Path analysis of sex difference, forebrain commissure area and brain size in relation to degree of laterality in selectively bred mice

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Abstract:
Male and female mice from HI and LO lines selectively bred by Collins for strength of lateralization were tested for paw preference and then studied histologically to assess size of forebrain commissures and myelination of the corpus callosum. When compared to LO line mice, HI line mice were more strongly lateralized for paw preference and had larger brains as well as greater cross-sectional areas of the anterior commissure and corpus callosum. A substantial sex difference was found only for body size. Myelination of the corpus callosum did not differ consistently between the lines or sexes. Path analysis indicated that the line difference in the anterior commissure was a consequence of the difference in brain size, but corpus callosum size was actually smaller in the HI line than the LO line when brain size was taken into account. However, the size of the corpus callosum relative to brain size was not related to strength of paw preference, whereas brain size relative to corpus callosum size was positively correlated with strength of paw preference. These results support the hypothesis that the large difference in brain size between the Collins HI and LO lines is an important cause of the difference in strength of behavioral lateralization.

Key words: Corpus callosum; Paw preference; Myelination; Morphometry; Anterior commissure; Selective breeding

Article:
INTRODUCTION

In laboratory mice selective breeding for the degree of behavioral lateralization, manifest as paw preference, produces divergent lines5, whereas selection for the sense (right vs left) or direction of paw preference does not4. Selection for degree of paw preference also effects changes in the size of the brain and possibly the degree of hemispheric asymmetry22. Selective breeding for one characteristic could occasion a change in another because they are both influenced by the same set of genes (pleiotropy) via different physiological or developmental pathways, or because the change observed in the brain actually mediates the change in behavioral laterality. Furthermore, a change in whole brain size could be a mere correlate of a functionally significant change in one major component of the brain such as the corpus callosum (CC), which is thought to play an important role in establishing hemispheric specialization2,6. It is expected that in populations of mice having no major hereditary defect of the CC, those with larger brains should have larger CCs19. If a line of mice with a high degree of paw preference shows both larger brains and larger CC sizes, how can the contribution of CC size be separated from the effect of whole brain size?

The present study addresses this question using a statistical approach, path analysis, developed originally by Wright23,24 to assess the strength of different pathways of causation among a system of 3 or more variables. Samples of the Collins lines of strongly and weakly lateralized mice were tested for paw preference, and then brain sizes and commissure areas were measured. Care was taken to distinguish between the CC proper and adjacent bundles of myelinated axons near the mid-sagittal plane which can easily be mistaken for portions of the splenium of the CC in unstained or poorly stained tissue, particularly the longitudinal striae of Lancisius, the dorsal commissure of the fornix and the superior fornix. The degree of myelination of the CC was also taken
into account to aid interpretation of results, because the size of the CC must reflect the extent of myelination as well as axon number, axon diameter and packing density. Postnatally, the number of CC 3Nons declines precipitously\textsuperscript{1,9,10} while myelination continues for several months\textsuperscript{1,12,17}. Axon number should affect the abundance of interhemispheric communications, whereas myelination should affect their speed.

**MATERIALS AND METHODS**

**Mice and paw preference testing**

The mice were from the 29th generation of an experiment involving selection for high (the HI line, 27 females, 25 males) and low (the LO line, 26 females, 26 males) degree of paw preference. Beginning with a population derived from an 8-way cross\textsuperscript{5}, selection was done for 11 generations and then resumed at generation 28. Random breeding within each line rather than selection was done from generations 12-27. Mice were weaned at 4 weeks after birth and housed with same sex littermates until testing for paw preference at 6-10 weeks of age. About 18 h before testing, each mouse was deprived of food (not water) and then placed into a 5 × 5 × 15 cm high cubicle where it could reach down a centrally placed horizontal tube to obtain Maypo oat flakes. The paw used for 50 leaches for food was observed and the number of right paw entries (RPF) was recorded. Mice which persistently failed to reach for food were not used in the study. The person observing paw entries did not know whether an animal was from the HI or LO line.

**Histology and morphometry**

Following paw preference testing, mice were housed individually until 10-14 weeks, when they were processed for histology by perfusion with 10% neutral buffered formalin, as described previously\textsuperscript{1}. The age at perfusion was variable because the mice were born at different times, but all were perfused in a 3-day period by one of the authors (B.C.) during a visit to the Jackson Laboratory. After 1 week in fresh fixative, each brain was gently blotted and weighed to the nearest mg. The two halves of the brain were separated by a longitudinal incision with the aid of a blocking jig, keeping the orientation of the brain with respect to the operator the same for all brains, and each half was then blotted and weighed. Frozen sagittal sections were then cut from the left half at 25 μm and stained with Sudan Black B for myelin. Areas of the corpus callosum proper (CC), hippocampal commissure (HC), anterior commissure (AC) and dorsal commissure of the fomix (DCF) were determined from tracings of the mid-sagittal section. The CC was also divided into 5 parts by constructing 4 lines perpendicular to the line joining the most distal portions of the genu (GCC) and splenium (SCC) at points equidistant along that line, and areas of each part were measured. All sectioning and measurements were done with coded brains so the technicians did not know strain, sex or littermates.

**Myelin staining intensity**

Relative myelin staining intensity was assessed by measuring the percent transmittance of the GCC at midplane, defined as the anterior fifth of the CC for a particular brain. This was done with an RCA TC 1005 vidicon
camera mounted on a Leitz Laborlux microscope with output processed by the Videtics, Ltd. (Waterloo, Ont.) image analysis system running on an IBM XT computer. The full field of 1024 by 512 pixels allowed definition of a rectangular window containing only the GCC and lightly stained background. The operator determined the gray level (0-63) which best discriminated between GCC and background, and then a program calculated the average gray level of the GCC. Certain artifacts resulting from variations in staining intensity were controlled by putting equal numbers of slides from each line and sex in a staining tray, and then expressing stain intensity for each brain as a deviation from the mean intensity for all brains in that tray.

RESULTS

Correction for age differences

Ages at perfusion ranged from 75 to 98 days with a mean of 87.7 days, and several measures increased slightly but significantly over this period. Consequently, linear regression was used to adjust the scores of each mouse to a common age of 90 days using the formula \( X' = X + b \) (90.0 — age), where \( X' \) is the adjusted measurement and \( b \) is the slope of the line regressing \( X \) on age within a group. Myelination\(^{17} \) and CC area\(^{19} \) increase gradually for many months in mice, but the rate of change diminishes with age. In the narrow age range of this study the change was essentially linear, so no quadratic term was included in the equation.

Paw preference

Fig. 1 shows the frequency distributions of RPE scores obtained for each line when the 51 possible RPE scores (0-50) are divided into 17 intervals of width 3. The distribution of RPE scores for the LO line is roughly unimodal, while that of the HI line is U-shaped. A two-factor ANOVA assessing effects of line, sex and the line-by-sex interaction revealed no significant effects (all \( P > 0.05 \)). Therefore, no group differences in direction of paw preference were evident. To assess the degree of paw preference without regard to its direction, the left side of each RPE distribution (scores 0-24) was folded and superimposed as a mirror image on the right side of each RPE distribution (scores 26-50) by the non-monotonic transformation of RPE scores into preferred paw entry (PPE) scores \([\text{PPE} = \text{abs}(\text{RPE} — 25) + 25]\). A logit transform\(^{5} \) was then applied to the PPE scores: \( \text{LPPE} = 0.5 \log \left( \frac{(\text{PPE} + 1/6)^{450} — \text{PPE} + 1/6}{\text{PPE} + 1/6} \right) \), which attenuated problems of restriction of measurement range and non-normality of PPE score distributions. The HI line had significantly higher raw PPE (\( P < 0.001 \)) and transformed LPPE scores (\( P < 0.0001 \)) than the LO line. In terms of LPPE score, the HI and LO lines differed by about 0.67 standard deviations. No sex differences or line-by-sex interactions in degree of paw preference were evident (\( P > 0.05 \)).

Fig. 2. Group mean differences on measures of body and brain size expressed as the number of standard deviations by which each group differed from the grand mean of the 4 groups (HI and LO males and females). Numerical values of group means are also specified. The standard deviation was obtained from the pooled within-group variation. The cross indicates significant sex differences (\( P < 0.0005 \)), whereas the cross indicates significant line difference (\( P < 0.0001 \)).
**Body and brain sizes**

Group means are compared in Figs. 2 and 3 in terms of standard deviations within groups, to provide a visual impression of the effect size ($d$) of the group differences. As indicated in Fig. 2, male mice were much heavier than females but there was no significant line difference in body weight. For brain weight, on the other hand, there was no sex difference but the HI line exceeded the LO line by about 43 mg or 10%, which is comparable to differences commonly observed among inbred strains,$^{15,21}$ although only half the size of the difference resulting from selection for brain weight itself. This brain weight difference of about 1.7 standard deviations between HI and LO lines was not an artifact of nutritional variation produced by litter size, which can be quite large,$^{20}$ because litter size did not differ significantly between lines. The line difference in whole brain size was evident for both the cerebellum and the remainder of the brain separated from the cerebellum (data not shown). Somewhat surprisingly, the tails of HI line mice were substantially longer than LO, even though body weights were similar.

**Asymmetry of brain**

Because the lines do not differ in direction of paw preference, it would not be surprising if the directional distribution of brain weight between the two halves was similar. Indeed, the lines did not differ in the percentage - of total cerebral weight in the right half and all group means were very close to 50% (see Fig. 2), nor were group differences in right half weight minus left half weight ($R - L$) significant. There was no significant correlation between $R - L$ and total cerebral weight ($p > 0.70$). For absolute asymmetry, expressed either as $abs((R - L)/(R + L))$ or $abs(R - L)$, no line differences were evident and mean asymmetries were all less than 1.0%. In fact, mean absolute asymmetry was not significantly different from zero for any group, suggesting that marked behavioral asymmetry can occur even in brains in which the two halves are of nearly identical weights. This is congruent with observations of region-specific but not general hemispheric asymmetries in these lines.$^{11}$ Consistent with earlier work,$^{22}$ there was no significant association between the direction of the cerebral asymmetry and direction of paw preference ($\chi^2 < 1.0$). However, it was apparent ($x^2 = 8.90, P < 0.01$) that the nature of the association between directions of behavioral and cerebral asymmetries differed between the lines. The heavier hemisphere was contralateral to the preferred paw in most HI line mice ($\chi^2 = 7.52, P < 0.01$) but was osilateral to the preferred paw in most LO line mice, although the ipsilateral trend in LO line mice was found in males ($\chi^2 = 4.35, P < 0.05$) but not females ($\chi^2 = 0.40, P > 0.75$) and therefore was not significant overall ($\chi^2 = 136, P > 0.1$).

![Fig. 3. Group mean differences in measures of forebrain commissures expressed as number of standard deviations away from the grand mean of the 4 groups (HI and LO males and females). Cross indicates significant line difference ($P < 0.0001$).](image)
**Forebrain commissures**

The total area of the CC was significantly larger \((P < 0.05)\) in the HI line mice relative to the LO line mice (by 0.058 mm\(^2\) or 8\%) but no effects due to sex or interaction were apparent (see Fig. 3). A small (1\%) but statistically significant \((P < 0.01)\) sex difference in the percentage of CC area lying in the posterior fifth of the CC was detected, with males having the higher percentage. Whether this result is due to a peculiarity of these two lines of mice, or to the exclusion of the DCF and much of the dorsal fornix from the measure of the splenium and posterior truncus remains to be determined. In any case, this sex difference in morphology was not paralleled by sex differences on the behavioral measures of interest and may, in fact, have little relation to any aspect of behavior.

The cross-sectional area of AC at midline was much larger (by 0.16 mm\(^2\) or 16\%) in the HI line than in the LO line. No sex difference or interaction involving AC area was found, nor were there group differences in midsagittal areas of the HC or DCE. However, the morphology of the DCF differed somewhat between the two lines. The DCF was comprised of a greater number of discrete commissural bundles in LO line mice than in HI line mice (see Fig. 4). In brains with large numbers (up to 10) of DCF bundles, several of the bundles were displaced anteriorly from the main DCF, sometimes crossing midline just above the most dorsal aspect of the HC, which suggested a marked line difference in the developmental dynamics of this brain structure and possibly the overlying CC.

**Myelination of CC**

Intensity of staining of the genu of the CC, expressed as average percent transmittance of the anterior fifth of the CC at midplane (Fig. 3), did not differ significantly between lines or sexes. The line-by-sex interaction was marginally significant \((F = 5.71, \text{df} = 1.99, P = 0.02)\), but the pattern of results made no sense in the context of the present study or published reports, and it could well have occurred because of sampling error. In any event, the 8\% line difference in area of the entire CC was probably not a reflection of differential myelination and was more likely the result of the 10\% line difference in whole brain size.

### TABLE 1

<table>
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<tr>
<th>Variable</th>
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<tr>
<td>Brain wt.</td>
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<td>3.28**</td>
</tr>
<tr>
<td>AC area</td>
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<td>3.94**</td>
</tr>
<tr>
<td>DCF area</td>
<td>-</td>
<td>0.13</td>
</tr>
<tr>
<td>HC area</td>
<td>-</td>
<td>0.48</td>
</tr>
</tbody>
</table>

* F ratio testing whether the amount of centroid separation added by each variable contributed significantly to the separation produced by the previously entered variables.

** Path analysis**

The HI line mice have larger brain size, larger CC area and larger AC area than LO line mice, and they also show stronger paw preference. The question thus arises whether the larger forebrain commissure areas are functionally related to behavioral lateralization or are merely consequences of larger brain size. This question was addressed by a step-wise discriminant analysis in an attempt to discriminate the 4 groups of mice (HI and LO males and females) on the basis of the midsagittal cross-sectional areas of their forebrain commissures and other variables that could plausibly explain the group differences. As indicated in Table I, the order of entry into the discriminant function was body weight, brain weight, CC area and AC area. Once these 4 measures were taken into account, adding HC or DCF areas did not improve discriminability of the groups significantly; and hence only the first 4 measures were used for path analysis. The path analytic model was developed through a series of multiple regression equations according to the general to specific theoretical rationale as explained by Pedhazur\(^{13}\). The suitability of these data for path analysis was supported by a statistical test of the homogeneity of dispersion matrices \((\text{Box's } M = 39.4, P = 0.20)\). Because many correlations were evaluated in the course of the multiple regressions, the significance level was set at \(\alpha = 0.01\) rather than 0.05, Because a line-
by-sex interaction term failed to improve prediction significantly, it was dropped from the model. All possible paths from more general to more specific variables were tested and non-significant paths were dropped, unless they were of central interest theoretically.

The results of this process are described in Fig. 5. The only direct effect of sex was on body weight, whereas the HI and LO lines were different on several measures of the brain quite apart from any line difference in body weight. Mice with larger brains tended to have greater CC areas, but when the line difference in brain size was taken into account, the LO line mice had a CC area 0.092 mm$^2$ larger (or 12%) than HI line mice, in contrast to the opposite direction of line difference in CC size without regard to brain weight (Fig. 3). There was no line effect on AC area apart from the line difference in whole brain size. Thus, although the HI line mice had larger areas of both CC and AC than the LO line, they also had larger brain sizes, such that CC size relative to brain size was actually smaller than the LO line. This interpretation of commissure areas derived from the more 'integrative path analysis demonstrates the inadequacy of assessing line differences in whole brain, CC and AC sizes separately.

Relation with paw preference
If selection for paw preference produced a small line difference in relative CC size, did this change in brain structure have any causal connection with a measure of lateralization? A regression equation predicting CC area from brain weight was computed and then the deviation (residual) of the CC area of each mouse from the value expected on the basis of its brain weight was determined. The deviation from expected CC area was not significantly related to LPPE score ($r = -0.02$, $P > 0.08$), which suggests CC size had little effect on paw preference. On the other hand, when CC area was used to predict brain size for all groups combined, mice with relatively large brain size in relation to CC area tended to be more strongly lateralized, as indicated by LPPE score ($r = 0.31$, $P < 0.002$). When line membership was also included in the equation, the strength of the relationship was attenuated ($r = 0.16$, $P > 0.1$), but no further reduction in correlation of relative brain size with LPPE occurred when sex and a sex-by-line interaction term were added to the equation. These results indicate that a large portion of the relation between LPPE score and brain size relative to CC area was attributable to differences between HI and LO lines, but that there was no effect of sex.

Other approaches
Linear regression is not the only possible approach to understanding the relation between whole brain size and commissure area. The ratio of CC area to brain weight may be appropriate if the Y-intercept of the regression line is zero ($Y = bX$), because the ratio will be a constant ($Y/X = b$). Of course, cross-sectional area of a structure increases as the square of the linear dimension, while volume increases as the cube, so the geometry
suggests CC area should increase as the 2/3 power of brain weight if different size brains are isometric\(^6\). Most often, however, parts of the brain are not geometrically similar but rather are allometric, increasing by some specific amount as whole brain size increases. The human corpus callosum in particular tends to exhibit a very small or even no allometric increase with brain size\(^8,14\). If two measures are completely unrelated statistically and groups differ on one (X) but not the other (Y), then the ratio Y/X will produce a group difference and may lead to different conclusions than the regression approach.

When ratio methods were used to compute relative CC area (CC area/brain weight and CC area/brain weight\(^{2/3}\)) or relative brain weight (brain weight/CC area and brain weight\(^{2/3}\)/CC area), no differences between the lines or sexes were apparent (all Ps > 0.32) and none of the measures correlated with either PPE or LPPE (Pearson r's from —0.12 to 0.11 with Ps > 0.38). The HI line mice, particularly males, had larger ratios of AC area to brain weight (P < 0.03) and brain weight\(^{2/3}\) (P < 0.0006) than the LO line mice but neither measure correlated significantly with PPE or LPPE (Ps > 0.36). Thus, the ratio method yielded somewhat different conclusions from the regression method.

Which approach is best? Each uses a different equation to correct CC area (Y in mm\(^2\)) for differences in brain weight (X in mg). The simple ratio method involves the straight line \(Y = 0.0018X\), and the second ratio method uses \(Y = 0.014X^{0.667}\). Asking whether groups differ on a ratio measure is equivalent to asking whether observed CC areas differ significantly from prediction using the two equations. On the other hand, the linear equation of best fit for the present data is \(Y = —0.282 + 0.0024X\). Its slope is greater than for the simple ratio and the intercept is less than zero. If there were compelling reasons a priori to use either of the ratio methods, this would be appropriate, but doubts in the literature about the true degree of allometry cause us to prefer the line fitted to the data at hand, as urged by Schmidt-Nielsen\(^16\), rather than rely on the inorganic geometry.

**DISCUSSION**

Compared to LO line mice, those of the HI line were more strongly lateralized for paw preference, had larger brains, longer tails, and greater cross-sectional areas of the AC and CC. Path analysis indicated that variation in AC area was a consequence of brain size, whereas the CC area of the HI line in relation to brain size was actually lower than in the LO line. However, the size of the CC relative to brain size was not related to paw preference, whereas brain size relative to CC area was positively related to strength of paw preference.

These findings support the hypothesis that the line difference in brain size is causally related to the line difference in behavioral lateralization, but they cannot, by themselves, exclude the possibility that the effects are produced by separate physiological processes. Path analysis is most likely to yield valid conclusions when the general structure of causal relations is known in advance, as is certainly true for body size, brain size and commissure areas. Interpretation is facilitated when certain group differences, in this case the line and sex of mice, are produced experimentally or present at conception, because this makes certain paths of causation impossible and thereby eliminates several potential models. Path analysis can be very misleading if arbitrary assumptions are made to eliminate paths or estimate certain parameters\(^18\), which was not done here, and it is also affected by the inevitable sampling error in data. According to Wright, the method does not allow patterns of causation to be deduced from correlations\(^24\). Rather, combination of knowledge of causal relations with observed correlations allows one to estimate the relative strengths of causal influences, provided effects are additive and linear\(^23\). If a hypothesized causal influence turns out to have zero strength, it may be reasonable to eliminate it from the model.

Of course, chance could play a role during the original selective breeding experiment, such that an excess of genes contributing to larger brains but unrelated to paw preference accumulated in the HI line. Because of this, it is always wise to seek other kinds of data to corroborate findings. If the brain size-paw preference relation is causal, then selective breeding for brain size might produce a correlated change in strength of paw preference. Likewise, treatments which substantially alter brain size relative to CC size, perhaps preweaning litter size or low protein diets, should affect behavioral lateralization. Alternatively, genetic correlation of brain size and paw preference could be assessed in a population with great genetic variability.
The lack of a line difference in brain asymmetry in this study conflicts with an earlier report\textsuperscript{22}. That report involved brains fixed in ethanol, which causes substantial shrinkage and may have differential effects on lipid-rich myelinated structures compared with cortical regions low in myelin, whereas we used buffered formaldehyde, which produces minimal shrinkage. It is conceivable that fixation and shrinkage of the cerebral hemispheres can magnify very small differences in asymmetry present in the living tissue. It is also possible that the neural asymmetry important for behavioral asymmetry is localized and will not be evident in measures of the whole brain, or that a very small neural asymmetry could yield a really large behavioral asymmetry.

One advantage of plotting results as in Figs. 2 and 3 is that the actual size of an effect of selective breeding can be readily distinguished from the statistical significance of the effect. When this is done, it is obvious that selection for strength of paw preference was accompanied by a large line difference in brain weight with no line difference in body weight, and that the change in CC size was relatively small. If another study reports a difference in asymmetry significant at the $P=0.05$ level, it will be important to note, the actual effect size, because a small effect will make its presence known with less consistency than the large effect evident in brain size. Presentation of results in terms of effect size should also facilitate comparisons among studies which used different numbers of subjects, because expected effect size, unlike statistical power, is not strongly affected by sample size.

**REFERENCES**