each set of comparisons. This approach can be used to test differences in group means, Y-intercepts or slopes (Marascuilo and Serlin, '88).

RESULTS

Body weight versus age

Axons tend to be longer in older mice. However, chronological or embryonic age (e.g., E17) is not the best indicator of maturity. There are often large differences in overall growth rate among littermates of the same chronological age. Different strains develop at different rates; hybrid fetuses develop faster than inbred fetuses; and fetuses develop faster in a hybrid than an inbred maternal environment (Wahlsten and Wainwright, '77). Treatments which modify the maternal environment sometimes alter the rate of development (Liu et al., '87). More consistent results not confounded by overall rate of organismic development can be obtained by comparing brains of mice at the equivalent morphological stage or, for the range of ages used in this study, the same body weight (Wahlsten, '87).

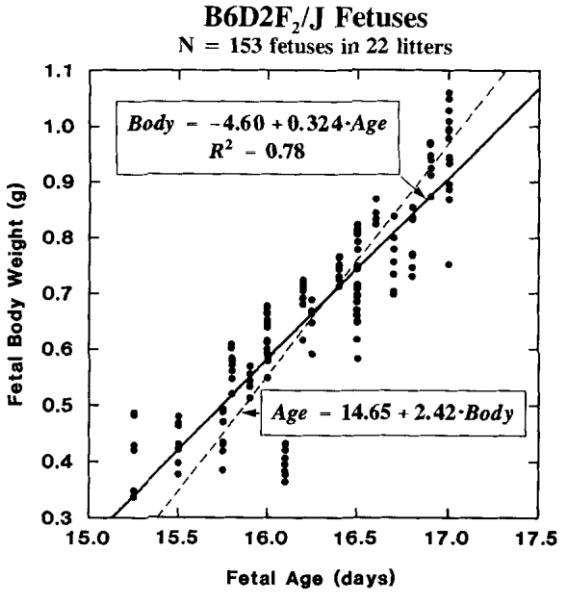

Fig. 1. Body weight versus chronological age from conception for B6D2F$_2$/J fetuses. Linear regression of body weight on age is shown by the solid line and age on weight is shown by the dashed line. About 78% of the variance in weight is attributable to its linear relation with age.

Although the growth of the body over several days is not linear (Goedbloed, '72), for the present data from E15.25 to E17.0, the relation is very nearly linear (Fig. 1). The fetus grows at about 0.32 g/day and growth of 0.1 *g* occurs in about 0.24 day (dashed line in Fig. 1). Different equations are computed to predict body size from age and vice versa. Adding a quadratic term to the equation does not improve the fit significantly ($P = .10$ in a litter means analysis; Abbey and Howard, '73). Therefore, the maturity of callosal axons in this study is assessed relative to fetal body weight. This simple metric allows convenient comparisons with studies in different laboratories.

DiA versus DiI

Although DiA diffuses along the axon slightly more rapidly than DiI in fixed tissue (Chua et al., '90), after a sufficient time both dyes should reach the growth cones and results should not depend on which dye was injected. Visual inspection revealed clear growth cones for both dyes in all fetuses analyzed in this report. Statistical analysis supported this finding. When a dummy variable for the kind of dye was added to the regression equation, no significant increase in goodness of fit was observed for either axon growth rate ($F = 0.14$, $P > .4$) or body size in relation to the distance of CC axons from midplane ($F = 2.51$, $P > .10$). Consequently, further analyses were done without regard to the kind of dye. Because various brains had either one, two, or three dye injection sites and a variety of pairs of sites was used when there were two sites, the unit

of statistical analysis in the following results is the dye injection site and cerebral hemisphere, not the individual mouse. Most mice contributed two values, and these two values are not strictly independent because they necessarily correspond to the same body weight. With 153 animals, any error from nonindependence should be a small sacrifice in the interest of economy of mice and double labelling. By treating injection sites as independent when certain of them are correlated, the $^{SS}_{Res}$ will tend to be a little larger, and tests of significance will tend to be somewhat conservative.

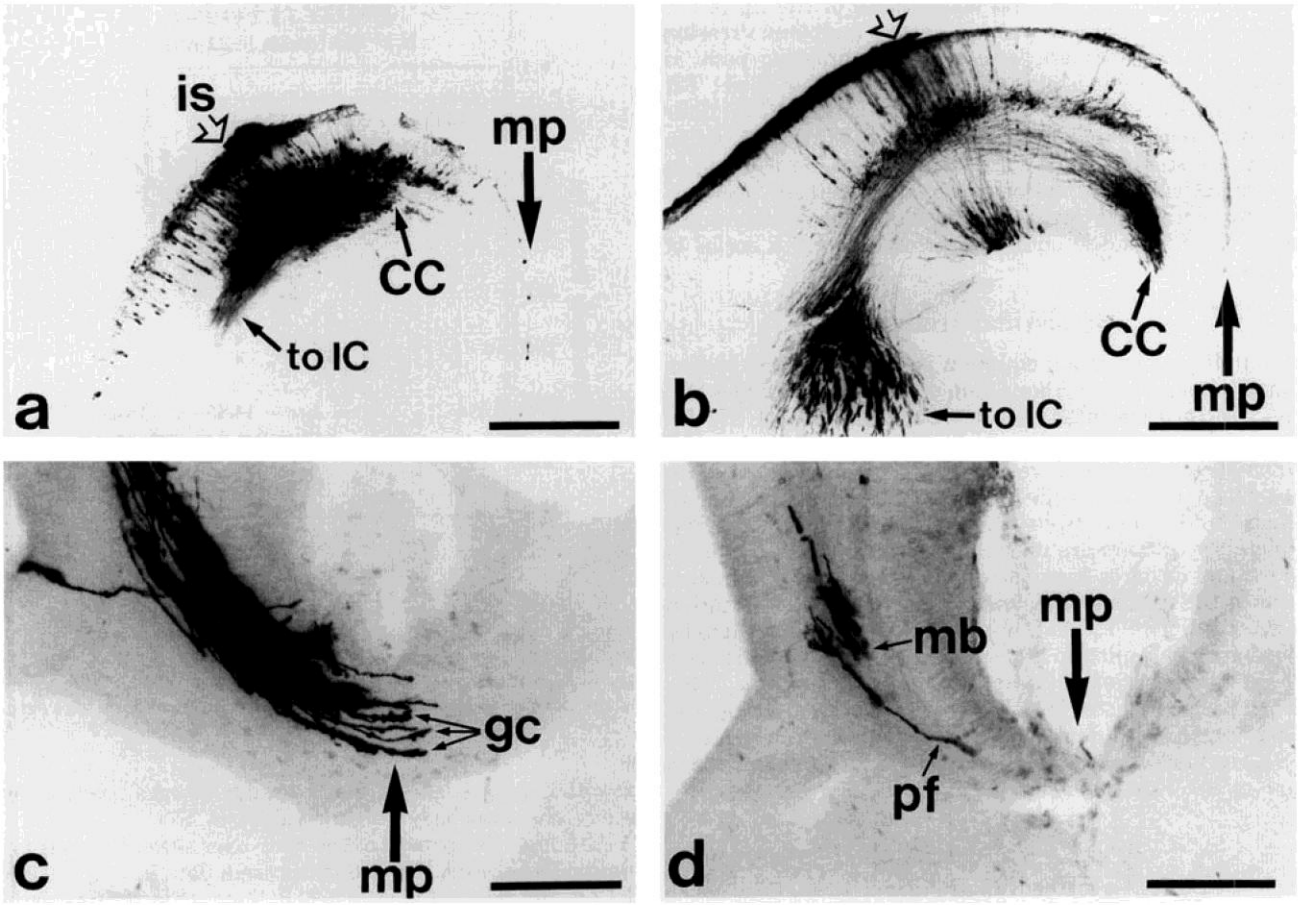

Fig. 2. Coronal sections of B6D2F$_2$/J fetal brains, showing corpus callosum (CC) axons labeled with DiA injected into frontal cortex. The location of the midsagittal plane (mp) is indicated by a large arrow. **a:** A 0.43 g fetus where CC axons have just emerged from the injection site (is; open arrow). Other axons are traveling ventrally toward the internal capsule (IC). **b:** A larger 0.59 g fetus where CC axons have grown a considerable distance towards midplane (mp). **c:** A 0.67 g fetus where CC axons have just crossed midplane. Growth cones (gc) are apparent. **d:** A 0.59 g fetus showing a pioneering fibre (pf) advancing ahead of the main bundle (mb) of the CC. Scale bars: (a), (b), 0.5 mm; (c), (d), 0.2 mm.

Emergence of CC axons

In all but a few of the youngest embryos near 0.4 body weight or E 15.5, axons had clearly emerged from the dye injection site and grown towards midplane through the external sagittal stratum (Crandall and Caviness, '84) and then the intermediate zone (Fig. 2a). Presumably some of these earliest axons originated from transitory subplate neurons (Koester and O'Leary, '91), but most appeared to be from cortical cells; their source could not be specified with confidence because of inconsistent retrograde diffusion of the dye and because many could have been from ipsilateral projections. The region of diffused dye made it difficult to perceive the earliest emergence of CC axons. This problem was addressed statistically. We measured the distance from the centre of the dye injection site to the leading edge of the main bundle of axons (ISMB). Because axon growth rate clearly differed among the four injection sites (see next section), separate equations were computed to predict body weight from ISMB (Fig. 3). Because the period of early emergence was relevant for this analysis, only embryos weighing 0.7 g or less were used. The expected value when ISM = 0 is the Y-intercept, the value of body weight when axons are just about to emerge. These expected values are shown in Table 1. The test of whether CC axons emerge at different degrees of maturity is equivalent to a test of whether the four Y-intercepts differ significantly, provided there are very few brains at the lower limit of ISMB = 0. With the Type I error probability for the six pairwise comparisons set at $\alpha = .01$, only the small difference between frontal and

temporal sites was significant. Compared with the large differences in arrival at midplane, differences in emergence of CC axons are minor. Larger samples might reveal more significant differences but would not increase the absolute magnitude of the small effect of injection site.

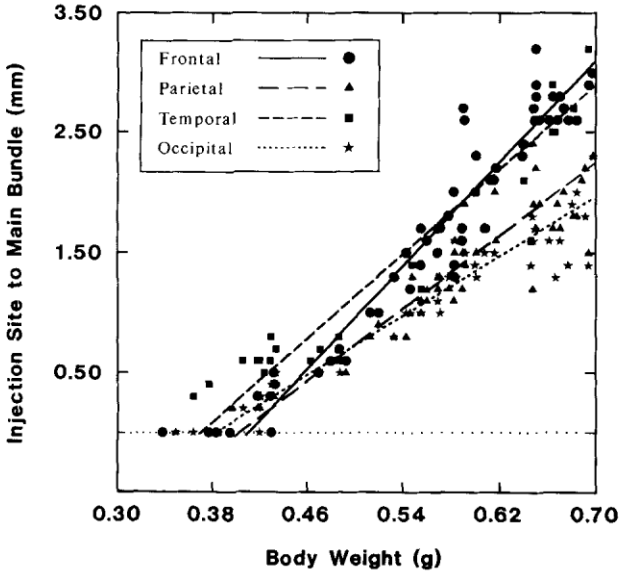

Fig. 3. Distance of the leading edge of the main bundle of callosal axons from the centre of the dye injection site, versus fetal body weight for 179 injection sites in fetuses weighing less than 0.7 g. Separate lines for each cortical injection site represent the regression of body weight on distance. The horizontal dotted line at a distance of 0.0 mm reveals the expected values of body weight when callosal axons are about to emerge from each site.

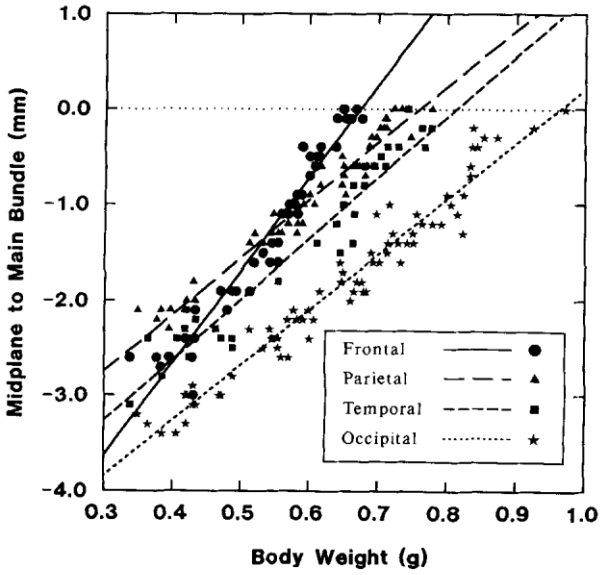

Fig. 4. Distance of the leading edge of the main bundle of callosal axons from the midplane crossing point, versus fetal body weight for 217 injection sites where the corpus callosum has not yet crossed midplane. Separate lines for each injection site represent the regression of body weight on distance. The dotted line at a distance of 0.0 mm reveals the expected fetal size when axons first reach the midplane.

Axon growth rate

Axon growth after the first CC emergence was very rapid (Fig. 2b). It is measured by the distance from injection site to the leading edge of the main bundle (ISMB). When ISMB was predicted from body weight plus three dummy variables for injection site, R^2 was 0.887. That is, about 89% of the total variance in axon distance from the injection is accounted for by the differences among sites and the gradual increase with body size. Differences in slopes were assessed by adding three terms expressing interaction with injection site, which increased R^2 significantly to 0.922 (F = 26.2, df = 3/171, $P < .001$). Therefore, a separate equation was computed for each site, and differences in axon growth rate were assessed by pairwise comparisons among the slopes of these lines. Growth of axons from frontal cortex was significantly faster than from the other three sites, and growth from occipital cortex was significantly slower than all but parietal cortex. Rates from parietal and temporal cortex did not differ. Slopes in the equations, expressed in mm/g body weight, were converted to mm /day in Table 1 using the equivalence of 0.24 day/0.1 g from Figure 1.

TABLE 1. Expected Values ±90% Confidence Limits of the Mean for Measures Related to Corpus Callosum Formation

Measure	Injection site			
	Frontal	Parietal	Temporal	Occipital
First emergence of axons				
Weight (g)	0.411 ± .012	0.404 ± .014	0.370 ± .016	0.384 ± .015
Age (days)	15.65 ± .02	15.63 ± .04	15.55 ± .04	15.58 ± .04
First crossing of midplane				
Weight (g)	0.686 ± .009	0.742 ± .009	0.771 ± .013	0.924 ± .015
Age (days)	16.31 ± .02	16.45 ± .02	16.52 ± .04	16.89 ± .04
Axon growth rate (mm/day)	3.22 ± .20	2.19 ± .19	2.62 ± .22	1.85 ± .19

TABLE 2. Ninety Percent Confidence Limits (Lower, Upper) for the Age (Days) at Which an Individual Mouse Brain Expresses the Characteristic of Corpus Callosum Axons

Measure	Limit	Injection site			
		Frontal	Parietal	Temporal	Occipital
First emergence of axons	Upper	15.77	15.76	15.68	15.71
	Lower	15.52	15.50	15.41	15.45
First crossing of midplane	Upper	16.49	16.63	16.70	17.07
	Lower	16.13	16.26	16.33	16.70

Axon growth from parietal and temporal sites almost directly lateral to the midplane crossing points of CC axons is relatively easy to measure because the entire extent of the bundle of CC axons is evident in one or a few thick sections. For sites anterior or posterior to the crossing point, growth medially as well as longitudinally must be evaluated. We tried to do this accurately, but measured axon length and hence growth rate from both frontal and occipital cortices is probably less precise and should be biased in the same direction. Nevertheless, rates from frontal and occipital cortices differed greatly, and frontal cortex CC axons grew faster than those from parietal and temporal cortex. These differences are not likely the results solely of artifacts in measurement.

All growth rates will tend to be underestimated slightly because the paths of individual axons are not perfectly smooth. Of course, the estimates of first CC axon emergence and first crossing of midplane will be less affected by errors of measuring distance because distances close to those sites are small.

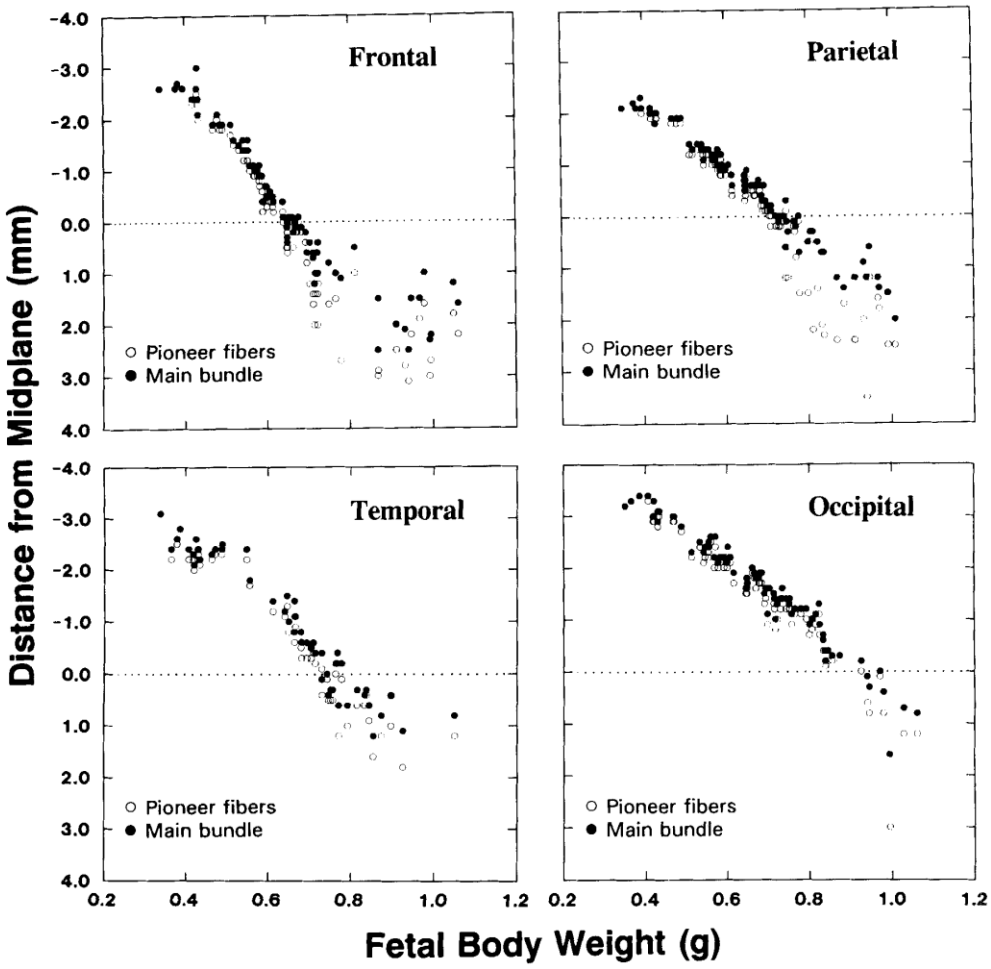

Fig. 5. Distances of pioneering callosal axons and the main bundle of the corpus callosum from midplane, versus fetal body weight for the entire sample of 295 injection sites. Values for axons emanating from the same injection site are plotted at the identical body weight. When no pioneering axons were apparent in advance of the main bundle or no callosal axons had yet emerged from the injection site, a single solid dot is shown. The dotted line at 0.0 mm indicates the midsagittal plane.

First crossing of midplane

The distance of the main bundle of axons from the midplane crossing point (CPMB) allowed us to determine when the CC axons first reached midplane, even when no single individual exhibited CC axon growth cones exactly at midplane (see Fig. 2c). For prediction of body weight from CPMB, the Y-intercept is the body weight of the embryo when axons have just reached midplane (CPMB = 0). Separate equations were computed for each site because axon growth rates differed (previous section). Of course, the CPMB measure is not a valid indicator of axon growth per se; it also reflects the simultaneous expansion of the brain between the main bundle of CC axons and the midplane crossing point. As shown in Table 1 and Figure 4, pairwise comparisons of Y-intercepts revealed that axons from frontal cortex crossed midplane first, followed in order by those from parietal, temporal, and occipital cortices. The difference for parietal and temporal cortices was marginally significant (t = —3.04 vs. critical t = —3.15, df = 287). Using the equation in Figure 1, the first crossing of CC axons at 0.686 g and 0.924 g body weight for frontal and occipital cortices corresponds to chronological ages of 16.3 days and 16.9 days, respectively, in B6D2F_2/J mice. These findings substantiate previous suggestions that the genu of the corpus callosum forms prior to the splenium (Silver et al., '82; Wahlsten, '84), and they confirm a rostral to caudal gradient of CC development (Floeter and Jones, '85, Lent et al., '90). The first CC axons from frontal cortex will not cross at 0.69 g or 16.3 days for every B6D2F_2 fetus. Instead, there is a range of likely values, expressed as a 90% confidence interval in Table 2.

Pioneer CC axons

In many instances, there were clearly one or a few pioneer axons growing far in advance of the main bundle (Fig. 2d), although in other cases the distinction between a pioneering axon and those at the leading edge of the main bundle was not so obvious. We defined the pioneer fibre as the one farthest from the injection site and the main bundle as the dense collection of numerous axons with growth cones emanating from the injection site. The measured distance between the pioneer fibre and the main bundle (PFMB) was sometimes very small (less than 100 μm) when there was no obvious pioneer. Figure 5 presents the distances of pioneering fibres and the main bundle from the midplane crossing point. Values for a single injection site are plotted at the same weight. A dummy variable in the equation indicated whether the main bundle had reached the midplane crossing point (MP = 1) or not (MP = 0). A simple regression equation with only MP as a predictor accounted for 44.3% of the variance in the distance between pioneers and the main bundle (PFMB), and adding three dummy variables for injection site did not improve the fit significantly (F = 2.80, df = 3/290, $P > .02$). As evident in Figure 5, the pioneer axons tended to extend far beyond the main CC bundle after the main bundle crossed midplane, and this occurred similarly for axons from all four injection sites. Prior to reaching midplane, the separation between pioneer axons and the main bundle increased gradually with body weight from about 100 μm to about 200 μm ($\widehat{R}^2$ = 0.258); the trend did not vary much when injection site was considered ($\widehat{R}^2$ increased to 0.295, $P > .01$). However, after the main bundle crossed midplane, the large separation between pioneers and the main bundle did not increase significantly with body size or depend on injection site, perhaps because of a smaller sample and greater individual variability. Pioneer fibres were often found 0.5 to 2.5 mm beyond the main bundle in the contralateral cortex. The most plausible explanation for these findings is that certain CC axons undergo a dramatic growth spurt when they encounter CC axons emanating from homotopic regions in contralateral cortex.

Growth cone morphologies

We observed various growth cone morphologies at the tips of developing callosal axons. Growth cone size and morphology appeared to differ along the callosal pathway. The larger growth cones with complex and bifurcated morphologies were found at the tips of more than half of the callosal axons proceeding within the dye-applied hemisphere (Fig. 6a) as well as in many of those crossing the midline (Fig. 6b). On the other hand, callosal axons in the white matter of the contralateral hemisphere generally had small growth cones with simple and flat morphologies (Fig. 6c) which could be interpreted as two small filopodia joined by a veil (Norris and Kalil, '90). Once the axons had crossed midplane, less than 5% showed complex morphologies and almost none of those growing a considerable distance into the contralateral hemisphere were complex. The change in morphology was gradual and highly variable as the growth cone approached, crossed and then departed the midplane region. There were no consistent differences in size or morphological complexity among growth cones of pioneer axons and those of the main bundle of callosal fibres. Generally speaking, it was difficult to perceive any clear boundaries between types of growth cones. Halloran and Kalil ('91) have observed that single growth cones in vitro continually change their shapes as they progress, which probably accounts for most of the variability we observed in fixed tissue. When there was branching of an axon in our material, this always occurred near the tip of the axon and was most likely a feature of the complex growth cone. No lengthy branches of callosal axons were seen near midplane.

Topography of axons in the CC

In addition to the gross rostrocaudal gradient of callosal development as mentioned above, callosal fibres from four cortical regions (frontal, parietal, temporal and occipital cortex) displayed a topographical arrangement along the rostrocaudal axis of developing corpus callosum. Callosal axons from frontal cortex first crossed the midline rostrally over the septal tissue, and axons from more caudal regions (parietal, temporal, and occipital cortex) were observed to add to the rostral fibres caudally according to their rostrocaudal sequence in later development. The most advanced fibres from each of these caudal regions could be seen to traverse the midplane in the ventral portion of the CC, intermingling with callosal fibres from the rostrally adjoining region which had already crossed. Axons from two distant regions such as frontal and occipital cortex, however, were completely separated from each other at the midline. For injection sites at the same anterior-posterior levels, axons from the more medial site occupied the more dorsal position in the body of the CC (Fig. 7). Thus, these

observations indicate that axons of the corpus callosum possess topographical information on their rostrocaudal and mediolateral organization from the beginning.

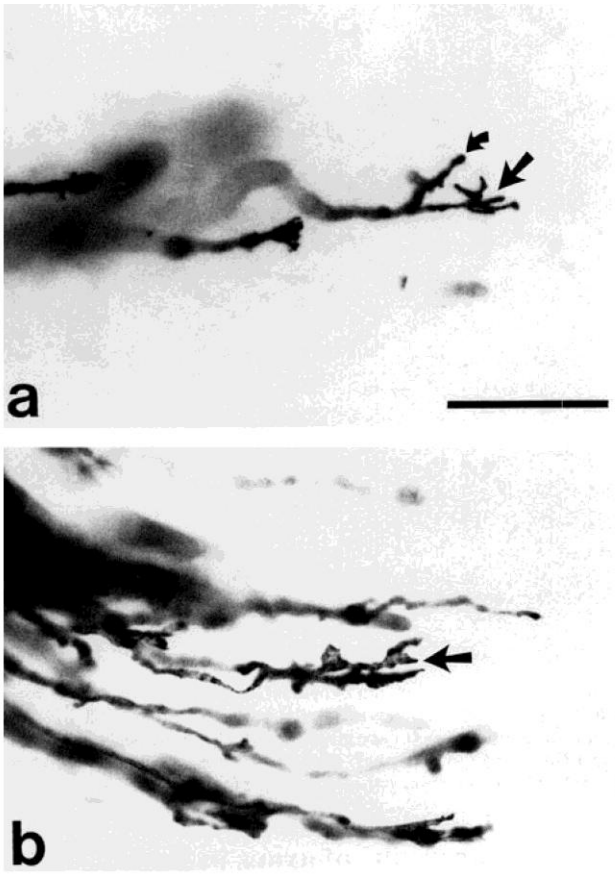

Fig. 6. Growth cones (arrows) of the corpus callosum in B6D2F$_2$/J fetal brains labelled with DiA. Fetal body weights range from 0.66 to 0.72 g. a: Complex growth cone on axon from parietal cortex shortly before crossing midplane. A branch of the same growth cone is indicated by a small arrow. **b:** Complex growth cone from frontal cortex that has just crossed midplane. This is the most dorsal of the three growth cones indicated in Figure 2c. **c:** Simple growth cone from frontal cortex after crossing midplane and entering the contralateral hemisphere. Scale bar indicates 50 μm and applies to a–c.

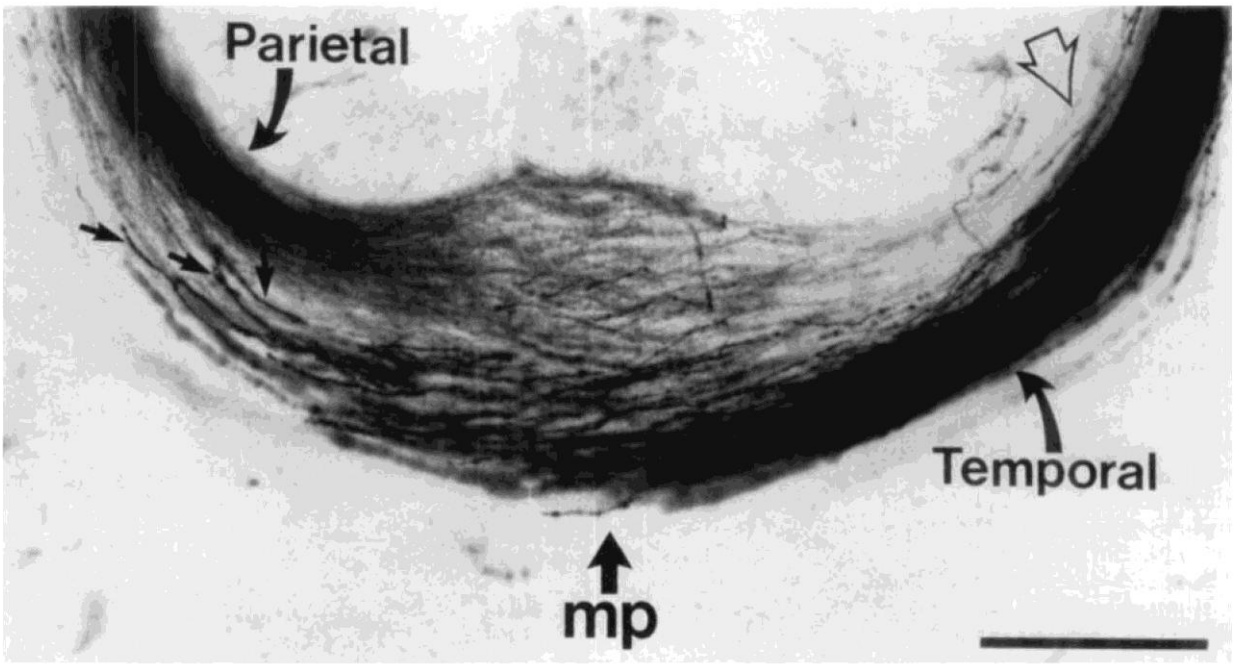

Fig. 7. Coronal section of a hybrid fetus at 0.75 g body weight where callosal axons from left parietal cortex are labelled with DiI and those from right temporal cortex are labelled with DiA. Photograph was made with FITC filtration. Although axons from temporal cortex are generally ventral to those from parietal cortex, a few temporal pioneer axons (small arrows) travel through the same region as the ventral portion of parietal axons. The location of a pioneer axon from parietal cortex is shown by an open arrow. Location of midplane (mp) is shown by the large arrow. Scale bar indicates 0.2 mm.

DISCUSSION

These results demonstrate large rostral-caudal differences in the contribution of regions of the cerebral cortex to formation of the early corpus callosum at the midsagittal plane. The differences occur mainly because of variations in axon growth rate combined with proximity to the earliest midplane crossing point, rather than time when CC axons first emerge from their cells of origin. Differences in axon growth rate may be caused by differences in the maturity of axonal pathways, which would also show a rostral-caudal gradient. A rostral-caudal sequence of cortical neurogenesis is well established (Bayer and Altman, '91). Apparently, callosal axon extension depends on the cellular milieu as well as time of last cell division. A medial-lateral gradient in cortical maturity, documented previously with rats (Floeter and Jones, '85), is weakly confirmed and not contradicted by the present data.

These results substantiate previous claims that the genu of the corpus callosum forms before the splenium. Because the earliest callosal axons cross just slightly anterodorsal to the hippocampal commissure, it has been asserted that the body of the corpus callosum forms prior to the genu or splenium (Berbel and Innocenti, '88), which is not supported by our findings. "Genu" and "splenium" are terms most appropriate for the gross morphology of the adult. The first callosal axons from occipital cortex cross midplane at about 0.9 g body weight in mice, and they are then located directly above the hippocampal commissure. No posterior extension of the corpus callosum beyond the hippocampal commissure exists at this point. Considering where in the cortex the axons originate, the occipital axons constituting the splenium are directly over the hippocampal commissure when they first cross. Later, as vast numbers of axons are added to all portions of the corpus callosum, the splenium moves far posterior to the hippocampal commissure in rodents (Wahlsten, '84), although it remains close to the hippocampal commissure in humans (Rakic and Yakovlev, '68). The topographic arrangement of callosal axons with respect to cortical origin seen in the adult (Olavarria and Van Sluyters, '86; Pandya and Seltzer, '86) exists from the outset in the fetal brain, but the entire corpus callosum is so small at that time that it sits atop the hippocampal commissure like a cap.

The stage when the first callosal axons arrive at midplane is probably somewhat before the body weight of 0.69 g (about E16.3 for F_2 hybrid fetuses) for axons from frontal cortex. Our dye placements were sufficiently lateral to avoid labelling axons from cingulate cortex, which also generates perforating fibres (Hankin and Silver, '88), and to prevent any nonaxonal diffusion of dye to the midplane region. Cingulate cortex may very well be the source of the earliest projections to reach midplane (Koester and O'Leary, '91). Our estimate of first CC axons at midplane between E16 and E17 is consistent with several previous estimates from mice based on silver stains (Glas, '75; Silver et al., '82) and electron microscopy (Zaki, '85), although the accuracy of the present estimate is much better. An earlier suggestion that CC axons were present at midplane in a 0.5 g hybrid fetus (Wahlsten, '87) was based on a general purpose stain (eosin) which could not definitively distinguish between callosal axons and those of the hippocampal commissure. Because the two commissures are so intimately associated early in development (Berbel and Innocenti, '88; Glas, '75), tract tracing is more convincing.

Even when fetuses were equated for body weight, individual differences were apparent in all measures. This reveals the peril of basing anatomical claims on one or two animals for each cortical injection site or age. A statistical approach that utilizes all the available data in one regression equation is less likely to yield erroneous conclusions about time of axon emergence, for example, than a descriptive study of prototypical cases.

The norms for early callosal development established by this study should facilitate future research. Attention can be focused on a narrow range of body sizes when an event of interest is likely to occur in mice. Results from different studies can be readily compared using body size as the common metric. The degree of abnormality can be assessed quantitatively in order to detect more subtle deficiencies which may not be visually obvious (e.g., Wahlsten and Smith, '89). Body weight is not suitable as an index of maturity postnatally because it is then very sensitive to litter size and less closely related to brain development (Wainwright et al., '89).

The exact locale of the first crossing of callosal axons is of course not definitively revealed when only the callosal axons are labelled. Compatibility of the carbocyanine dye method with certain immunohistochemical

methods (Elberger and Honig, '90) should make it possible to document specific antigens important for axon guidance. Likewise, photo-oxidized DiI can be detected with electron microscopy (Kadhim et al., '91), which should tell much more about the surroundings of pioneering axons known unequivocally to be part of the corpus callosum from a specific region of cortex.

These results with carbocyanine dyes demonstrate that pioneering axons are present in advance of the main bundle of callosal axons, especially after crossing midplane. Prior to that time, the "pioneers" do not usually extend very far ahead of the main bundle and there is no assurance that the leading axon at one time will remain in the vanguard a few hours later. Growth of an individual axon can be saltatory. It seems likely that the failure of previous studies to find pioneer axons (Floeter and Jones, '85; Lent et al., '90; Valentino and Jones, '83) was a consequence of the HRP method. When the pioneering fibres reach midplane, their growth cones are highly variable and often complex, and the axons are not closely fasciculated (Norris and Kalil, '90). At or near midplane they suddenly make contact with callosal axons arriving from homotopic points in the opposite hemisphere, and these fibres probably provide efficient cues that prompt some of the pioneering axons to extend rapidly beyond the main bundle.

LITERATURE CITED

Abbey, H., and E. Howard (1973) Statistical procedure in developmental studies on species with multiple offspring. Dev. Psychobiol. 6:329-335.

Atchley, W.R., and W.M. Fitch (1991) Gene trees and the origins of inbred strains of mice. Science 254,554-558.

Bayer, S.A., and J. Altman (1991) Neocortical Development. New York: Raven Press.

Berbel, P., and G.M. Innocenti (1988) The development of the corpus callosum in cats: A light- and electron-microscopic study. J. Comp. Neurol. *276:132-156.*

Chua, C.K., R.J. Balice-Gordon, and J.W. Lichtman (1990) Differential labeling of terminal arbors of multiple axons innervating the same target all with DiI and a new lipophilic tracer, 4-Di-16-ASP. Soc. Neurosci. Abstr. *16:1004.*

Crandall, J.E., and V.S. Caviness, Jr. (1984) Axon strata of the cerebral wall in embryonic mice. Dev. Brain Res. *14:185-195.*

Dehay, C., H. Kennedy, J. Bullier, and M. Berland (1988) Absence of interhemispheric connections of area 17 during development in the monkey. Nature *331:348-350.*

Elberger, A.J., and Honig, M.G. (1990) Double-labeling of tissue containing the carbocyanine dye DiI for immunocytochemistry. J. Histochem. Cytochem. *38: 735-739.*

Floeter, M.K., and E.G. Jones (1985) The morphology and phased outgrowth of callosal axons in the fetal rat. Dev. Brain Res. *22:7-18.*

Glas, P. (1975) Onderzoek naar de vroege ontwikkeling van de commissuren in het mediane gebied van het telencephalon bij de witte muffs. Groningen: Drukkerij Van Denderen B.V.

Godement, P., J. Vanselow, S. Thanos, and F. Bonhoeffer (1987) A study in developing visual systems with a new method of staining neurones and their processes in fixed tissue. Development 101:697-713.

Goedbloed, J.F. (1972) The embryonic and postnatal growth of rat and mouse. I. The embryonic and early postnatal growth of the whole embryo. A model with exponential growth and sudden changes in growth rate. Acta Anat. 82:305-336.

Halloran, M.C., and K. Kalil (1991) Video microscopy of growth cones extending in the corpus callosum of a hamster cortical brain slice. Soc. Neurosci. Abstr. 17:532.

Nankin, M.H., and J. Silver (1988) Development of intersecting CNS fiber tracts: The corpus callosum and its perforating fiber pathway. J. Comp. Neurol. *272:177-190.*

Heimer, L., and L. Zaborszky (eds) (1989) Neuroanatomical Tract-Tracing Methods 2. Recent Progress. New York: Plenum Press.

Hogan, D., and N.E.J. Berman (1990) Growth cone morphology, axon trajectory and branching patterns in the neonatal rat corpus callosum. Dev. Brain Res. 53:283-287.

Honig, M.G., and R.I. Hume (1989) DiI and DiO; versatile fluorescent dyes for neuronal labelling and pathway tracing. Trends Neurosci. 12:333341.

Kadhim, H.J., P.G. Bhide, W.C. West, and D.O. Frost (1991) Axonal branching in the developing hamster corpus callosum. Soc. Neurosci. Abstr. *17:765.*
Koester, S.E., and D.D.M. O'Leary (1991) Subplate cells in medial cortex send the first axons across the corpus callosum. Soc. Neurosci. Abstr. 17:41.
Lent, R., C. Hedin-Pereira, J.R.L. Menezes, and S. Jhaveri (1990) Neurogenesis and development of callosal and intracortical connections in the hamster. Neuroscience 38:21-37.
Lipp, H.-P., and R. Waanders (1990) The acallosal mouse strain I/ Ln: Behavioral comparisons and effects of cross-breeding. Behay. Genet. 20:728-729.
Liu, L., S.M. Russell, and C.S. Nicoll (1987) Growth and differentiation of transplanted rat embryos in intact, diabetic and hypophysectomized hosts: Comparison with their growth in situ. Biol. Neonate 52:307-316.
Marascuilo, L.A., and R.C. Serlin (1988) Statistical Methods for the Social and Behavioral Sciences. New York: Freeman.
Norris, C.R., and K. Kalil (1990) Morphology and cellular interactions of growth cones in the developing corpus callosum. J. Comp. Neurol. 293:268-281.
Olavarria, J., and R.C. Van Sluyters (1986) Axons from restricted regions of the cortex pass through restricted portions of the corpus callosum in adult and neonatal rats. Dev. Brain Res. *25:309-313.*
Ozaki, H.S., T.H. Murakami, T. Toyoshima, and M. Shimada (1984) Agenesis of the corpus callosum in ddN strain mouse associated with unusual facial appearance (flat face). Neurosci. Res. *1.81-87.*
Ozaki, H.S., K. Iwahashi, and M. Shimada (1989) Ipsilateral corticocortical projections of fibres which course within Probst's longitudinal bundle seen in the brains of mice with congenital absence of the corpus callosum: A study with the horseradish peroxidase technique, Brain Res. 493:66-73.
Pandya, D.N., and B. Seltzer (1986) Topography of commissural fibres. In F. Lepore, M. Ptito, and H.H. Jasper (eds): Two Hemispheres-One Brain. Functions of the Corpus Callosum. New York: Alan R. Liss, pp. 47-73.
Rakic, P., and P.I. Yakovlev (1968) Development of the corpus callosum and cavum septi in man. J. Comp. Neurol. *132:45-72.*
Senft, S.L. (1990) Prenatal central vibrissal pathways labeled with Dil and DiA. Soc. Neurosci. Abstr. *16:1215.*
Silver, J., S.E. Lorenz, D. Wahlsten, and J. Coughlin (1982) Axonal guidance during development of the great cerebral commissures: descriptive and experimental studies, in vivo, on the role of preformed glial pathways. J. Comp. Neurol. *210:10-29.*
Taylor, B.A. (1972) Genetic relationships between inbred strains of mice. J. Herod. *63:83-86.*
Valentino, K.L., and E.G. Jones (1983) The early formation of the corpus callosum: A light and electron microscopic study in foetal and neonatal rats. J. Neurocytol. 11:583-609.
Wahlsten, D. (1981) Prenatal schedule of appearance of mouse brain commissures. Dev. Brain Res. 1:461-473.
Wahlsten, D. (1984) Growth of the mouse corpus callosum. Dev. Brain Res. 15:59-67.
Wahlsten, D. (1987) Defects of the fetal forebrain in mice with hereditary agenesis of the corpus callosum. J. Comp. Neurol. *262:227-241.*
Wahlsten, D., and G. Smith (1989) Inheritance of retarded forebrain commissure development in fetal mice: Results from classical crosses and recombinant inbred strains. J. Hered. *80:11-16.*
Wahlsten, D., and P. Wainwright (1977) Application of a morphological time scale to hereditary differences in prenatal mouse development. J. Embryo'. Exp. Morphol. *42:79-92.*
Wainwright, P., C. Pelkman, and D. Wahlsten (1989) The quantitative relationship between nutritional effects on preweaning growth and behavioral development in mice. Dev. Psychobiol. *22:183-195.*
Zaki, W. (1985) Le processus degeneratif au tours du developpement du corps calleux. Archiv. Anat. Micr. Morphol. Expel-. *74:133-149.*