

Genetic Analysis of Variation in Neuron Number

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Abstract

There are large differences in neuron number both within and between species. This variation in neuron number results from both non-genetic and genetic factors. Non-genetic factors, such as litter size and parity, and genetic cofactors, such as sex, age, and body weight, are known to generate variation in neuron number. However, the cumulative variance in neuron number that can be explained by the summation of these factors is unknown. Genetic variation has been shown to explain a substantial portion of the variation in neuron number. However, the identity of the genetic factors and the manner in which they influence neuron number are not known. In this dissertation, I have analyzed the components of environmental and genetic variation contributing to variation in neuron number among inbred strains of mice. I have focused on variation in neuron number on a large scale, by using the surrogate measure of whole brain weight, and variation within a distinct neuron population, retinal ganglion cells.

Brain weight ranges from 403 mg to 495 mg among standard inbred strains of mice. I have assessed the relative importance of environmental and genetic factors in the variation of brain weight in 27 standard inbred strains, two sets of recombinant inbred strains, and four F2 intercrosses. I first estimated the portion of variance in brain weight due to sex, age, body weight, litter size, and parity by regression analysis. Sex, age, parity, and body weight account for approximately half of the variance in brain weight, with body weight being the most important variable. However, the remaining variance in brain weight is substantial. Significant genetic variation was evident from the significantly lower variance in brain weight within strains compared to across strains, or within heterogeneous mice. Heritability of brain weight in the inbred strains is 0.50. The broad platykurtic and nearly bimodal probability density distributions of two intercrosses indicate that genes with major effects on brain weight are

segregating within these crosses. In addition, my estimates of the minimum number of genes modulating brain weight within the crosses ranged from one to six. The high heritability and low effective gene number indicate that it should be possible to map some of the genes modulating brain weight between strains. Genes that produce variation in a quantitative trait, such as brain weight, are called quantitative trait loci (QTLs).

I mapped QTLs responsible for variation in brain weight using two recombinant inbred sets (BXD and AXB/BXA) and one F2 intercross (ABXDF2, $n = 517$). Using linkage analysis, with composite interval mapping, I detected four significant QTLs affecting brain weight on Chrs 7, 11, and 14. The brain weight QTLs have been named *Brain size control 1, 2, 3, and 4* (*Bsc1*, 2, 3, & 4). *Bsc1* maps to proximal Chr 11 at 12 cM and has a LOD score of 8.4. *Bsc2* maps to distal Chr 7 at 65.2 cM and has a LOD score of 6.7. *Bsc3* and *Bsc4* map to Chr 14 at 25 cM and 59.1 cM and have LOD scores of 6.8 and 5.9, respectively. Secondary brain weight QTLs were mapped to Chrs 1, 5, 8, 11, 14, 18, and X. In the ABXD5F2 cross, three QTLs, *Bsc3* and *Bsc4*, and a secondary QTL on Chr 18, were each estimated to explain between 4% to 6% of the variance in brain weight. These three QTLs account for 60% of the total genetic variance and 15% of the total phenotypic variance in brain weight in the ABXDF2 cross.

Retinal ganglion cell numbers range from 50,600 to 69,000 among standard inbred strains of mice. I examined the contribution of environmental and genetic factors to the variation in ganglion cell number among 17 standard inbred strains, 26 BXD recombinant inbred strains, and two F2 intercrosses. Variation in ganglion cell number was not correlated with age, sex, or body weight, within any of the inbred strains. However, ganglion cell number was significantly correlated with brain weight across strains and within heterogeneous mice, accounting for 20% to 30% of the variance in ganglion cell number. Genetic variation was evident from the significantly larger variance in ganglion cell number among strains compared to within strains. A bimodal probability distribution of ganglion cell number from 57 inbred strains, in addition to the estimate of one to three genes modulating ganglion cell number, indicates that there are genes with large effects on ganglion cell number. The heritability of retinal ganglion cell number in the standard inbred mouse strains is 0.48. These results indicate

that it should be feasible to map some of the QTLs modulating ganglion cell number among inbred strains.

I used the BXD recombinant inbred set and two F2 intercrosses (CCASF2, n = 112, 32CASF2, n = 140) in linkage analysis, with composite interval mapping, to map genes responsible for variation ganglion cell number. I mapped four significant QTLs affecting ganglion cell number to Chrs 1, 7, 11, and 16. The ganglion cell QTLs have been named *Neuron number control 1, 2, 3, and 4* (*Nnc1, 2, 3, & 4*). *Nnc1* maps to Chr 11 at 57 cM and has a LOD score of 6.7. *Nnc2* maps to Chr 7 at 65 cM and has a LOD score of 5.9. *Nnc3* maps to 82 cM on Chr 1 and has a LOD score of 9.3, and *Nnc4* maps to Chr 16 at 41.5 cM and has a LOD score of 6.0. Thyroid hormone receptor alpha (*Thra*) was identified as a superb candidate gene for *Nnc1*. I tested *Thra* as a candidate by comparing the ganglion cell number in transgenic mice carrying a null transgene at the *Thra* locus with ganglion cell number from mice carrying a wild-type *Thra*. The mice with the null *Thra* transgene had significantly lower ganglion cell numbers compared to the wild-type mice. The result supports *Thra* as a candidate gene for *Nnc1*.

Finally, I examined the developmental mechanisms responsible for the differences in ganglion cell among strains of mice. I estimated ganglion cell production in strains with high ganglion cell number and strains with low ganglion cell number by counting ganglion cells at postnatal day zero. Approximately 77% of the variation among adult strains result from differences in the production of ganglion cells. Thus, the variation in adult ganglion cell number among inbred mouse strains results predominantly from differences in cell production. Collectively, the results indicate that some of the *Nnc1, 2, 3, & 4*, QTLs are likely to modulate ganglion cell number by influencing cell production.

In summary, I have demonstrated, 1) the proportion of variance in brain weight and ganglion cell number explained by genetic and non-genetic factors, 2) the location of QTLs producing variation in brain weight and ganglion cell number, and 3) the predominant mechanism generating variation in ganglion cell number is cell production. Finally, of significance, the mapping studies prove that it is possible to map the genes that are responsible for both global and discrete quantitative variation within the mouse brain.

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Chapter 1: Introduction

Throughout mammalian evolution brain size has varied from a massive 6800 gm in whales down to a meager 0.10 gm in shrews (Count, 1947; Jerison, 1973) yet the regional organization has remained virtually the same (Butler and Hodos, 1996). Indeed, upon microscopic examination of the mouse and human brain Ramon y Cajal, a Nobel Laureate recognized for his detailed descriptions of the nervous system, stated: "In my opinion there are only quantitative differences, not qualitative differences between the brain of a mouse and that of a man." The genetic differences responsible for the vast differences in brain size are unknown.

Differences in brain size between species ultimately depend on the accumulation of micro-evolutionary changes among the individuals within a species (Butler and Hodos, 1996). Variation within a species can be substantial. For example, normal variation in brain weight among humans ranges two-fold, from 900 to 1,800 gm (Cobb, 1965). Even among closely related strains of inbred laboratory mice brain weight differs by as much as 13%, from 429 mg in C3H/HeJ to 490 mg in C3H/HeSnJ. Examining the genetic and developmental bases for variation in brain size within a species can reveal bases for the large diversity in brain size found across species.

Variation in brain weight results primarily from differences in glia and neuron number. This has been demonstrated by finding a high correlation between brain weight and total brain DNA (Zamenhof and van Marthens, 1978). Also, neocortical neuron number in humans is correlated with brain weight ($r = 0.56$) and with neocortical volume ($r = 0.69$) (Pakkenberg and Gundersen, 1997). Not surprisingly, estimates of neuron numbers for discrete structures within the brain are also highly variable. For example, the volume and cell number in the visual cortex of humans and other primates ranges almost three-fold (Gilissen and Zilles, 1996; Suner and Rakic, 1996). Large variation in neuron numbers have also been found among inbred mouse strains in numerous cell populations, e.g., midbrain dopaminergic neurons (Ross et al., 1976); forebrain cholinergic neurons (Albanese et al., 1985); and granule cell number in hippocampus area dentata (Wimer et al., 1978).

Variation in neuron number is the result of both environmental and genetic factors. A key question is how much of the variation is attributed to the environment and how much is attributed to genetic factors. A heritability estimate expresses the magnitude of the genetic contribution and is measured as the ratio of the genetic variance over the total phenotypic variance (V_G/V_P). The heritability of brain size in humans has been estimated to be as high as 94% (Bartley et al., 1997). Heritability estimates for brain weight and neuron number in mouse, are also high, 0.67 for brain weight (Roderick et al., 1973) and 0.80 for granule cell number (Wimer and Wimer, 1989). These high heritability estimates indicate that variation in brain size and neuron number is generated predominantly by genetic variation. Identifying the genetic variation that modulates neuron number can expose specific molecules and the developmental and regulatory mechanisms controlling neuron number.

Forward genetic approach

The first step to determine the genetic bases of variation is to map the genomic location of the genes responsible for the phenotypic variation. Using phenotypic variation to map the responsible genes is known as the forward genetic approach. One method for mapping a gene is linkage analysis. The ability to perform linkage analysis requires genetic variation between individuals. The genetic sites where DNA sequences differ between individuals are called polymorphic loci, with each variant form representing an allele type. Linkage analysis involves testing for an association between the inheritance of a particular allele and the inheritance of a particular phenotype. A strong association between an allele and a particular phenotype indicates that a gene that contributes to the phenotypic variation maps near the allele. The ability to use alleles as genetic markers to infer the presence of a nearby gene is dependent on the physical linkage of loci on chromosomes. The closer the physical proximity between two loci the less likely they are to form a chiasmata between them and recombine with their homologous chromosome during meiosis. The frequency that genetic loci recombine translates into units of genetic distance called centimorgans (cM). The frequency of recombination can be determined from the percentage of non-parental allele combinations in the offspring.

Gene mapping before recombinant DNA technology required mutations that exhibited prominent visible or biochemical phenotypes in the offspring to carry out linkage analysis. For example, the first linkage demonstrated in the mouse was between the albino (c) mutation and the pink-eyed dilution (p) mutation ([Haldane et al., 1915](#)). In the last decade a dense map of genetic markers called microsatellites and their ease of typing by the polymerase chain reaction (PCR) has revolutionized genetic mapping. Microsatellites or simple sequence repeats (SSR) consist of di- or tri-nucleotide repeats, such as CACACA ([Dietrich, 1992](#); [Love, 1990](#)) and are dispersed at a high frequency throughout mammalian genomes. Over 6,000 microsatellites have been genetically mapped in the mouse and recently 4,000 of these markers were physically mapped ([Dietrich et al., 1994](#)). Microsatellites have been typed in 12 inbred mouse strains and have been found to be highly polymorphic between strains.

Gene mapping has been greatly facilitated by the production of genetic variants produced by x-ray, chemical, and P-element induced mutations ([Sentry et al., 1994](#)). In *Drosophila* and zebrafish, the production of mutant phenotypes by mutagenesis and careful screening has lead to the identification of many genes involved in neurogenesis ([Sentry et al., 1994](#)). Large-scale mutagenesis screens are now underway in mouse and are expected to yield a plethora of mutant phenotypes for study ([Kasarskis et al., 1998](#)). However, one drawback of induced mutations is that they have pleiotropic effects, and many mutations will affect the viability of the animal, making it difficult to study.

Exploiting natural genetic variation

Of the roughly 40,000 genes expressed in the human brain, approximately 10–20% are expected to have multiple alleles ([Silver, 1995](#)). Almost all of the heritable variation between individuals results from random segregation of different alleles that produce subtle quantitative effects ([Falconer and Mackay, 1996](#)). A quantitative trait, such as height, has a continuous range of phenotypes in a population and is controlled by multiple genetic and environmental factors. In contrast, qualitative traits have distinct phenotypes, such as coat color in mice, and are controlled by one or two genes with little environmental influence. Genes with allelic variants that produce quantitative variation are called quantitative trait loci (QTLs). Quantitative traits

such as neuron number can be used in a forward genetic approach to identify the location of the QTLs. Mapping a QTL is different from mapping a gene with qualitative expression in that there is no one-to-one relation between a genotype and a phenotype. This is because the effects of other genes and environmental variation combine to produce the expressed phenotype. A QTL is mapped by comparing the phenotypic means from the progeny in each genotype class at every marker across the genome. Linkage is suggested when there is a significant difference between the means of the genotypic classes.

Mice are the ideal animal for genetic analysis, especially if the goal is to extrapolate to humans. Although, the evolutionary divergence of mice and humans occurred over 60 million years ago, there is still a close correspondence between the genomes of mice and humans. Large segments of the human and mouse genomes are conserved and the differences found today can be explained by breaking the mouse genome into roughly 130 segments and shuffling them into a new order ([Nadeau and Taylor, 1984](#)). The existence of a great variety of inbred mouse strains, which represent a wealth of quantitative variation, is crucial in a quantitative genetic analysis. Mice from a particular inbred strain are genetically identical or isogenic. Phenotyping a number of isogenic mice to obtain a strain average can minimize the variance due to environmental factors, measurement errors, or developmental factors, resulting in a more accurate mean phenotype. Mice are usually inbred by at least 20 generations of brother and sister mating. Inbreeding forces the alleles to become homozygous at all loci. Finally, in consideration of time and cost, mice reproduce quickly and are relatively inexpensive to house.

Dissertation research

In my dissertation research, I use the forward genetic approach to assess and dissect genetic sources of variation in neuron number. I have focused on variation in neuron number on a global scale by using the surrogate measure of whole brain weight. I have also focused on variation within a discrete neuron population, the retinal ganglion cells. I began my studies by determining the relative proportion of variance in brain weight and ganglion cell number that is due to environmental and genetic factors ([Chapters 2 and 4](#)). Subsequently, I used linkage analysis with composite interval mapping techniques to pursue QTLs that are responsible for

genetic variation in brain weight and ganglion cell number ([Chapters 3 and 5](#)). To identify the developmental mechanisms generating variation in neuron number I have examined the role of cell production and cell death in the generation of ganglion cell variation among mouse strains ([Chapter 6](#)).

In the next section I address the behavioral significance of variation in brain size and neuron number. Finally, I conclude this introduction with a summary of molecular neural development to acquaint the reader with the processes in which QTLs might be involved to produce variation in neuron number.

Please note that although I present the work in [Chapters 4 and 5](#) using the first person singular pronoun, this work is actually the result of collaboration with Robert Williams, Dan Goldowitz, and Dennis Rice. For the most part, the methods and results in [Chapters 4 and 5](#) match those in recent published papers. Differences include the addition of new strains and larger numbers of cases per strain and as a result I have also reanalyzed most of the data.

Importance of neuron number

Brain weight

Brain tissue is metabolically costly and an increase in brain size should be counterbalanced by an increased capacity for behavioral adaptations ([Williams and Herrup, 1988](#)). Increased neuron number is usually associated with more elaborate neural networks that have evolved for novel sensory or behavioral adaptations required for exploiting new ecological niches. The behavioral significance of variation in brain size is readily apparent across species, where more complex behaviors are generally associated with larger brains ([Aboitiz, 1996](#)). However, within species behavioral associations with variation in brain size are less evident. Controversial findings have been reported in humans, with positive correlation between cerebral volume measured by MRI and IQ in children ([Reiss et al., 1996](#)), but the absence of a correlation between forebrain volume, also measured by MRI, and IQ in young adults ([Tramo et al., 1998](#)). The disparity may have to do with the differences in the age of the subjects. Older subjects harbor the effects of years of environmental variance, as well as an extended period of brain growth, which could dissolve any initial correlation between brain size and IQ.

In mice, high and low brain weight lines, generated by selective breeding, initially showed behavioral differences in field activity, but these differences disappeared in later generations (Wimer et al., 1969). A recent study with rats reported a high correlation between general intelligence and brain weight (Anderson, 1993). In summary, an association with brain weight and behavior within a species is inconclusive and the outcome of individual studies may depend on the particular structures responsible for differences in brain size and the appropriateness of the behavioral tests.

Discrete populations

Behavioral associations are found with the size of discrete neuronal populations. The relative size of the hippocampus has been shown to be associated with spatial memory capacity in species of passerines, cowbirds (Healy and Krebs, 1996; Reboreda et al., 1996), and kangaroo rats (Jacobs and Spencer, 1994). In males of many seasonally breeding songbird species the volume of the song nuclei, hyperstriatum ventralis, pars caudalis (HVC) and archistriatum (RA), increases along with their singing ability during each breeding season (Smith et al., 1997). In male zebra finches, neuron number and the volume of HVC and RA in an individual correlates positively with the number of tutor syllables copied (Ward et al., 1998). Moreover, in humans, cerebellum volume significantly correlates with the individual's verbal memory and fine motor dexterity (Paradiso et al., 1997). In conclusion, there is a strong association between the number of neurons in a discrete population and its behavioral output, particularly if the behavior is important for survival.

Numbers of retinal ganglion cells range from fifty thousand in nocturnal rodents to several million in diurnal birds and primates (Rager and Rager, 1978; Rakic and Riley, 1983). Variation is also marked within species: numbers range from 0.7 to 1.5 million in humans (Curcio and Allen, 1990), and from 40,000 to 80,000 in mice (Williams et al., 1996). It is not known whether visual acuity differs in animals with high and low ganglion cell number. However, visual acuity in glaucoma patients is compromised after a small decrease in ganglion cell number (Quigley et al., 1989). The difference in visual acuity will depend on what classes of ganglion cells are variable. There are three major morphological classes of ganglion cells in the

mammalian retina (alpha, beta, and gamma) (Rodieck and Brening, 1983). The extent of variability within the represented classes of ganglion cells has not been studied. However, the numbers of beta and gamma cells are likely to be the most variable since they are the most numerous, with each representing roughly 45% to 50% of the ganglion cell population in several species (Wässle and Boycott, 1991; Williams et al., 1993). The high density and short dendritic fields of the gamma ganglion cell class are thought to set the limit for spatial resolution (Wässle and Boycott, 1991) and thus, even moderate variation in the density of this class should affect visual acuity. The two-fold variation in ganglion cell number among mice is significantly associated with retinal area ($r = 67$, $t = 269$, $P < 0.001$) (Rice et al., 1995), and thus cells per degree of visual angle, the critical factor determining visual acuity, may not change with increased cell number.

Developmental control of neuron number

Neuron number in the brain

The number of neurons in the adult central nervous system results from a balance between cell proliferation and developmental cell death. While there is considerable cell death in most vertebrate brains, the major process defining brain size in a species is neurogenesis. Neural progenitors originate from the embryonic ectoderm. The neural fate is the default pathway for embryonic ectoderm. The switch from the neural fate into the epidermal fate is initiated by an endogenous inhibitory signal within the ectoderm, possibly BMP-4 (Hemmati-Brivanlou and Melton, 1997). Release from the neural inhibitory signal may be mediated by follistatin, secreted from the mesoderm.

Once cells are committed to the neural fate they coalesce to form the neural plate. The neural plate then invaginates along the neuroaxis, folds inward, and fuses to form the neural tube. In the neural tube, neuroepithelial cells divide in the ventricular zone, leave the cell cycle, and migrate outward to reside in the appropriate cell layers. In neurogenesis, a complex molecular cascade controls the progressive hierarchy of cell fate competence that ultimately leads to a specific cell type in the appropriate place and numbers.

In mice, neocortical neurons are generated from a founding population in the pseudostratified ventricular epithelium (PVE) between embryonic day 11 (E11) and E17. During this interval of neurogenesis there are an average of eleven cell cycles, which increase the number in the founding population by 140 fold (Takahashi et al., 1995). At the onset of neurogenesis neural progenitors undergo symmetrical divisions in which both daughter cells return to the cell cycle (Rakic, 1988). During this time, cells increase in number exponentially. As development proceeds the neural progenitors begin to divide asymmetrically, with one daughter cell leaving the cell cycle and differentiating while the other daughter cell continues to divide. Gradually, as the neurogenesis proceeds, both daughter cells will cease to divide and differentiate. The portion of the population that remains proliferative represents the P fraction, and is equal to 1 when all cells are dividing, while the portion of cells leaving the cell cycle represents the Q fraction, for quiescent, and is equal to 1 when all cells have ceased to divide (Takahashi et al., 1997).

Developmental mechanisms for variability. Variability in neuron numbers could result from differences in the (1) number of founding neural progenitors, (2) parameters of the cell cycle, and (3) duration of cell proliferation. The variation of Purkinje cell number among chimeric mice produced between wildtype mice and *lurcher*, a mouse mutant whose Purkinje cell population is completely absent, appears to occur in quantum units equal to 10,200 cells. The integral units suggest that variation in Purkinje cell number results from differences in the initial proliferative fraction of wildtype progenitors (Wetts and Herrup, 1983). Quantum differences in the Purkinje cell number in chimeras made between the mouse strains C57BL/6 and AKR/J has also demonstrated that variation can result from differences in the initial clone size of progenitors (Herrup, 1986). These studies suggest that differences in cell number can originate from differences in the number of early progenitors, probably arising during regionalization of the neural plate or neural tube.

Variability in parameters of the cell cycle. Variation in neuron number could result from differences in the parameters of the cell cycle. One such parameter is the rate at which proliferative fraction Q approaches 1, which could be a major factor in controlling the number of neurons generated (Takahashi et al., 1997). In the mouse neocortex the Q fraction equals 0.5 at

cell cycle number 8. However, if the cell cycle at which Q equals 0.5 were delayed from 8 to 10 the population would expand 5-fold. The number of neurons generated is also determined by the progressive increase in the length of the cell cycle. During the period of neocortical neurogenesis in mouse, the length of the cell cycle increases from ~8.1 hours to ~18.4 hours. This increased cycle time is a result of a 4-fold increase in the length of G1 (Takahashi et al., 1995). A broad range in the length of G1 has been found among different cell populations (Jacobson, 1991).

In primates the striate visual cortex has a higher density of neurons compared to other cortical areas. The differences in cell number between the striate and extrastriate cortex was shown to result from a higher proliferative fraction, but also from a higher portion of cells cycling, as shown by 3H-thymidine pulse labeling (Dehay et al., 1993). The proliferative portion, known as the labeling index, was 50% higher in the striate cortex than in the extrastriate. The authors concluded that differences in the mitotic rate must have produced the difference in the labeling index, however an equally plausible explanation is a difference in the progression of Q. Nevertheless, it is not known whether variability in the rate of Q or variability in the length of the cell cycle contributes to variation in cell number.

Variability in the number of cycles can have a large impact on cell number. For example, if the number of cell cycles doubled while the relative progression rate of P to Q was the same, the population would increase 60-fold. Of course, an increase in the cell cycle number, and consequently the duration of neurogenesis, means that the over all progression of Q is slower. It has been known for some time that the large differences in neuron number found between species is best explained by differences in the period of neurogenesis (Passingham, 1975). A recent study confirmed this finding by finding a high correlation between the peak day of neurogenesis and the size of 51 brain structures among 7 different mammals (Finlay and Darlington, 1995).

Extrinsic signals in cell cycle control. Although much is known about cell cycle mechanics and the molecular components, little is known about the regulatory mechanisms governing variation in the exit from the cell cycle (Ross, 1996). In vertebrates the decision of a neural precursor to leave the cell cycle during development involves the Notch signaling

pathway (Beatus and Lendahl, 1998). Notch, a transmembrane protein, and its ligands, Delta and Serrate, are the mediators of cell-cell interactions that control the competence of neighboring cells. Through a process called lateral inhibition, a prospective neuron expressing Delta or Serrate on its cell surface binds the Notch receptor on a neighboring cell thereby activating the cell's Notch pathway, which inhibits cell differentiation and holds them in a proliferative state. Growth factors may regulate the expression of genes in the Notch pathway. For example, the epidermal growth factor (EGF), which can induce widespread proliferation of neuronal precursors in the brain (Kuhn et al., 1997), was recently shown in the developing retina to down regulate Mash-1, a proneural factor within the Notch signaling pathway (Ahmad et al., 1998). A connection between EGF and Notch suggests that one way growth factors may act is by down regulating the Notch pathway, thereby holding cells in a proliferative state. Other extrinsic factors such as retinoic acid and thyroid hormone influence the extent of cell proliferation in the brain by inducing the withdrawal from the cell cycle and subsequent differentiation (Jacobson, 1991; Kelley et al., 1994). How the expression patterns and concentration levels of specific growth factors and hormones are regulated to set proliferative limits to defined populations of neurons is not known.

Finally, extrinsic factors ultimately regulate the progression of the cell cycle by inhibiting or promoting the buildup of cyclins and the activity of their dependent kinases (Hutchinson and Glover, 1995). The complete pathway between the proliferative effects of growth factors and hormones and their effectors within the cell cycle during neurogenesis remain to be mapped out. In summary, the developmental control of neuron number involves a complex collaboration of molecular pathways that converge to determine the progression of the cell cycle through specific checkpoints.

Retinal ganglion cell development

In mouse the neural retina is part of the brain, comprising ~0.4% of the brain's weight. The eyes begin to form in the mouse around E9 with the bilateral out-pocketing of the forebrain into a structure called the optic vesicle (Mann, 1964; Pei and Rhodin, 1970). The optic vesicle grows outward toward the surface ectoderm and then invaginates to form the optic cup. The

optic cup then separates into an inner portion, the neural retina and an outer portion, the pigment epithelium.

Proliferating retinal cells migrate in a to-and-fro pattern, moving to the inner retina to synthesize DNA and then back to the outer retina to divide. During retinal development the percentage of proliferating cells (P) decreases as cells from asymmetric divisions leave the cell cycle, migrate toward the vitreal surface and differentiate. The neural retina develops into three distinct cell layers, a ganglion cell layer, a bipolar layer and an inner photoreceptor layer. The onset of differentiation for retinal cells occurs in a temporally-ordered manner, with retinal ganglion cells, amacrine cells, and horizontal cells beginning to differentiate early, while photoreceptors, bipolar cells and Müller glia cells begin to differentiate later (Sidman, 1961). Although the generation of cell types begin and peak at different times the cell types are being generated simultaneously throughout most of the developmental period. In mouse, ganglion cells start to differentiate on E11 and continue until just before birth (Dräger, 1985). Developmental cell death of ganglion cells begins at, or just before birth, peaks between postnatal days 4–6, and is essentially complete by P12 (Linden and Pinto, 1985; Young, 1984). Variation in ganglion cell number could result from differences in the extent of ganglion cell production or ganglion cell death.

Variability in ganglion cell production. Variation in ganglion cell production could result from differences in: (1) numbers of retinal progenitor cells, (2) the kinetics and duration of progenitor cell proliferation, or (3) bias in cell fate determination in progenitors. The variance of retinal cell numbers in embryonic chick to peaked during the exponential phase of cell division and were lowest after cell differentiation (Morris and Cowan, 1984). A regression analysis of the variance in cell number at the start of incubation and growth rate indicated that the variation in the exponential phase was due mostly to the variance in the number of retinal progenitors at the start of incubation. However, variation in retinal cell number at the start of incubation could result from subtle differences in the correspondence of the developmental stage and not necessarily to the variability in the number of retinal progenitors

Variance in numbers of multipotent retinal progenitors should have consistent effects on the number of many retinal cell types. However, a comparison of horizontal cell and ganglion

cell numbers for six strains has demonstrated that ratios in these early-generated cell types are not always matched (Williams et al., 1998). In fact, preliminary data indicates a weak negative correlation exists between the number of ganglion cells and photoreceptors within inbred strains (Williams et al., 1998). If true, that would suggest that a reciprocal relationship exists between early- and late-generated retinal cell types and that variation in ganglion cell number is produced by differentiation bias. An example of the reciprocal relationship between retinal cell types is found when the inhibition of Notch signaling results in an increase of early-generated cell types at the expense of later-generated cell types (Dorsky et al., 1997). It is possible that a genetic bias in the Notch signaling pathway may play a role in executing the differentiation ratios of retinal cell types.

Variation in the ganglion cell number could result from differences in the proliferation kinetics of progenitors that give rise to ganglion cells. The rates of mitosis may be influenced through inhibitory molecules and pathways. One example is dopa—a tyrosine metabolite that normally has inhibitory effects on cell genesis in the retina. The absence of dopa in albino rats leads to an anomalous up-regulation of ganglion cell production followed by an increase in the severity of cell death (Ilia and Jeffery, 1999). Finally, as with brain weight, large differences between species within a defined population is best explained by differences in the duration of neurogenesis. For example, a two-fold difference in the duration of retinal neurogenesis can explain the near two-fold difference in retinal ganglion cell number between gerbil and hamster (Wikler et al., 1989).

Variability in ganglion cell death. Differences in cell death can produce large differences in cell number across species. Estimates of retinal ganglion cell death range from none at all in fish to 40% in chicken, 50–60% in rat (Lam et al., 1982), and up to 80% in cat (Williams et al., 1986). The ganglion cell population in the domestic cat is 30% lower than that of wildcat—the species from which the domestic cat has evolved. Retinal ganglion cell number in a Spanish wildcat fetus and domestic cat fetus obtained at E38, were remarkably close, suggesting that cell death rather than cell production generates the marked species differences (Williams et al., 1993).

Variation in the severity of cell death may result from differences in titers of neurotrophic factors. The neurotrophins—BDNF and neurotrophin-3/4—have been found to increase survival of retinal ganglion cells in chicken and rat (Ma et al., 1998; Rosa et al., 1993). Neuregulin, found on the cell surface and as a secreted protein, can also increase survival of neonatal rat retinal ganglion cells (Bermingham-McDonogh et al., 1996). Differences in the concentration or expression time of these neurotrophic factors, their receptors, or components within their signaling pathways could produce variation in the severity of naturally occurring ganglion cell death.

In summary, the generation of variation in cell number could involve any of the molecular processes described above. Determining the genetic bases of variation in neuron number could identify key regulatory genes in neurogenesis, which may lead to a greater understanding of cell-cycle regulation. In addition, examining the molecular variants in the QTLs that produce natural variation in neuron number will lead to a better understanding of the genetic bases of quantitative variation and provide insight into the molecular nature of brain evolution.

Chapter 2: Genetic and Environmental Control of Brain Weight Variation

Introduction

Variation in brain weight among different mouse strains could result from genetic variation in genes that are expressed only within the brain. Variation in brain weight can also result from environmental factors, such as nutritional differences resulting from variable litter size and from genetic factors, such as the expression of hormones relating to the sex of the animal or from body weight genes with pleiotropic effects. In order to estimate the proportion of variance in brain weight that results from assessable factors, I have performed linear regression analyses with brain weight and the cofactors, sex, age, body weight, litter size and parity. In fitting a regression equation, the approximate change in brain weight for a unit change in a cofactor can be estimated. To minimize the variance in brain weight attributed by these factors, brain weights were standardized to the mean age, body weight, litter size and parity. Although a number of studies have examined the effects of variation in these variables on brain weight, this study is the first comprehensive analysis of the effects of multiple factors and a comparison of these variables between strains.

In this study I have also tested the feasibility of mapping genes that control variation in brain weight among mice. Before launching a mapping study, it is necessary to establish that there is an appreciable amount of heritability for the trait of interest. Studies have reported brain weight to be highly heritable, ranging from 0.70 in mice (Atchley et al., 1984; Roderick et al., 1973), to 0.75 in a primate colony of rhesus macaques (Cheverud et al., 1990). However, those estimates are only relevant to the particular population and the environment from which they were measured. Thus, as a prelude to mapping, I have estimated the heritability of brain weight among inbred strains and within mapping crosses. The accuracy of the heritability estimate depends on the precise estimate of the environmental variance within an isogenic group. For example, if the environmental variance is exceedingly high as a result of age-related variance then the genetic component could be underestimated. To compute precise heritability estimates and to estimate the number of genes producing variation in brain weight, I used corrected brain weights in which the variance due to known cofactors has been minimized.

Materials and methods

Mice

Most of the inbred mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Environmental variation was minimized by housing the mice in a pathogen-free environment, maintained at 20-24°C on a 14:10 hr light/dark cycle, with all mice being fed a consistent diet of 5% Agway Prolab 3000 rat and mouse chow.

Standard inbred strains. The standard inbred strains were derived from domesticated hybrids generated from crosses between *M. m. domesticus*, *M. m. musculus*, and *M. m. castaneus* early in the 20th century ([Bonhomme, 1992](#)). The hybrids strains were produced by inbreeding through at least 20 generations of brother x sister matings and most have now been maintained by successive sibling matings for greater than 80 generations. Brain weights were obtained for 27 standard inbred mouse strains ([Table 2.1](#)). [Figure 2.1](#) shows the lineage chart of the genus *Mus* with fixed brain weights for strains, species, and subspecies of mice. Inbred strains are shown in the yellow panel.

Subspecies of *M. musculus*. Brain weights were obtained from four wild subspecies of *M. musculus*: (1) *M. m. castaneus* (CAST/Ei and CASA/Rk), a Southeast Asian subspecies; (2) *M. m. musculus* (CZECHII/Ei), a commensal Eastern European mouse; (3) *M. m. molossinus* (MOLD/Rk, M, MOLF/Ei, MOLC/Rk), a Japanese hybrid of *M. m. musculus* and *M. m. castaneus*; (4) *M. m. domesticus* (WSB/Ei), a commensal and widely dispersed subspecies of Western Europe and the Americas. The phylogeny and native territory of these species are reviewed in [Bonhomme \(1992\)](#).

Species of *Mus*. Brain weights were obtained from inbred representatives of four wild species of *Mus*, [Fig. 1](#), bottom panel: (1) *M. musculus*, the common house mouse, a wide-ranging and highly adaptable commensal species from which the standard strains are derived; (2) *M. spretus* (SPRET/Ei), a short-tailed field mouse distributed around the Western Mediterranean; (3) *M. spicilegus* (PANCEVO/Ei), a colonial mound-building species from Eastern Europe, (4) *M. caroli* (CARL/ChGo), a small tropical East Asian species. The mouse CARL/ChGo is outbred and has been maintained since the mid 1970's in a colony of 5 to 10 breeding pairs with specific

avoidance of sibling matings. [Table 2.2](#) lists the mean brain weight, body weight, age and numbers sampled for each subspecies and species of *Mus*. The lineage of the subspecies and species of *Mus* is shown in the bottom panel of [Fig. 2.1](#).

Isogenic F1 hybrids I obtained brain weights from eleven isogenic F1 hybrids, (ABXD5F1, AC3HF1, C3HAF1, B6D2F1, D2B6F1, BXD32x5F1, PLSJF1, 32CASTF1, CCASF1, CAF1/J, CB6F1). The hybrid CAF1/J, was obtained from the Jackson Laboratory. All others were generated in the University of Tennessee mouse colony. Data for the F1 hybrids can be found in [Table 2.4](#).

Recombinant mice

In this study, I used two types of recombinant progeny, recombinant inbred strains and F2 intercross progeny. Recombinant inbred strains (RI) are made by first mating mice from two inbred strains and then crossing the F1 hybrids to produce F2 progeny. F2 mating pairs are then chosen at random and their descendants are inbred through 20 or more generations of brother and sister mating. Each F2 mating leads to an isogenic strain, homologous at all genetic loci, but with a unique mosaic of chromosomal segments derived from the two progenitors. Brain weights were obtained from 26 BXD strains and 24 AXB/BXA strains. The sex ratio is approximately equal across all BXD and AXB/BXA cases, although within some strains the sex ratio is not well balanced. The average age and the number of mice sampled within each BXD and BXA/AXB strain are shown in [Tables 3.1](#) and [3.2](#).

Brain weights were obtained from four F2 intercrosses: ABXD5F2 (n = 517), AC3HF2/C3HAF2 (n = 360), CCASF2 (n = 112), 32CASTF2 (n = 141). The brain weights for these mice are not shown. The AC3HF2 and C3HAF2 progeny were produced from reciprocal F1 hybrids produced by interchanging the sexes. The sex ratios within these crosses are close to 50:50.

Fixation and processing of tissue

Adult mice were anesthetized with Avertin (1.3% 2,2,2-tribromoethanol and 0.8% tert-pentyl alcohol in water, 0.5–1.0 ml ip). Mice were transcardially perfused with 0.9% sodium

phosphate buffered saline (pH 7.4, room temperature), followed with 1.25% glutaraldehyde and 1.0% paraformaldehyde in 0.1 M PB (pH 7.3). Fixed brains were dissected at low magnification using a dissecting microscope. The brains were transected at the caudal margin of the cerebellum. Brains were weighed immediately after dissection to the nearest 0.1 mg. Paraflocculi or any other brain tissue torn away during dissection was added to the weigh pan. Small corrections were made when tissue was lost, usually a paraflocculus at ~2.5 mg.

Quantitative trait genetics

The two major components of phenotypic variance (VP) are genetic variance (VG) and environmental variance (VE). Examples of environmental factors are nutrition, uterine spacing, litter size, obstetrical complications, parity or age of mother and prenatal exposures (temperature, pathogens) (Jacobson, 1991). Environmental variance can also result from stochastic internal variation during development, known as developmental noise. Factors contributing to total genetic variance (VG) can be partitioned into additive gene effects (VA), dominance deviation (VD), and gene interaction (VI) (Falconer, 1989). Additive genetic variation results from allelic substitutions at genes and is estimated by the degree of resemblance among relatives. Dominance deviation occurs when one allele produces a larger effect with respect to the other. Interaction variance, or epistasis, results when different genetic loci interact and produce complex non-linear effects. Another factor contributing to phenotypic variance is the technical variance (VT), which results from measurement error. Taking all these factors into account, phenotypic variance consists of, $VP = VA + VD + VI + VE + VT$.

Regression analysis

To estimate the effects of environmental factors on brain weight I used DataDesk 6 (Data Description, Ithaca, NY) to perform least-squares regression with the variables, sex, age, body weight, litter size, and parity. Body weight, sex and age were recorded at the time of perfusion. In the ABXD5F2 progeny, parity and litter size were also recorded. Animal records, including parentage information, cage identification, litter size, birth dates were stored in a record keeping program called Mendel's Lab 4.

Heritability

In this study, I estimated the variance attributable to genetic factors by subtracting the environmental variance found among isogenic animals from the variance found among a genetically heterogeneous population, (VP – VE) (Crusio, 1992). Because inbred strains tend to show greater variability due to the fixation of deleterious alleles, the environmental variance (VE) is best calculated from the weighted average of the variance within the parentals and the F1 hybrid (VE = 1/4VP1 + 1/4VP2 + 1/2VF1) (Wright, 1968). Heritability in the broad-sense measures the proportion of the total phenotypic variation in a population attributable to all genetic factors, including dominance deviation and genetic interaction. Heritability in the narrow sense measures the proportion of phenotypic variance due to additive gene effects, (VA/VP). A good approximation of narrow-sense heritability can be estimated by comparing the nongenetic variance found within inbred strains to the variance found between strains (Hegmann and Possidente, 1981).

$$h^2 = 1/2 \text{ VA} / [(1/2 \text{ VA}) + \text{VE}]$$

The estimate of VA can approximate additive genetic variance because no dominance deviation exists within homozygous inbred strains. However, the accuracy of this estimate depends on the absence of variance from genetic interactions, which can not be guaranteed (Crusio, 1992). Note that the variance between inbred strains is divided in half because inbreeding results in the loss of intermediate heterozygotes, which produces a two-fold increase in genetic variation (Plomin and McClearn, 1993).

Gene number

The number of genes affecting brain weight in F2 intercross progeny can be estimated using the Castle-Wright formula: $(p_1 - p_2)^2 / 8V_G$; where $p_1 - p_2$ equals the difference between the two parents and V_G is equal to the phenotypic variance in the F2 intercross minus the phenotypic variance in the F1 (Wright, 1966). Modifying the formula to reflect the genetic variance among RI strains the equation for the number of effective genes among RI strains: $D^2 / 2V$, where D is the difference between the highest and lowest strain mean and V is the

variance of the RI strain means ([Bailey, 1981](#)). The accuracy of this computation relies on the assumptions of additive gene effects, unlinked genetic factors, polarized genetic factors in the parental strains (i.e. all increasing or decreasing alleles), and constant environmental effects. If these criteria are not met, the number of effective genes can be underestimated. At this stage, it is not possible to know if these criteria are met, and therefore the computations provide an estimate of the minimum number of effective genes. The number of genes involved in the variation of a trait can also be inferred from the phenotypic distribution of a segregating population.

Results

Variation among strains, species and subspecies

Average brain weights for 27 standard inbred strains range between 403 mg to 495 mg ([Table 2.1](#)). The average coefficient of variation (CV) for brain weight corrected with respect to sex, age and body weight within inbred strains is 3.9% and is 5.1% with the uncorrected brain weights. Brain weights for the wild *Mus* subspecies and species range from 303 to 454 mg, and the average CV for corrected brain weights is 4.5% and is 5.1% with uncorrected brain weights ([Table 2.2](#)). Note that the CV in the outbred strain CARL/ChGo is much higher 6.8%. The average phenotypic variance found within inbred strains is significantly less than the variance found between strains ($F(24,323) = 49.7, p = 1.85E-93$).

Table 2.1. Average brain weights for 27 standard inbred strains corrected for sex, age, and body weight.

Strains	Brain wt (mg) ± SE	<i>n</i>	Body wt. (gm).	Mean age (days)
129/SvJ	437.6 ± 4.1	10	18	40
129/J	440.8 ± 3.4	10	20	89
A/J	422.5 ± 3.9	25	22	190
AKR/J	439.8 ± 7.6	12	25	65
BALB/cByJ	447.0 ± 4.4	23	23	95
BALB/cJ	483.5 ± 3.3	61	32	142
CBA/CaJ*	452.6 ± 4.3	12	17	39
CE/J	452.3 ± 4.8	15	31	236
C3H/HeJ	428.7 ± 3.3	10	19	64
C3H/HeSnJ	490.3 ± 6.0	17	29	138
C57BL/6J	487.0 ± 2.8	72	24	108
C57BL/10J	451.7 ± 5.2	10	21	57
C57L/J	426.8 ± 5.3	7	17	37
C57BLKS/J	463.1 ± 4.2	22	22	80
C58/J	438.0 ± 4.9	10	18	43
DBA/1J	416.6 ± 4.6	10	19	70
DBA/2J	424.7 ± 3.7	35	23	149
FVB/NJ	479.9 ± 1.9	26	20	47
LG/J	435.5 ± 6.9	14	34	92
LP/J	402.7 ± 5.2	16	21	120
NOD/LtJ	477.7 ± 8.8	15	26	136
NZB/BinJ	491.4 ± 5.8	12	26	112
NZW/LacJ	457.5 ± 5.9	11	25	112
PL/J	464.5 ± 5.0	12	17	39
SJL/J	429.0 ± 5.2	16	17	46
SM/J	495.2 ± 2.8	27	18	107
SWR/J	417.1 ± 2.5	9	15	43

*Pooled together 12 CBA/CaJ and 5 CBA/J mice

Table 2.2. Brain weight for *Mus* species and subspecies corrected for sex, age and body weight.

Strain	Species	Brain wt. $\pm$ SE	<i>n</i>	Body wt.	Mean Age
CASA/Rk	<i>M. m. castaneus</i>	388.9 $\pm$ 7.9	6	13	46
CAST/Ei	<i>M. m. castaneus</i>	395.2 $\pm$ 4.6	23	15	252
CZECHII/Ei	<i>M. m. musculus</i>	367.1 $\pm$ 4.1	7	12	133
MOLD/Rk	<i>M. m. molossinus</i>	341.2 $\pm$ 9.6	2	13	51
MOLF/Ei	<i>M. m. molossinus</i>	302.7 $\pm$ 4.0	9	14	162
MOLC/Rk	<i>M. m. molossinus</i>	354.8 $\pm$ 9.5	5	14	166
WSB/Ei	<i>M. m. domesticus</i>	432.3 $\pm$ 6.5	8	16	163
PANCEVO/Ei	<i>M. spicilegus</i>	454.1 $\pm$ 4.1	6	14	191
SPRET/Ei	<i>M. spretus</i>	356.0 $\pm$ 8.9	5	16	143
CARL/ChGo*	<i>M. caroli</i>	450.9 $\pm$ 12.4	6	20	294

*outbred sample

The probability density distribution for 27 inbred strains has a platykurtic shape and kurtosis value of -1.0 , indicating that there are many extreme values spread out over a wide range (Fig. 2.2). A platykurtic distribution implies the presence of two or more normally distributed populations with different means. The distribution is also positively skewed ($+0.71$), indicating that a disproportionate number of strains with high brain weights. Using the Kolmogorov-Smirnov goodness of fit test the inbred strain distribution was found to be significantly different from a normal distribution ($p < 0.0001$).

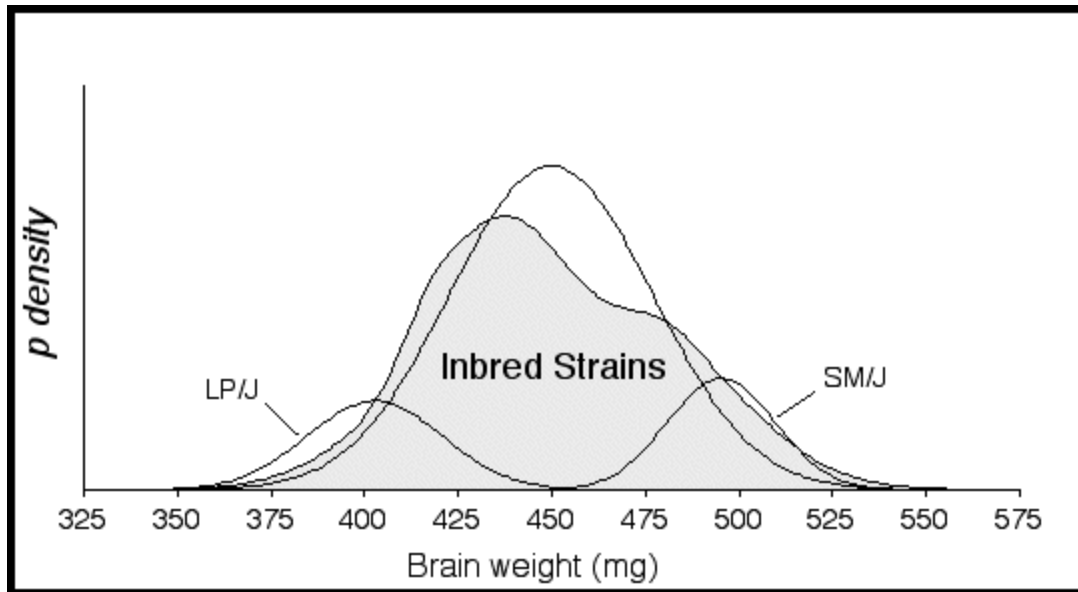


Figure 2.2. Probability density distribution of corrected brain weights for 27 inbred strains. The large shaded function represents a summation of individual probability densities for 27 inbred strains. The two small functions represent the strains LP/J with the lowest mean brain weight at 402.7 ± 18 mg and SM/J with the highest mean brain weight at 495 ± 14 mg. Brain weight means and SD were calculated from weights regressed with respect to sex, age, and body weight. The following probability density function was used to compute the probabilities:

$$P(x) = \frac{1}{n} \sum_{i=1}^n \frac{1}{s_i \sqrt{2\pi}} e^{-\left[\frac{(x - \bar{X})^2}{2s_i^2} \right]}$$

$P(x)$ is the normalized probability for a particular count x , the mean is a strain average and S_i is the SE of the mean .

In table 2.3, the column labeled d shows the differences between the F1 strain averages and their mid-parental values. The measure d estimates the net dominance effect of the genes on brain weight. The means of four F1 hybrids are close to their mid-parental values. The means of five F1 hybrids exhibit a slight to moderate dominance toward the high parental strain, while the brain weights of 32CASTF1 and CCASF1 exhibit overdominance. There was no significant difference in brain weight between the reciprocal F1 hybrids used to generate AC3HF2 and C3HAF2, although, there may be too few AC3HF1 cases for a reliable comparison. A significant difference was found between the reciprocal hybrids B6D2F1 and D2B6F1 (Difference (D) = 12.68 mg, $t = -3.196$, 26 df, $p = 0.0036$). The reciprocal B6D2F1 means are close to those

previously reported by Seyfried and Daniel (1977). The average coefficient of variation for the F1 hybrids is 4.2%. This is not substantially less than that of the inbred strains and suggests that for brain weight the heterozygous state is not a significant buffer against environmental effects.

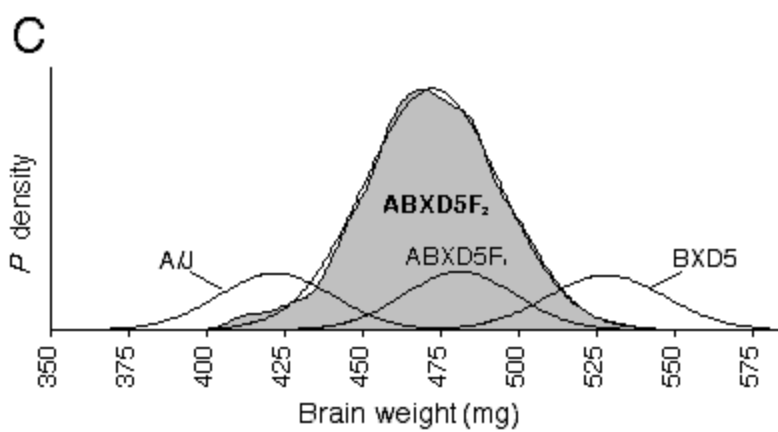
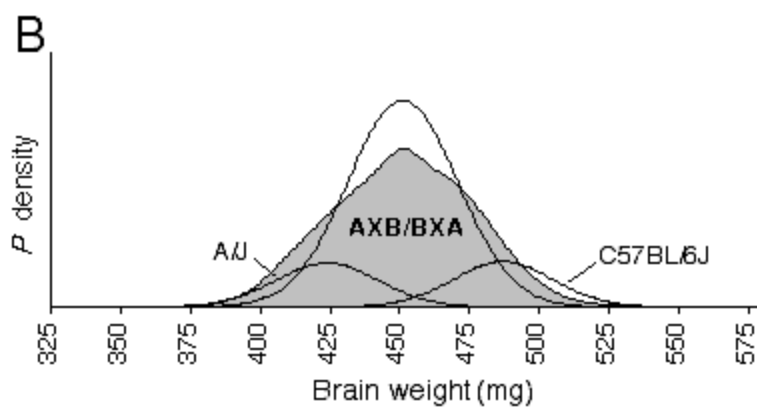
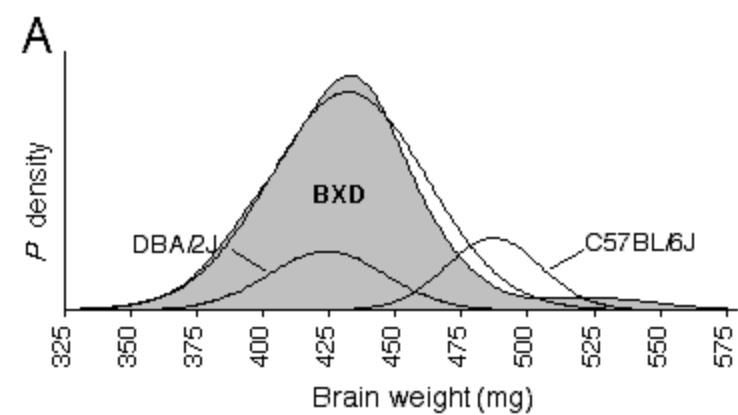
Table 2.3. Average brain weight for F1 hybrids and their parental strains

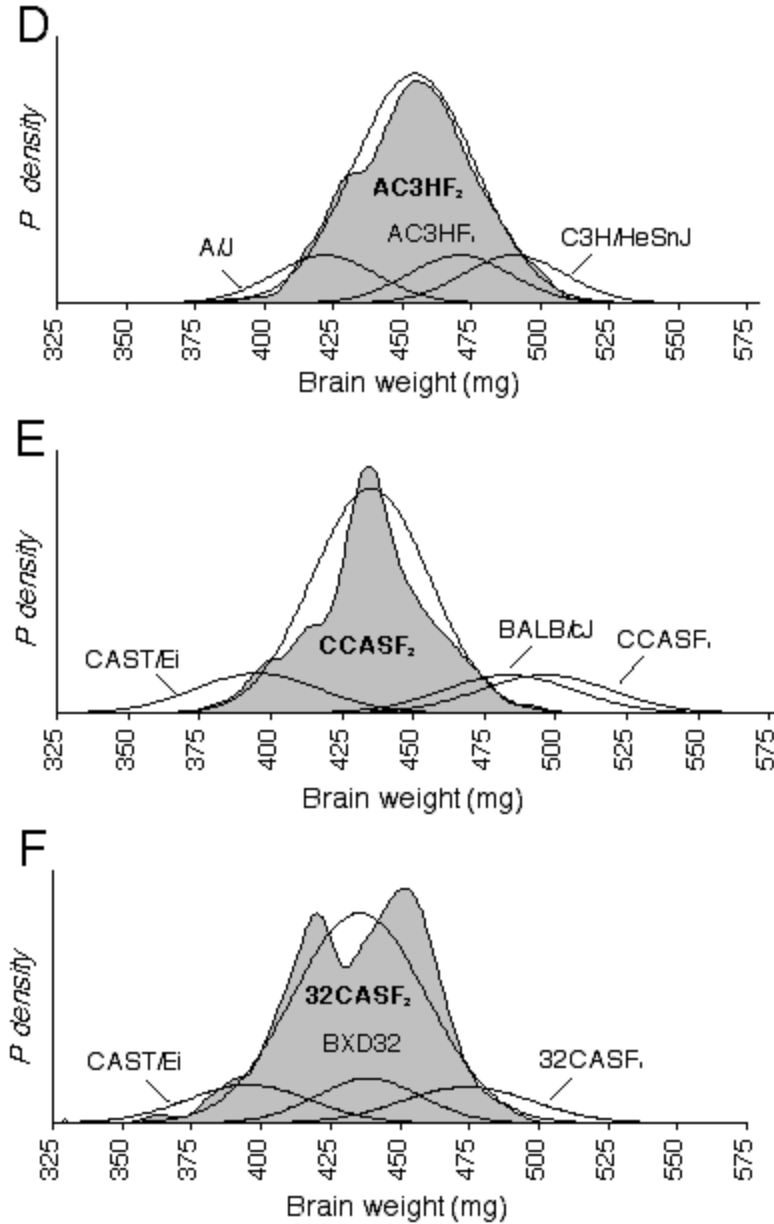
F ₁ Strains	Brain wt. ± SE	<i>n</i>	Maternal strain	Brain wt. ± SE	Paternal strain	Brain wt. ± SE	Mid	d
C3HAF ₁	470.6 ± 3.8	32	C3H/HeSnJ	490.3 ± 6.0	A/J	422.5 ± 3.9	456.6	14.6
AC3HF ₁	468.3 ± 6.6	7	A/J	422.5 ± 3.9	C3H/HeSnJ	490.3 ± 6.0	456.5	11.5
PLSJF ₁	448.0 ± 4.7	8	PL/J	464.5 ± 5.0	SJL/J	429.0 ± 5.2	446.5	1.5
B6D2F ₁	450.4 ± 4.7	18	C57BL/6J	487.0 ± 2.8	DBA/2J	424.7 ± 3.7	455.9	-5.5
D2B6F ₁	462.8 ± 5.8	18	DBA/2J	424.7 ± 3.7	C57BL/6J	487.0 ± 2.8	455.9	6.9
ABXD5F ₁ *	494.1 ± 4.1	25	A/J	422.5 ± 3.9	BXD5	519.0 ± 9.0	470.8	23.4
32CASTF ₁	474.1 ± 6.8	12	BXD32	433.0 ± 2.5	CAST/Ei	395.2 ± 4.6	414.1	60.0
BXD32X5F ₁	475.8 ± 3.3	4	BXD32	433.0 ± 2.5	BXD5	519.0 ± 9.0	476.0	-0.2
CAF ₁ /J	478.4 ± 5.7	8	BALB/cJ	483.5 ± 3.3	A/J	422.5 ± 3.9	453.0	25.4
CB6F ₁	503.2 ± 12.9	6	BALB/cJ	483.5 ± 3.3	C57BL/6J	487.0 ± 2.4	485.3	18.0
CCASF ₁ *	496.1 ± 3.2	16	BALB/cJ	483.5 ± 3.3	CAST/Ei	395.2 ± 4.6	439.4	56.8

*Corrected F₁ brain weights. Strains not corrected did not have significant effects from sex, age or body weight.

The CV for corrected brain weights is 6.8% between BXD strains and averages 3.8% within strains. The CV for corrected brain weights averages 4.9% in the intercrosses, and ranges from 5.5% in 32CASTF2, 4.6% in ABXD5F2, and 4.6 % in CCASTF2. The degree to which the brain weight CV were reduced by corrected weights varied from only 0.2% in AC3HF2/C3HAF2 to 3.2% in CCASF2. A significant difference in the variance between segregating and non-segregating generations is necessary to establish that genetic differences are responsible for brain weight variance. Using the variance ratio test, I examined whether brain weight variance in the recombinant crosses was significantly different from the pooled variance of their parents and F1 hybrid. The genetic variance in the ABXD5F2 cross is just barely significant at $p = 0.05(F_{0.05}(1), 516, 65 = 1.33)$, while the genetic variance in 32CASTF2 is highly significant ($F_{0.005}(1), 144, 69 = 1.75$). The highly significance variance in the 32CASTF2 is expected considering the genetic diversity between the parental strains.

In the AC3HF2 probability density plot, brain weight probabilities are slightly negatively skewed ([Fig. 2.3](#)). The probability density of CCASF2 is very narrow and indicates low brain weight variance within this cross. The probability density plots of AXB/BXA and 32CASTF2 appear platykurtic, where as, two modes are actually evident in the 32CASTF2 plot. Platykurtic distributions, especially those with two modes resolved, indicate that a small number of genes with large effects on brain weight are segregating within the cross. The location of the F1 function in the four intercrosses demonstrates partial or complete dominance, and, in the case of 32CASTF1 and CCASF1, overdominance.





Figures 2.3. shows probability density distributions of corrected brain weight for recombinant progeny and their parental strains. The variation in brain weight demonstrated in these graphs is independent of the covariance associated with body weight or other significant cofactor, such as age, sex, litter size, and parity. The large shaded function is a summation of individual normalized probability densities. Each individual function in an F₂ distribution is derived from the corrected brain weight of one mouse and a standard deviation of 5 mg based on the estimated measurement error. In the RI distributions, the individual functions are derived from a strain mean and its SD. The thin black line is the function of the expected Gaussian distribution based on the mean and SD of the recombinant progeny. The smaller probability functions describe the mean and SD of parental strains. The BXD distribution includes 26 RI strains and the mean is 432 ± 30 mg (A). The AXB/BXA distribution includes 14 AXB and 14

BXA RI strains and the mean is 451 ± 20 mg (B). The mean of the ABXD5F2 distribution is 475 ± 21 mg (C). The AC3HF2 distribution includes both AC3H and C3HA and the mean is 454 ± 22 mg (D). The mean and SD of the CCASF2 distribution is 439 ± 20 mg (E). The mean of the 32CASTF2 distribution is 435 ± 24 mg (F).

The average brain weights in the intercrosses should equal the mid-parental values if brain weight is only under additive gene effects ([Falconer and Mackay, 1996](#)). From the F1 values, I anticipated the means for the 32CASTF2 and CCASF2 crosses to be above the mid-parental value, indicating dominance. While the 32CASTF2 mean did indicate dominance ($d = 42$), the mean of the CCASF2 cross was not well matched with the mid-parental value. This latter result is difficult to explain but could result from epistatic interactions being broken up during the segregation of the F2.

Regression analyses

Using multiple regression, brain weight was regressed with respect to the cofactors, sex, age, body weight, and fixation quality across inbred strains. Sex, age, and body weight all have significant effects on brain weight and together explain 44% of the variance in brain weight ([Table 2.4](#)). Body weight alone had the largest effect on brain weight, explaining 36% of the variance. No significant sex difference was found in absolute brain weight across strains (females: 451 ± 48 , males: 453 ± 48). Male mice weigh on average 2.5 grams more than female mice, thus, when differences in body weight between the sexes are controlled for, females are significantly brainier, having on average an 11-mg heavier brain than males ([Table 2.4](#)). Brain weight is found to increase by almost 0.1 mg per day across strains and age differences can explain 5% of the variance in brain weight. However, when brain weight is controlled for with respect to body weight, brain weight relative to body weight decreases with age ([Table 2.4](#)). Fixation quality was assessed after dissection and the assigned score was used in a regression analysis to estimate the effects of fixation. The score was based on a five point scale: no fix = 0, poor fix (just post-fixed) = 1, marginal fix (just paraformaldehyde) = 2, adequate fix = 3, good fix = 4 and excellent fix = 5. Fixation quality had significant effects on brain weight. Brain weights were found to shrink by 2.4 mg for each increase in the fix quality rating.

Table 2.4. Inbred strain multiple regression analysis for brain weight.

Variable	Coefficient	SE	t-ratio
Age	-0.1584	0.022	-7.21**
Sex	11.7391	3.272	3.59*
Body wt	5.5182	0.326	16.90**
Fix	-2.4054	1.439	-1.67
$r^2 = 44\%$			

* $p = 0.0004$, ** $p < 0.0001$

A within strain regression analysis was performed in three strains (BALB/cJ, $n = 61$; C57BL/6J, $n = 67$; DBA/2J, $n = 35$). As expected, the effect of specific variables on brain weight differs between strains. In BALB/cJ and DBA/2J, body weight was an important variable, explaining 16.3% and 34.4%, respectively, while body weight was not a significant factor in C57BL/6J. There were no sex differences within any of these strains. While no age effects were found in DBA/2J and C57BL/6J, brain weight was found to increase by ~ 0.1 mg per day in BALB/cJ and explained 5% of the variance. Interestingly, no single factor had significant effects on brain weight in C57BL/6J. However, regressing sex, age, and body weight together explained 22% of the variance in brain weight in C57BL/6J, with body weight becoming significant only after being corrected with respect to sex and age.

A regression analysis in the inbred wild strains demonstrated an age-effect similar to that found in the standard inbred strains, ~ 0.1 mg per day. However, age explained almost 10% of the variance in brain weight in wild strains, while the effect of body weight on brain weight accounted for only 10% of variance in brain weight. The body weights of the wild inbred strains are 40% less than that of the standard inbred strains, 14.2 ± 3.5 gm compared to 23.3 ± 6.8 gm.

Among CCASF2 mice, variation in sex, age and body weight explains 52% of the variance in brain weight, with body weight accounting for the majority of the variance, 49.5% ([Table 2.5](#)). Absolute brain weight is significantly different between sexes ($D = 19.48$, $t = -2.89$, p

= 0.0047, $r^2 = 7.6\%$), however sex becomes an insignificant factor when body weight is controlled. Brain weight significantly increases with age in the CCASF2 cross, but after controlling for body weight, age becomes insignificant. Brain weight increased ~2 mg per day over the narrow age range between 30 to 55 days. In the CCASF2, variation in brain weight is significantly correlated with coat color. Coat colors were transformed into a numerical code, with the albino color given a 0, the light agouti a 0.5 and agouti a 1. The coat color coefficient of -12.41 in [Table 2.5](#) indicates that brain weight decreases by 12.41 mg going from the albino-0 to the agouti-1. Thus, albino strains have a brain weight that is on average 12.4 mg heavier than the pigmented strains. A positive albino effect is expected since the BALB/cJ strain, which carries the albino allele, has the heavier brain weight. Differences in coat color explain only 3.1% of the variance in brain weight. Nonetheless, this is an important finding because it indicates that a gene modulating brain weight maps close to the gene tyrosinase on chromosome 7.

Table 2.5. CCASF2 multiple regression analysis for brain weight.

Variable	Coefficient	SE	t-ratio
Age	0.62	0.48	1.30
Sex	12.04	4.67	2.58*
Body wt	8.78	0.88	9.91**
Coat color	-12.41	5.02	-2.47*
$r^2 = 53.9\%$			

* $p < 0.01$; ** $p < 0.0001$

Among 32CASF2 body weight is the only significant factor influencing brain weight, explaining 13.6% of the variance in brain weight ([Table 2.6](#)). Absolute brain weight is significantly different between sexes in 32CASF2 ($D = 10.53$ mg, $t = -2.45$, $p = 0.015$, $r^2 = 4.1\%$), however again, sex becomes an insignificant factor when body weight is controlled. There is no significant change in brain weight with age in this cross. A non-significant age coefficient does

not necessarily mean that age has no effect on brain weight, but could rather reflect the narrow age range within the cross. The age range of 32CASF2 is from 30 to 54 days with the mode at 39 days. Data from this narrow of an age range may be insufficient to detect a gradual change in brain weight with age.

Table 2.6. 32CASF2 multiple regression analysis for brain weight.

Variable	Coefficient	SE	t-ratio
Age	-0.18	0.41	-0.450
Sex	1.16	5.14	0.225
Body wt	3.94	1.09	3.600*
$r^2 = 14\%$			

* $p = 0.0004$

In ABXD5F₂ progeny sex, age, body weight, litter size, and parity all had significant effects on brain weight ([Table 2.7](#)). Body weight alone explains 32.2 % of the variance in brain weight ([Fig. 2.4](#)).

Table 2.7. ABXD5F₂ multiple regression analysis for brain weight.

Variable	Coefficient	SE	t-ratio
Age	0.69	0.28	2.50*
Sex	11.01	2.41	4.57**
Body wt	5.63	0.53	10.7**
Litter	-1.76	0.48	-3.64*
Parity	-1.16	0.52	-2.22*
$r^2 = 40\%$			

* $p < 0.05$, ** $p < 0.0001$

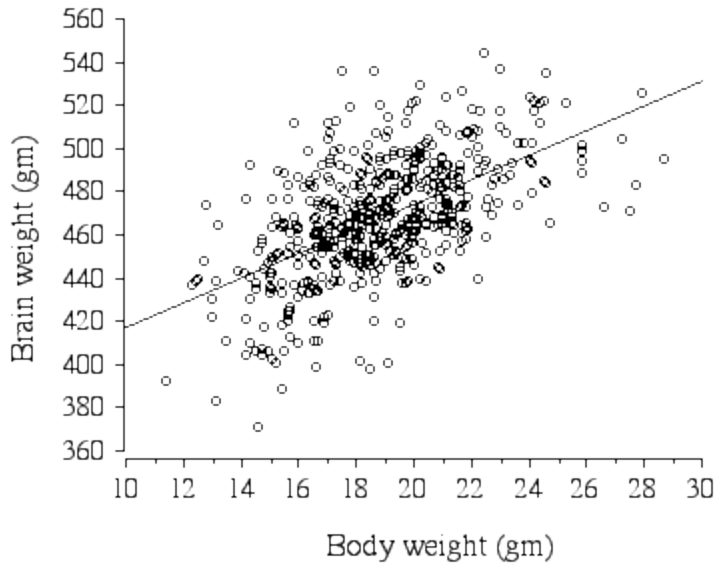


Figure 2.4. Regression of ABXD5F₂ brain weight and body weight.

There is a small significant difference in brain weight between the sexes ($D = 5.78$ mg, $t = -2.38$, $p = 0.01$, $r^2 = 1.1\%$). Most remarkably, brain weights of the ABXD5F₂ mice increase on average a whopping 2.4 mg per day, even within the narrow age range of 34–56 days, and explains 13.8% of the variance in brain weight ([Fig. 2.5](#)). When brain weight is regressed with multiple factors, age is still a positive factor, increasing brain weight on average by 0.69 mg a day! Note that the growth curve of ABXD5F₂ brain weight in [Fig. 2.5](#) appears to reach an asymptote around 48 days, suggesting that the preceding growth rate is an extension of the post-natal growth period.

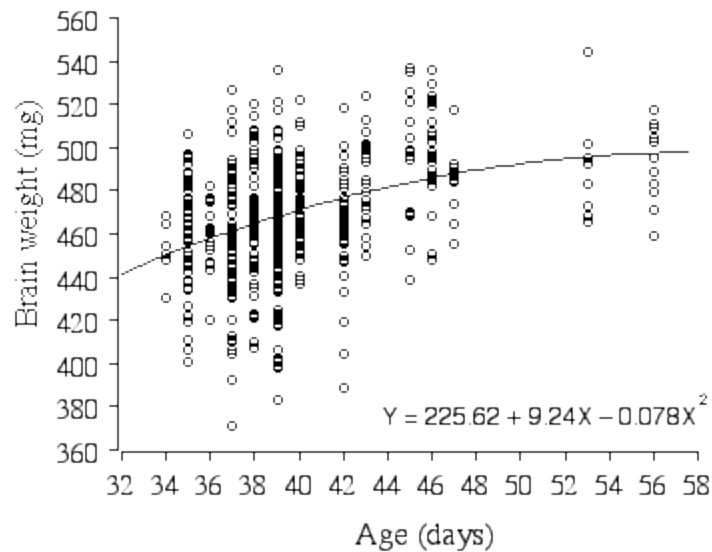


Figure 2.5. Polynomial regression of ABXD5F₂ brain weight and age.

Litter size in the ABXD5F₂ cross typically ranged between 5 to 15, with the average litter size being 10. Strikingly, the average brain weight of litters is stable over a 2-fold range in the number of pups per litter, 7 to 14 pups ($F = 0.95$, $df = 46$, $p = 0.47$). Only in the smallest (5 and 6 pups) and largest litters (15 pups) is brain weight affected. Parity, however, has a significant negative effect on brain weight, with brain weight decreasing by 2.4 mg with each successive litter ([Fig 2.6](#)). The parity effect persists even when the first and last litters were dropped from the analysis.

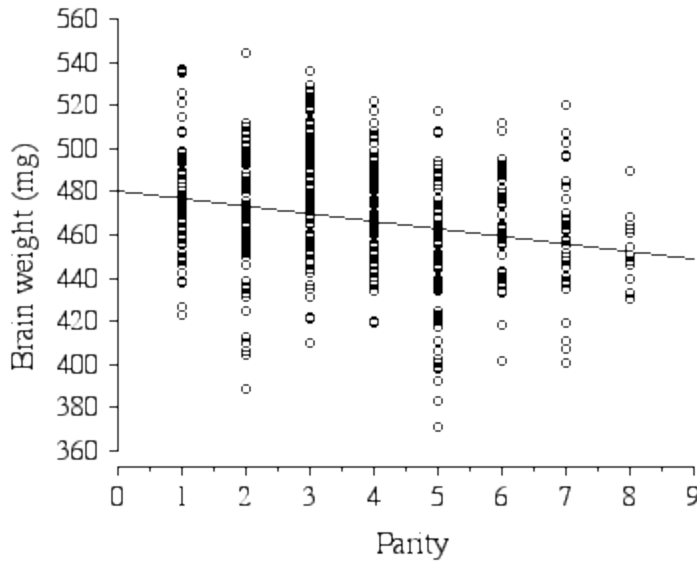


Figure 2.6. Regression of ABXD5F₂ brain weight and parity.

To determine whether genetically identical ABXD5F₁ mothers produce F₂ offspring with significant differences in brain weight, I performed an analysis of variance, with the single factor being the identity of the mother. Litter averages for each of the eight dams were computed from individual brain weights corrected for differences in sex, age, body weight, litter size, and parity. There were no significant differences in the litter averages between dams, ($F = 1.14$, $df = 8$, $p = 0.36$).

In AC3HF₂ mice, sex, body weight, litter size, and parity all have significant effects on brain weight ([Table 2.8](#)). In C3HAF₂ mice only body weight, sex and age significantly affect brain weight ([Table 2.9](#)). Body weight alone is responsible for 10% of the variance in brain weight in AC3HF₂, but is responsible for 27% of the variance in C3HAF₂. The polarity of the sex difference is different between AC3HF₂ ($D = 8.78$ mg, $t = 2.39$, $p = 0.01$, $r^2 = 4.2\%$) and C3HAF₂ ($D = 8.08$ mg, $t = -2.61$, $p = 0.01$, $r^2 = 3.0\%$). However, when body weight is controlled, the sex differences between crosses are closely matched, 22.5 mg and 19.8 mg. The reciprocal AC3HF₂/C3HAF₂ crosses are significantly different, with means and SD of 464.0 ± 21.4 mg and 458 ± 23.6 mg, respectively, ($t = -$

2.53, $n = 358$, $p = 0.01$). The regression coefficients for two maternal effect variables (litter size and parity) also differ between the reciprocal AC3H/C3HA F₂ crosses.

Table 2.8. AC3HF₂ multiple regression analysis for brain weight.

Variable	Coefficient	SE	t-ratio
Age	-1.05	0.61	-1.73
Sex	22.52	3.69	6.10***
Body wt	5.87	0.86	6.85***
Litter	-2.22	0.92	-2.41*
Parity	-3.45	1.30	-2.65**
$r^2 = 36\%$			

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$

Table 2.9. C3HAF₂ multiple regression analysis for brain weight.

Variable	Coefficient	SE	t-ratio
Age	-2.41	0.56	-4.34*
Sex	19.76	3.50	5.65*
Body wt	8.63	0.75	11.6*
Litter	-0.35	0.60	-0.59
Parity	-1.73	1.49	-1.16
$r^2 = 41\%$			

* $p < 0.0001$

In the BXD strains, body weight and age, are the only significant cofactors influencing brain weight ([Table 2.10](#)). Variation in body weight accounts for 17.4% of the variance in brain weight. The age of mice in the BXD strains ranged from 29 days to 444 days. Across all BXD strains, brain weight was found to increase by 0.16 mg per day, with variance in age explaining 2.5% of the variance in brain weight. The age-effect could be influenced by genetic differences, for example, if the individuals in the high

brain weight strains were older. To eliminate genetic differences, I computed z-scores for individual brain weights based on the mean and SD within the strain. The age-effect with z-scores was significant and in fact, the effect was increased to 0.23 mg per day. There are no significant differences in absolute brain weight between sexes.

Table 2.10. BXD multiple regression analysis for brain weight.

Variable	Coefficient	SE	t-ratio
Age	-0.18	0.05	-3.28*
Sex	10.44	3.40	3.07*
Body wt	4.43	0.44	9.97**
$r^2 = 20\%$			

* $p < 0.01$, ** $p < 0.0001$

In the AXB and BXA strains, body weight explains most of the variance in brain weight, accounting for 35% of the variance in the AXB group and 27% of the variance in the BXA group ([Table 2.11](#) and [Table 2.12](#)). There is no difference in absolute brain weight between sexes in AXB/BXA. Brain weight was found to increase significantly with age, equivalent to an increase of 0.28 mg per day. Since body weight also increases with age, controlling for body weight eliminates the age effect on brain weight. In the BXA strains, but not the AXB strains, there is a significant correlation between brain weight and coat color, ($p = 0.0004$). The coat coefficient of -15.85 indicates that albino strains have a brain weight that is on average 15.9 mg less than the pigmented strains. This effect is opposite to that found with the albino linkage in the CCASF₂, but then a negative effect transmitted from A/J, which carries the albino allele, is expected since it has the lower brain weight. Coat color explains only 5.2% of the variance in brain

weight. The reason a coat color correlation was not found in the AXB strains is because there are a disproportionate amount of pigmented strains in the set.

Table 2.11. AXB multiple regression analysis for brain weight.

Variable	Coefficient	SE	t-ratio
Age	-0.18	0.08	-2.37*
Sex	10.74	4.69	2.29*
Body wt	5.94	0.80	7.46**
Coat color	-1.25	2.61	-0.478
Fix	1.05	4.78	0.220
$r^2 = 36\%$			

* $p < 0.05$ ** $p < 0.0001$

Table 2.12. BXA multiple regression analysis for brain weight.

Variable	Coefficient	SE	t-ratio
Age	-0.01	0.06	-0.23
Sex	6.42	3.52	1.82
Body wt	3.20	0.49	6.48*
Coat color	-15.82	4.26	-3.71**
Fix	-8.29	3.35	-2.47*
$r^2 = 33\%$			

* $p < 0.01$, ** $p < 0.001$

Heritability and gene number

The heritability of brain weight within inbred strains is 0.58. When minimizing the effects of variation in sex, age, and body weight on brain weight by multiple regression heritability of brain weight in the inbred strains is reduced to 0.51. The

heritability of brain weight measured in the mapping crosses ranges from 0.15 to 0.39 ([Table 2.13](#)). The minimum number of genes modulating brain weight in these crosses ranges from one to six ([Table 2.13](#)).

Table 2.13. Heritability, brain and body weight correlation, and gene number for BXD, AXB/BXA, and F₂ intercross mice.

Strain	<i>n</i>	Heritability	Correlation Brain & Body	Gene Number
BXD*	26	0.45*	0.46	4.0
AXB, BXA*	24	0.15*	0.52	6.0
ABXD5 F ₂	522	0.24	0.58	3.0
CCAST F ₂	123	0.27	0.74	2.0
32CAST F ₂	144	0.32	0.36	1.0
AC3H F ₂ , C3HAF ₂	460	0.28	0.52	6.0

*Hegmann and Possidente method

Discussion

The significant difference in the variance of brain weight between and within inbred strains and the lower CV for brain weight within inbred strains (3.9%) compared to the outbred strain CARL/ChGo (6.8%) and F₂ crosses (4.9%), demonstrates that genetic variation contributes to variance in brain weight across strains. The CV in inbred strains would probably have been lower had there been more environmental consistencies. For example, the CV in the heterogeneous crosses are minimized by being sacrificed at nearly the same age, being reared in the same room, with many individuals born from the same parents and in some cases even coming from the same litter. In

contrast, the individuals making up an inbred strain often vary widely in age, with some bred at the University of Tennessee while others were bred at The Jackson Lab.

The probability density distributions demonstrate that brain weight is a complex trait influenced by multiple factors ([Figs. 2.2](#) and [2.3](#)). There are obvious differences in the genetic complexity between the crosses. The nearly bimodal distribution of the 32CASTF₂ cross indicates that one to two genes have major effects on brain weight. The broad platykurtic distribution of AXB/BXA also suggests the segregation of a few major genes is modulating brain weight. The distributions reveal the genetic complexity of brain weight variation and indicate that there are genes with major effects on brain weight segregating among the inbred strains!

Regression analyses

Sex and brain weight. There was no difference in absolute brain weight between sexes within the inbred strains and in the BXD and AXB/BXA strains. This finding was unexpected since Roderick and colleagues had reported that the brains of females were on average 10 mg heavier than males ([Roderick et al., 1973](#)). Their analysis was based on a sample of 250 mice belonging to 25 inbred strains—17 of which I have also examined. When I examined these same 17 strains, I failed to detect a sex difference. Roderick and colleagues used retired breeders—animals that were probably older than 200 days. This discrepancy could be explained if a sex difference developed with increasing age. Indeed, an examination of the age effect between sexes within inbred strains revealed that brain weight increases in females by 0.06 mg more per day than in males and explained twice as much of the variance! However, dividing the sexes into groups, younger than 100 days and older than 100 days, still did not result in a significance sex difference in absolute brain weight. The increased age-effect in females could be related to the effects of sex hormones. For example, estrogen has been found to play a protective role in neurodegenerative diseases ([Beyer, 1999](#)). Recently, a study

reported finding no difference in brain weight between the sexes in mouse, but did find a significant sex difference in rat (Bishop and Wahlsten, 1999). Incidentally, the mice in this study were young, 21-60 days, and the rats were older, 110 days. Brain weight in humans has been consistently reported to be higher in males compared to females (Pakkenberg and Gundersen, 1997; Peters, 1991). In conclusion, a difference in brain size between the sexes may not be a universal phenomenon in mammals and should be investigated further with age-related changes in mind.

There were absolute differences in brain weight between sexes in the crosses CCASF₂, 32CASTF₂, ABXD5F₂ and AC3HF₂/C3HAF₂. The sex differences in these crosses suggest sex-specific influence on brain weight. A sex difference could result from the higher dosage of the X chromosome from one parental strain versus the other in the F₂ females. The lower female brain weight, on average 5 to 10 mg less than males, is associated with a lower female body weight, on average 3 grams less than males. Thus, it is likely that the difference in brain weight results as pleiotropic effects from body weight factors during development. A body weight QTL accounting for 25% of the divergence between mouse lines has been mapped to chromosome X (Rance et al., 1997).

Age and brain weight. A second important finding was that brain weight continues to increase with age in some mice even after reaching sexual maturity. Adult brain weight in mouse has been reported to reach its adult size by 2 weeks of age (Hahn et al., 1983; Kobayashi, 1963). However, in ABXD5F₂ mice brain weight increased on average 2.4 mg per day between the ages of 34–56 days and in CCASF₂ mice brain weight increased 1.1 mg per day between the ages of 29–55 days. In BXD and inbred strains, brain weight had a more modest increase, averaging 0.16 mg and 0.10 mg per day, respectively. In contrast, brain weights of 32CASTF₂ and AC3HF₂/C3HAF₂ did not increase with age. Differences in the magnitude of the age effects between groups could be related to differences in the age range, e.g., the age range within inbred strains is

much broader than in the F₂ crosses. It should also be noted that the age effects within a narrow interval do not describe age effects at older ages. In the inbred strains, a large contribution of the age effect results from a high growth rate between 30 to 40 days. Dropping these cases reduces the explained variance by half. In contrast, brain weight in the BXD strains appears to occur linearly as a function of age. The differences between strains suggest that the mouse brain can have a persistent linear phase of postnatal growth that is genetically determined. In addition, a generality can not be made as to when the mouse brain stops growing, but as for body weight, brain growth is a continuum with inflection point that divides an exponential and linear phase.

Why does brain weight increase with age? The ventricular volume in human brains increases with age (Celik et al., 1995), and an increased volume of cerebrospinal fluid within enlarged ventricles might increase brain weight. However, when brains from SM/J mice were dehydrated and weighed, the dry brain weight increased with age (personal communication from Robert Williams). It is not known whether the increase in dry brain weight results from glia proliferation, increased myelin content, or changes in mean cell size. Although one study reports that brain weight and DNA content in mouse remain constant from age 60 to 470 days (Howard, 1973), there has been no report of increasing brain weight past maturity in mouse. Numerous studies in humans have reported a reduction in brain weight and cortical neuron number with age (Flood and Coleman, 1988; Pakkenberg and Gundersen, 1997). Much of the decrease in brain weight in humans is associated with a decrease in white matter. In conclusion, an increase in brain weight with age in mice suggests that a decrease in brain matter is not a necessary consequence of aging.

Body and brain weight. Variation in brain weight is associated with body weight among all animals studied. This correlation exists, both within strains, in which isogenic mice with heavier bodies tend to have heavier brains, and across strains, in which strains with heavier bodies tend to have heavier brains. In these mice body weight and

brain weight is also highly correlated within the sexes. The correlation within strains is due to factors that affect the body and brain, such as maternal effects and parity. Contrary to mouse, brain weight in humans is not correlated with body weight within the sexes (Pakkenberg and Gundersen, 1997).

In mouse, the correlation between brain and body originates during the embryonic and early postnatal period when the body and brain grows isometrically from a widespread circulation of growth-promoting factors (Riska and Atchley, 1985). For example, insulin growth factor (IGF)-I and -II are known to affect the growth of multiple organs including brain (D'ercole, 1993). Although IGF-I and -II are primarily synthesized within their target organs their expression is regulated by growth hormone, an endocrine hormone that is released from the pituitary and transported through the bloodstream. Thyroxine also circulates throughout the body during development and is known for its mitogenic effects in the brain and body (Morreale de Escobar et al., 1987).

Litter size, parity, and brain weight. Litter size had a significant negative effect on brain weight in ABXD5F₂ mice, explaining 9.4% of the variance in brain weight. However, when the extreme litter sizes were dropped, brain weight was stable across a two-fold range in litter size. In contrast, Wahlsten and Bulman-Fleming (1987) found litter size to have a highly negative *linear* effect on brain weight in study of 67 BALB/cJ litters ranging in size from 2 to 11 (Wahlsten and Bulman-Fleming, 1987). In another study, litter size explained 8% of brain weight variance found within inbred strains (Leamy, 1992). A study rearing rats in small and large litters found postnatal litter size to have a significant effect on brain weight (Sara et al., 1979). The effects of litter size could result from differences in postnatal nutrition as well as *in utero* nutrition. The stability of brain weight across litter sizes in the ABXD5F₂ could be explained by the strong reproductive performance typical of F₁ hybrids.

The number of successive litters, or parity, was found to have a negative effect on brain weight. This result is not attributed to the small litter sizes occurring with the first

and last parity, because when these litters were dropped parity still had a significant effect on brain weight. The effect of parity on brain weight could be related to the age of the mother, since parity and maternal age is highly correlated. It is conceivable that a decrease in the mother's serum levels of brain growth-promoting factors, such as IGF-I, which occurs with progressing age in mice and humans, has repercussions on the developing fetal brain (Xu et al., 1995) (Klein et al., 1996)

The regression coefficients and the amount of variance in brain weight explained by sex, age, body weight, litter size, and parity, differ between different groups of mice. Differences are even present between the reciprocal F₂s from AJ and C3H/HeSnJ matings. Although the F₁ hybrids have identical genomes, the X chromosome originates from two different strains in the reciprocal crosses—A/J and C3H/HeSnJ. Thus, differences between reciprocal crosses could result from sex-specific loci. In fact, there was a significant difference in brain weight between reciprocal F₂ females ($D = 13.9$, $t = -4.2$, $p < 0.0001$, $n = 193$). There was also a large difference in the brain weight variance between reciprocal F₂ females, (555 compared to 460 mg). However, a significant difference in brain weight was also found between reciprocal F₂ males, albeit much smaller, although variance was alike in reciprocal males. Differences between reciprocal F₁ males can also reveal the presence of a sex-linked trait. Unfortunately, there are too few reciprocal F₁ males to make a reliable comparison. Differences between reciprocal crosses could result from maternal effects, such as cytoplasmic differences or genomic imprinting.

In summary, the regression analyses demonstrate that body weight, age, sex, and parity, can explain a substantial portion of the variance in brain weight.

Genetic variation

The brain weights of F₁ hybrids can reveal the cumulative additive or dominant action of brain weight QTLs. Genes with additive effects will combine in the hybrid and

produce a mid-parental phenotype. This occurred in 4 of the 11 hybrids studied. When alleles are directionally dominant, the F₁ hybrids will resemble one parent more than the other. Positive dominance occurred in 5 of the 11 F₁ hybrids studied. When the phenotype of F₁ hybrids exceed the phenotype of either parental strain there is overdominance. Overdominance was exhibited by two hybrids, CCASF₁, and 32CASF₁. It is interesting that both of these hybrids include CAST/Ei as the parental. These two F₁ hybrids are from an inter-subspecific cross and demonstrate the effect of hybrid vigor or heterosis. Overdominance can occur when genes of the same polarity disperse among the parents and brought together in the F₁ hybrid. In the case of CB6F₁ note that both parents have high brain weights and that, the average brain weight for CB6F₁ is higher than either parentals. The overdominance in CB6F₁ could result if the parents had different genes with increaser alleles that behaved dominantly in the F₁. The association of dispersed brain weight QTLs probably causes the ambi-directional dominance in two BXD strains ([Table 3.1](#)). The strain BXD5 has an average brain weight of 527 mg, while BXD27 has an average brain weight of 382 mg, both of these strains are more extreme than their parental strains. Overdominance can also result from epistasis, when alleles from two genes interact to produce a larger effect than their individual effects combined. Evidence that epistasis is involved in brain weight variation among mice comes from a study which found the generation means and variances from C57BL/6J and DBA/2J reciprocal crosses (backcross and intercross) were not adequately explained by an additive-dominance model ([Seyfried and Daniel, 1977](#)). The over-dominance exhibited in the F₁ hybrids suggests that ancestors of these mice were subjected to selection pressures for high brain weight in the past and that brain weight must have been important for fitness at one time ([Crusio, 1992](#)).

Genetic factors account for as much as 39% of the variation in brain weight in BXD strains and 51% in the inbred strains. This result is not surprising, given the number of studies that have reported genetics to be the major determinant of brain size

(Bartley et al., 1997; Roderick et al., 1973; Wimer et al., 1969). Estimates of the number of genes producing variation in brain weight among the mice studied ranged from 1 to 6. Together, the high heritability of brain weight and low gene number indicate that mapping a major brain weight QTL is feasible. Indeed, the regression analysis with brain weight and coat color in the BXA strains and CCASF₂ mice have tentatively located a brain weight QTL to chromosome 7, near the gene tyrosinase!

Chapter 3: Mapping Genes Controlling Brain Weight Variation Using Recombinant Inbred strains and F₂ Intercross Progeny

Introduction

The high heritability of brain weight together with the estimate of a relatively small number of genes affecting this trait, indicate that it should be possible to map at least several responsible for variation in brain weight. In this study, I map quantitative trait loci that are responsible for variation in brain weight between mouse strains using the recombinant inbred (RI) strains, BXD and AXB/BXA, and an F₂ intercross.

Belknap and colleagues (1992) used the BXD RI strains to map QTLs responsible for brain weight differences between DBA/2J and C57BL/6J ([Belknap, 1992](#)). In the Belknap study, only 20 RI strains were used, whereas my BXD analysis includes 26 strains. Also, Belknap's study uses 360 markers, whereas I use a data set that includes 529 markers. The increase in both strain number and marker density greatly improves the power of detecting significant associations. In addition, this study combines two sets of RI strains and an F₂ intercross for the purpose of QTL confirmation.

The same genetic interval could be linked to variation in brain weight in more than one cross. Identifying the same QTLs is especially likely in this study since there are some common parental origins between the crosses, ABXD5F₂, AXB/BXA, and BXD. For example, ABXD5F₂ share the A/J parental with the AXB/BXA RI strains, DBA/2J is a parental for the BXD strains, including BXD5, which is a parental in ABXD5F₂. In addition, all of the crosses have C57BL/6J origins in common. The detection of the same linkage interval in multiple crosses will provide QTL verification. I also expect to identify unique QTLs within each cross since there will be different allele segregating between the crosses.

There are several advantages in using RI strains for QTL analysis. First, since the individuals of each RI strain are isogenic an infinite number of mice can be phenotyped to obtain a strain average. Obtaining a strain average serves to reduce the environmental and technical variance, that can obscure the association between a phenotype and a genotype. Second, with RI strains there is no genotyping necessary since there are hundreds of marker loci already typed for each RI strain. Another advantage with RI strains is that during the generation of each RI strain, there were multiple opportunities for recombination, resulting in a 4-fold increase in resolving power for determining linkage distance.

The F₂ intercross has the advantage of being able to detect genes with a lower effect size, which are not detected in an RI analysis due to the limited number of strains in a set (Johnson et al., 1992). However, detecting and positioning QTLs with reasonable accuracy requires genotyping and phenotyping a large number of intercross progeny. The number of progeny necessary to genotype can be reduced by first phenotyping and then selecting the animals from the phenotypic extremes for genotyping (Darvasi, 1997; Lander and Botstein, 1989). This technique is called selective genotyping and in addition to saving time, the progeny with phenotypic extremes will have parental genotypes, which are the most informative for linkage detection (Tanksley, 1993).

Materials and methods

Mapping progeny

The BXD set consisted of 26 strains that were generated from parental strains, DBA/2J and C57BL/6J, which have a 73 mg difference in brain weight. All the BXD mice were obtained directly from the Jackson Laboratory (Bar Harbor, ME). The AXB, BXA RI strains were produced from reciprocal crosses between the inbred strains A/J and C57BL/6J. The strains A/J and C57BL/6J have an 80 mg difference in brain weight. Brain

weights were obtained for 24 AXB/BXA strains. Mating pairs for each of the 24 AXB/BXA RI strains were obtained from the Jackson Laboratory and the majority of the mice were generated in the mouse colony at the University of Tennessee. The ABXD5F₂ progeny were generated from a cross between A/J and BXD5 ($n = 542$). The strains A/J and BXD5 have a 106 mg difference in brain weight. The BXD5 strain has one of the heaviest brain weights of all inbred strains—528 mg, while A/J has one of the smallest—422.5 mg.

Corrected brain weights

Fixed brain weights were obtained as described in [Chapter 2](#). The effects of the variables sex, age, body weight, litter size and parity on brain weight were determined by multiple regression as in [Chapter 2](#). Standardizing brain weights to the average body weight, age, litter size, parity, and female sex can minimize the effects of these variables on brain weight. Mapping with standardized or corrected brain weights, increases the probability of mapping only brain weight QTLs. The BXD brain weights were standardized to that of a 21.5 gm, 79-day-old female mouse (the body weight and age average across BXD strains), using the regression coefficients for body, age, and sex shown in [Table 2.10](#). Litter size and parity data were not available for the RI strains. The sex code for a female mouse is 1 and is 0 for a male mouse. For example, the equation to standardize brain weight would be:

$$\text{Corrected brain weight} = \text{fixed brain weight} + 4.43*(21.5 - 20) + 10.44*(1-0) + -0.18*(79 - 40).$$

In the equation, the coefficients for body weight, sex, and age, are italicized. Corrected brain weight averages for the 26 BXD strains can be found in [Table 3.1](#). Similarly, AXB/BXA brain weights were standardized using their respective coefficients, to a 19.5 gm, 78.8-day-old female. Corrected brain weight averages for the 24 AXB/BXD strains can be found in [Table 3.2](#). The ABXD5F₂ brain weights were

standardized to that of a 40-day-old, 19 gm, female mouse, coming from a litter size of 10, and born in a litter with a parity of three.

Table 3.1. Corrected brain weight and body weight data for BXD strains and parentals, C57BL/6J and DBA/2J.

Strain	Brain wt. (mg) $\pm$ SE	<i>n</i>	Body wt. (gm)	Mean age(days)
C57BL/6J	487.0 $\pm$ 2.8	72	24	108
DBA/2J	424.7 $\pm$ 3.7	35	23	149
BXD1	465.7 $\pm$ 3.7	16	21	71
BXD2	424.3 $\pm$ 4.0	17	23	62
BXD5	527.6 $\pm$ 5.5	20	22	123
BXD6	419.7 $\pm$ 5.9	12	22	193
BXD8	460.7 $\pm$ 5.9	29	23	170
BXD9	432.2 $\pm$ 3.3	21	24	82
BXD11	442.2 $\pm$ 4.4	10	21	66
BXD12	436.2 $\pm$ 5.6	14	23	116
BXD13	406.1 $\pm$ 3.4	21	22	57
BXD14	442.5 $\pm$ 3.3	14	24	85
BXD15	436.0 $\pm$ 3.6	10	26	129
BXD16	462.5 $\pm$ 6.4	11	21	61
BXD18	421.6 $\pm$ 2.4	15	23	73
BXD19	434.1 $\pm$ 4.0	22	19	88
BXD20	407.4 $\pm$ 3.2	20	19	74
BXD21	457.6 $\pm$ 5.8	10	25	114
BXD22	450.4 $\pm$ 3.3	16	23	84
BXD23	439.8 $\pm$ 3.5	16	20	83
BXD24	399.7 $\pm$ 4.7	16	24	189
BXD25	433.9 $\pm$ 2.6	19	18	77
BXD27	382.6 $\pm$ 4.2	32	21	99
BXD28	408.9 $\pm$ 2.6	33	22	68
BXD29	398.7 $\pm$ 5.6	18	20	73
BXD30	392.8 $\pm$ 3.4	18	18	80
BXD31	417.4 $\pm$ 5.9	11	23	75
BXD32	432.7 $\pm$ 3.0	55	26	129

Table 3.2. Corrected brain weight and body weight data for AXB/BXA strains and parentals, A/J and C57BL/6J.

Strains	Brain wt. (mg) $\pm$ SE	<i>n</i>	Body wt. (gm)	Mean age (days)
C57BL/6J	487.0 $\pm$ 2.8	72	24	108
A/J	422.5 $\pm$ 3.9	25	22	190
BXA1	450.3 $\pm$ 5.3	19	20	56
BXA2	455.2 $\pm$ 3.0	19	20	67
BXA4	417.4 $\pm$ 3.5	18	19	80
BXA7	457.9 $\pm$ 4.2	15	22	79
BXA8	436.5 $\pm$ 3.1	14	21	80
BXA11	465.2 $\pm$ 6.5	14	21	72
BXA12	464.1 $\pm$ 4.4	19	17	81
BXA13	463.3 $\pm$ 3.1	38	17	56
BXA14	414.7 $\pm$ 3.4	19	23	66
BXA16	437.7 $\pm$ 4.1	17	19	73
BXA17	462.8 $\pm$ 6.4	5	25	134
BXA24	436.5 $\pm$ 3.9	15	21	80
BXA25	474.7 $\pm$ 16.1	2	24	176
BXA26	430.7 $\pm$ 3.6	21	19	67
AXB1	428.4 $\pm$ 4.0	22	20	56
AXB2	475.9 $\pm$ 6.3	10	21	92
AXB4	450.9 $\pm$ 3.0	4	19	73
AXB5	460.7 $\pm$ 6.1	19	20	71
AXB6	467.9 $\pm$ 4.9	11	23	93
AXB8	441.8 $\pm$ 2.4	16	18	67
AXB10	475.0 $\pm$ 4.6	18	20	79
AXB12	414.1 $\pm$ 3.1	14	17	81
AXB13	480.9 $\pm$ 6.5	4	21	129
AXB15	430.1 $\pm$ 2.4	23	19	58
AXB18	468.4 $\pm$ 4.4	9	18	89
AXB19	466.6 $\pm$ 3.0	22	21	69
AXB20	463.2 $\pm$ 4.4	15	18	85
AXB24	440.4 $\pm$ 4.5	19	19	77

Genotyping

The BXD and AXB/BXA RI strains have already been genotyped at approximately 600 and 700 loci, respectively. (Marshall et al., 1992; Sampson et al., 1998). For the ABXD5F₂ progeny, I performed a genome-wide screen by typing 126

microsatellites dispersed at an average interval length of 30 cM across the entire genome, Chr Y excepted ([Fig. 3.1](#)). Genomic DNA was isolated from spleens of the intercross progeny using the simplified DNA isolation procedure described by [Laird et al. \(1991\)](#), with minor modifications. Details of the genotyping procedure are available on the WWW at (<http://nervenet.org/papers/PCR.html>). Microsatellites were typed using the polymerase chain reaction (PCR). The PCR protocol was set up as described by [Dietrich et al. \(1992\)](#). A PCR reaction contained (10 µl): 1X PCR buffer, 1.92 mM MgCl₂, 0.25 U/µl Taq polymerase (0.5 U), 0.2 mM dNTPs, 132 nM Mit map pairs and 50 ng genomic DNA. The map pairs were purchased from Research Genetics, Huntsville, AL. A loading dye (60% sucrose, 1.0 mM cresol red, 11% in-assay) was added to the reaction before the PCR. Polymerase chain reactions were carried out in 96-well microtiter plates, which allowed the use of a 12-channel multichannel pipettor to dispense reagents and load gels. I used the high stringency thermocycling protocol called "touchdown" described by [Don et al. \(1991\)](#). The protocol is 94° for 3 min., followed by 5 cycles of (94° for 30 sec, 60°C and then -2°C with each cycle, and 72° for 2 min). The protocol then continues with 30 cycles of 94° for 30 sec, 50° for 2 min. and 72° for 2 min., with a final cycle at 72° for 7 min.

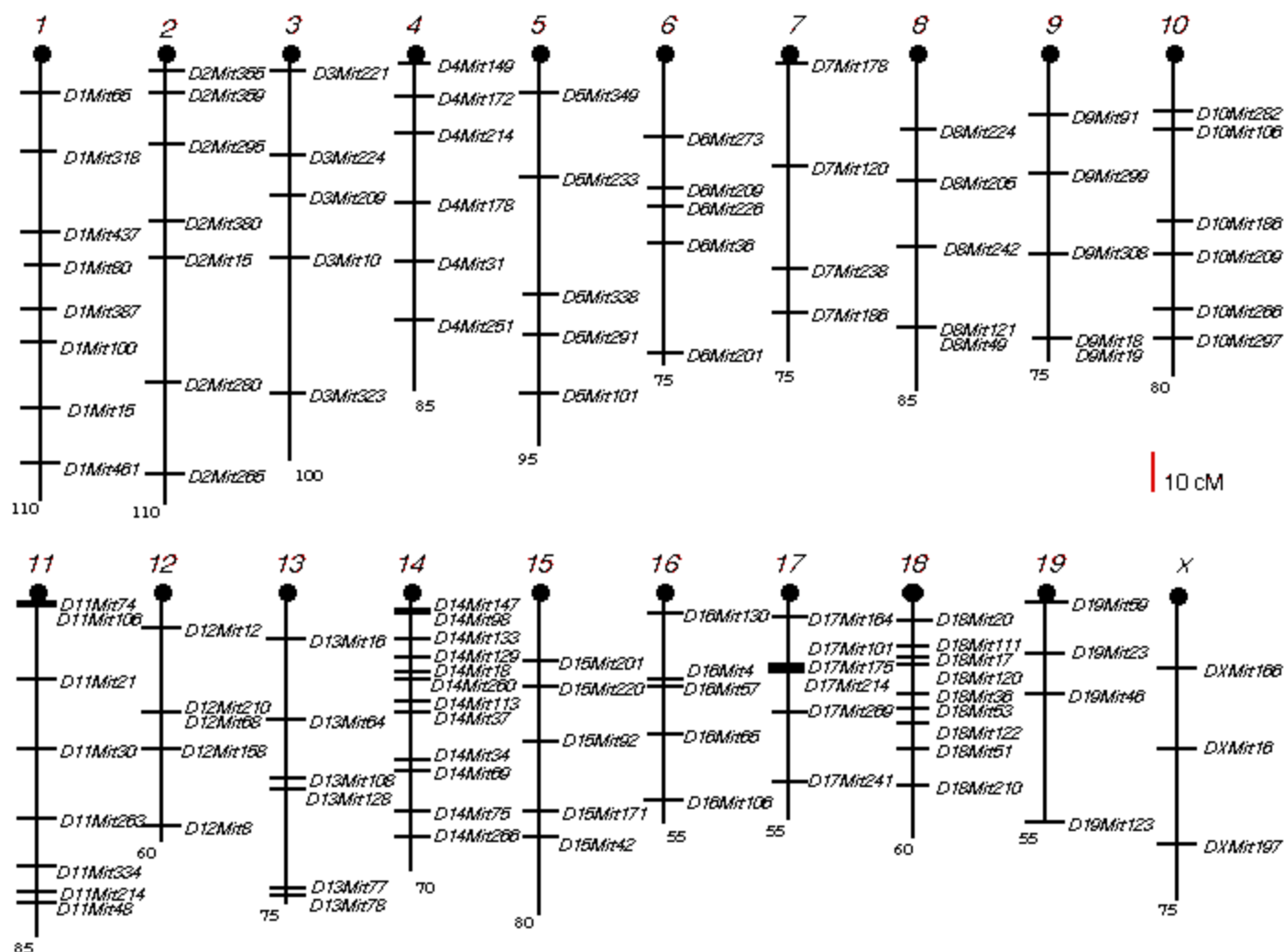
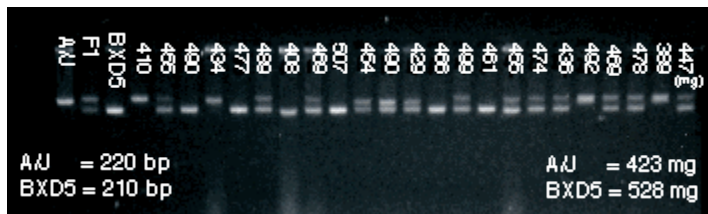


Figure 3.1. Microsatellite marker positions for ABXD5F₂ intercross screening.

The PCR products were separated by electrophoresis in 3% Metaphor agarose gels (FMC Bioproducts, Rockland ME.) in 1X TBE buffer. Gels were generally run for 90 min at 170 V, and maintained below 30 °C with cooled circulating buffer. PCR products were visualized by in-gel staining with ethidium bromide (Fig 3.2). Most loci could be scored as homozygous or heterozygous with complete confidence. To detect QTLs in the ABXD5F₂ progeny, I genotyped ~4% of with the lowest corrected brain weights and ~4% with the highest corrected brain weights (the lowest 22 cases and the highest 23 cases). These 45 extreme cases were chosen because with the addition of the two

parentals and F₁ DNA the number of DNA samples equaled 48, which used exactly half a microtiter plate, and allowed the testing of two markers per plate. To determine the effect size of a particular QTL, all the F₂ progeny were genotyped.



markers. The predicted additive or dominant deviation coefficients are computed across the interval and compared to the observed additive deviation coefficients and at each position a residual sum of squares (RSS) is calculated (Haley and Knott, 1992). A QTL effect and positional assignment can be seriously skewed by the presence secondary QTLs outside the QTL interval. To eliminate this interference I performed composite interval mapping, in which QTLs mapping outside the interval were fitted as cofactors in a multiple regression analysis (Jansen, 1993; Zeng, 1994). I used Map Manager QT to perform composite interval mapping.

Significance levels

In testing multiple markers in a genome-wide scan one will obtain significance associations just by chance. Therefore, it is necessary to determine the probability of a significant linkage in genome-wide test. The genome-wide probability thresholds can be determined with a permutation test. A permutation test shuffles the trait data, reassigning the data to new individuals and then compares this "null distribution" with the genotypic pattern (Churchill and Doerge, 1994). The trait data is then reshuffled from 1,000 to 10,000 times and after each permutation the single best linkage statistic is recorded. The genome-wide significance thresholds are computed from the incidence of test statistics in the complete permutation test. For example, if the highest linkage statistic was reached only once in 1000 permutations, then the probability of obtaining that high a linkage statistic by chance is 1 in 1000. The statistics obtained with the experimental data are compared to the distribution of permutation scores to determine their genome-wide probability. The false positive rates of 0.63, 0.05, and 0.001 correspond to a suggestive, significant, and highly significant linkage. The advantage of using permutation tests is that the threshold values are specifically tailored to the parameters of a given data set, e.g., chromosome number, genetic length, genetic marker density, and trait distribution. I used the program Map Manager QT to perform

the permutation tests ([Fig. 3.3](#)). I computed conditional empirical thresholds (CET) to control for the effects of secondary QTLs ([Doerge and Churchill, 1996](#)).

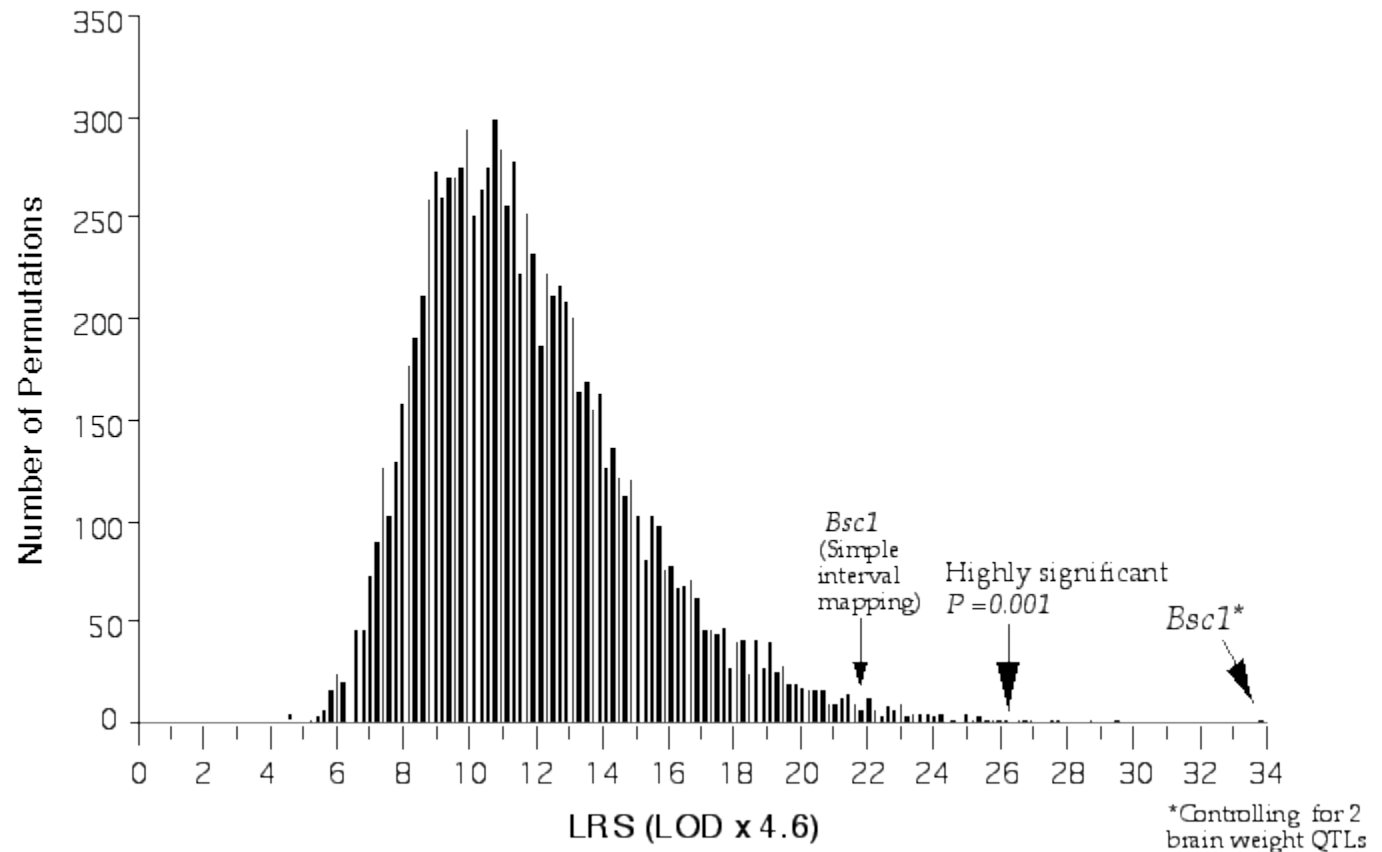


Figure 3.3. Permutation of BXD brain weight data for conditional genome-wide significance testing. The BXD data set was permuted 20,000 times at 1 cM intervals across the genome with conditional loci, *R1Rik100* and *Iapls2-13*. The suggestive LOD, ($p = 0.63$), is 1.2, the significant LOD, ($p = 0.05$), is 3.1 and the highly significant LOD, ($p = 0.001$), is 5.7.

Results

Recombinant inbreds

The best match with variation in brain weight was found at *D11Mit53*. The correlation coefficient between brain weight and alleles, assigned either a 1 and 0 at *D11Mit53*, is +0.90 ([Table 3.3](#)). The underlined alleles indicate discordance with the brain weight phenotype. In [Table 3.3](#), the allele assignments are based on the strain's

average brain weight resemblance to that of C57BL/6J (B) or DBA/2J (D). The U stands for unknown genotype and is assigned for phenotypes between that of the parentals. There are five strains that have intermediate genotypes. Refer back to [Fig. 2.3A](#) for the parental brain weight distribution.

A linkage analysis with corrected BXD brain weights resulted in a LOD score of 4.7 at *D11Ncvs74* at 0 cM on proximal Chr 11 and at 12 cM a LOD score of 4.5 at *D11Mit53*. Interval mapping on Chr 11 increases the LOD score to 5.2 between *Glns-ps1* and *D11Mit53*. *Glns-ps1* and *D11Mit53* map to 8.6 cM and 12.1 cM, respectively. The concordance of brain weight variation and alleles at *Glns-ps1* and *D11Mit53* is shown in [Table 3.3](#). To map secondary QTLs that may not be detected due to the interference of *D11Mit53*, I tested for linkage while controlling for the variance associated with *D11Mit53*. This resulted in detecting secondary QTLs on distal Chr 1 at *D1Rik100* with an LOD of 2.8 and on mid Chr X at *Iapls2-13* with LOD 2.8.

Table 3.3. BXD strain distribution pattern for brain weight and genotypes at five loci on Chr 11

Locus	cM	BXD strains																										
		1	2	5	6	8	9	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	3	3	3		
								1	2	3	4	5	6	8	9	0	1	2	3	4	5	7	8	9	0	1	2	
D11Mit2	5.4	B	B	B	D	B	D	B	B	D	D	B	B	D	B	D	B	B	D	D	B	D	D	D	B	D	D	
D11Ncvs73	6.4	B	B	B	D	B	D	B	B	D	D	D	B	D	B	D	B	B	D	D	B	D	D	D	B	D	D	
Glns-ps1	8.6	B	D	B	D	B	D	B	B	D	D	D	B	D	<u>B</u>	D	B	B	<u>D</u>	D	B	D	D	D	D	D	D	
Brain weight		B	D	B	D	B	D	U	U	D	U	U	B	D	D	D	B	B	B	D	U	D	D	D	D	D		
D11Mit53	12.1	B	D	B	D	B	<u>B</u>	B	B	D	D	D	B	D	D	D	B	B	B	D	B	D	D	D	D	D	D	
D11Mit51	15.5	D	D	B	D	B	B	B	B	D	D	D	B	D	B	D	B	B	B	B	B	D	D	D	D	D	D	

Composite interval mapping on Chr 11 with the two secondary loci as cofactors resulted in an LOD score of 5.1 at *D11Ncvs74* and a peak LOD score of 8.4 between *Glns-ps1* and *D11Mit53*, at 12 cM ([Fig. 3.4](#)). A 2 LOD confidence interval (CI) will have a 99% probability of containing the QTL. The 2 LOD CI for the QTL linked to *D11Mit53* is between 11–16 cM. Correcting for the variance of the secondary loci increases the ability to detect QTLs and to accurately map and estimate QTL effects. A permutation performed on this data set defined the conditional empirical threshold for a highly significant linkage to be 5.7 ([Fig. 3.3](#)). The brain weight QTL mapped to proximal Chr 11 at 12 cM, exceeds the highly significant threshold. Thus, there is less than 0.001 probability that the linkage is spurious in a genome-wide scan! This QTL has been named *brain size control 1* (*Bsc1*).

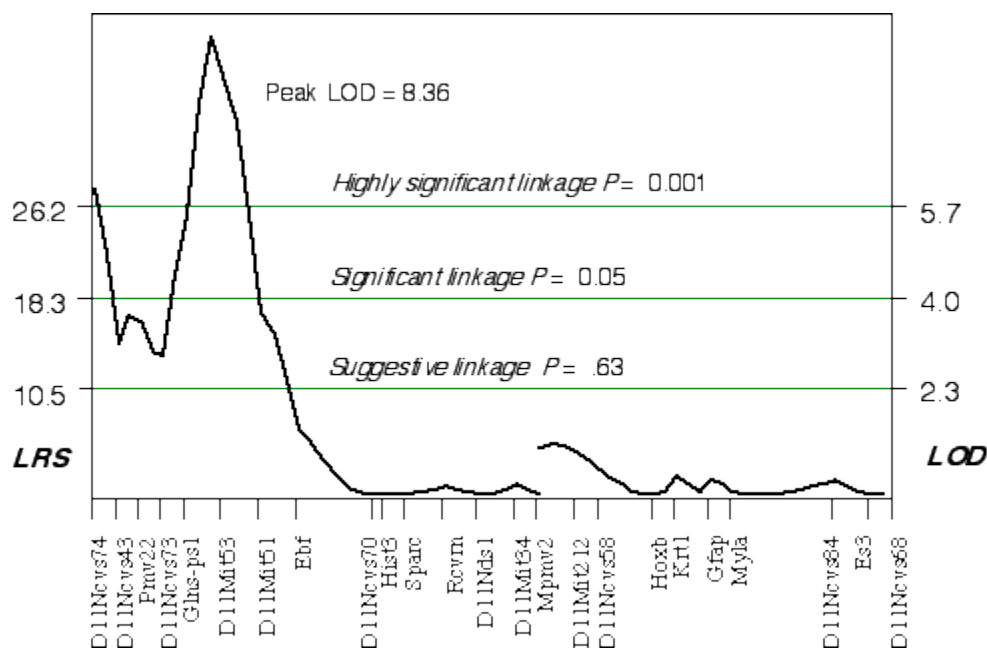


Figure 3.4. Linkage map demonstrates the QTL *Bsc1* on Chr 11 in the BXD data set. Linkage statistics were computed at 1 cM intervals while controlling for *D1Rik100* on distal Chr 1, and *Iapls2-13* in middle of the X Chr.

Linkage analysis in the AXB/BXA strains indicated that a gene modulating brain weight is close to a marker on distal Chr 7. Using simple linear regression, the peak LOD on distal Chr 7 at *D7Mit71* was 3.8. The correlation coefficient for alleles at *D7Mit71* and brain weight is +0.72. Controlling for this QTL interval on Chr 7 indicated a linkage on proximal Chr 14 (*D14Mit14* and *D14Mit48*), with an LOD of 3.0 using interval mapping. Composite interval mapping on Chr 7, while controlling for the proximal Chr 14 region increased the LOD score on distal Chr 7 at *D7Mit71*, to 6.7 (Fig. 3.5). Permuting the trait data 10,000 times with the conditional locus on Chr 14, resulted in only 1 in 1000 tests as significant as the strain distribution pattern at *D7Mit71*. The QTL mapped to proximal Chr 7 at 65.2 cM, with a 2 LOD CI between 64-72 cM, exceeds the highly significant threshold! This QTL is named *Brain size control 2*, (*Bsc2*).

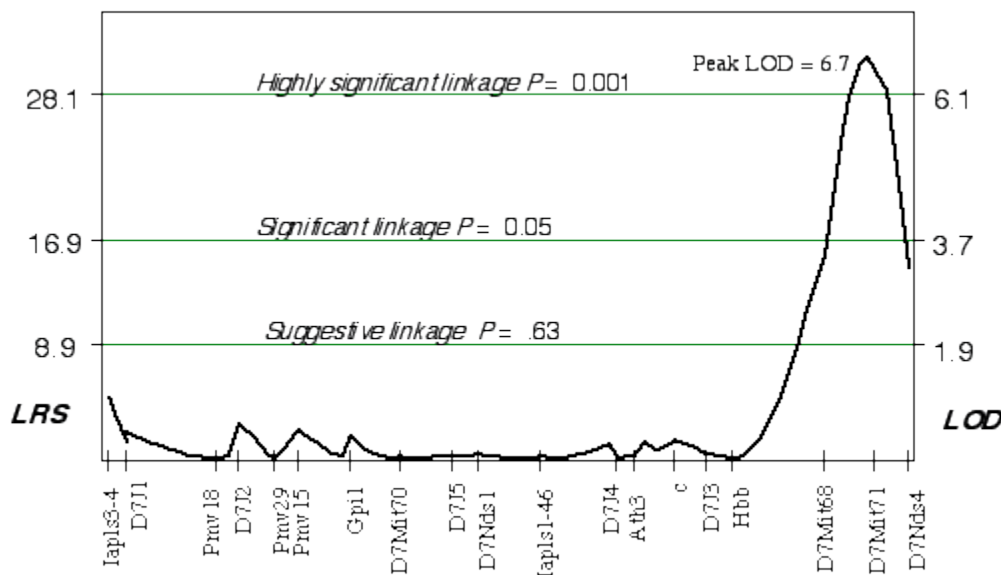


Figure 3.5. Linkage map demonstrates the QTL *Bsc2* on Chr 7 in the AXB/BXA data set. Linkage statistics were computed at 1 cM intervals while controlling for loci *D14Mit14* and *D14Mit48*.

ABXD5F₂ intercross progeny

An association was found between brain weight variation in the 45 extreme ABXD5F₂ progeny and their genotypes at two loci on Chr 14. Linkage testing resulted in LOD scores on Chrs 14 of 5.8 at *D14Mit260* and 5.4 at *D14Mit69*. Three secondary QTLs were identified, one on mid Chr 8 at *D8Mit242* with an LOD score of 4.3, one on distal Chr 5 at *D5Mit291* with an LOD score of 3.6, and one on proximal Chr 18 at *D18Mit36* with a LOD score of 3.0. Controlling for the Chr 14 interval increased only the LOD score at Chr 18 at *D18Mit36* to 3.7. A permutation test defined the LOD score required for a significant linkage to be 4.7. Thus, the linkage at *D18Mit36* is only suggested. Composite interval mapping while controlling for *D18Mit36* boosts the LOD score at *D14Mit260* and *D14Mit69* to 6.8 and 5.9, respectively ([Fig. 3.6](#)). The QTLs on Chr 14 map to 25 cM with a 2 LOD CI between 20–30 cM and to 59.1 cM, with a 2 LOD CI between 50–69 cM. Permuting the ABXD5F₂ trait data 5000 times with the conditional locus on Chr 18, resulted in only 0.7% and 1.6% of the tests with LOD scores as high as *D14Mit260* and *D14Mit69*, respectively. Thus, these two QTLs exceed the significant threshold, $p < 0.05$ and have been named *Brain size control 3* (*Bsc3*) and *Brain size control 4* (*Bsc4*).

In the AXB/BXA cross, a secondary QTL was detected on proximal Chr 14 between markers *D14Mit14* and *D14Mit48*, loci that map to 0 cM and 13 cM, respectively. The peak linkage statistic was at 9 cM. The Chr 14 committee report places *D14Mit48* a little more distal at 16.5 cM. The Chr 14 QTLs in the ABXD5F₂ cross, *D14Mit260* and *D14Mit69*, map to 25 cM and 59.1 cM, respectively. These are close to the positions reported by the Chr 14 committee, 29 cM and 43 cM, respectively. The secondary QTL identified in AXB/BXA strains is too proximal to be the *Bsc2* in ABXD5F₂. However, the secondary QTL in AXB/BXA does give credence to the small increase in the linkage statistic proximal to the *D14Mit260*, between markers *D14Mit98* and *D14Mit144* ([Fig. 3.6](#)).

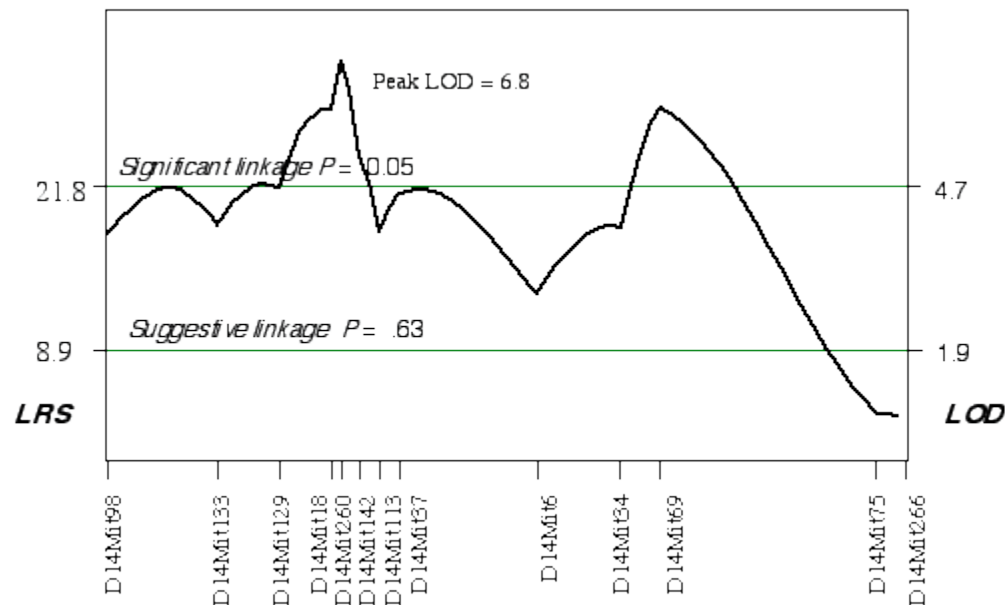


Figure 3.6. Linkage map demonstrates the QTLs *Bsc3* and *Bsc4* on Chr 14 in the ABXD5F₂ data set. Linkage statistics were computed at 1 cM intervals while controlling for the locus *D18Mit17*.

QTL effect size

To examine the effect size of QTLs on Chr 14 and Chr 18 in the ABXD5F₂ cross I performed a regression analysis on the brain weight z-scores for each genotype class. A single allelic substitution at the QTL linked to *D14Mit260* is responsible for an effect size of 0.3 SD units, with a 95% confidence interval equal to ± 0.21 . An analysis of variance determined that the regression was highly significant ($p < 0.0001$) and that variance at *D14Mit260* accounts for 5.9% of the variance in brain weight. The standard deviation of the corrected brain weights for ABXD5F₂ is 21.7. Thus, each allelic substitution at *Bsc3* translates into ~6.5 mg difference in brain weight. The effect of a single allelic substitution at the QTL near *D14Mit69* is also equal to $0.3 \text{ SD} \pm 0.23$, explains 5.1% of the variance, and each allelic substitution translates into ~ 6.5 mg difference in brain weight. The effect of a single allelic substitution at the QTL near *D18Mit20* is equal to $0.25 \text{ SD} \pm 0.23$, explains 3.5% of the variance, and produces a ~5.4 mg difference in brain weight.

The heritability estimate for ABXD5F₂, reported in [Chapter 2](#), is 24%. Adding the explained variance of the three QTLs equals 14.5%, therefore, together they explain 60% of the genetic variance and 15% of the total phenotypic variance in brain weight among ABXD5F₂ progeny.

Discussion

I have mapped four QTLs that have major effects on brain weight in mouse to Chrs 7, 11, 14. These brain weight QTL have been named *Bsc1*, 2, 3, and 4. *Bsc1* maps proximally to Chr 11 at 12 cM, *Bsc2* maps distally to Chr 7 at 65.2, and *Bsc3* and *Bsc4* map to Chr 14 at 25 cM and 59.1 cM, respectively. Secondary brain weight QTLs were mapped to Chrs 1, 5, 8, 11, 14, 18, and X.

In [Chapter 2](#), a correlation between albino coat color and brain weight in the AXB/BXA strains indicated that a QTL controlling brain weight mapped near the albino allele on distal Chr 7. The significant linkage between brain weight and *D7Mit71* on distal Chr 7 in the AXB/BXA cross confirms the linkage between coat color and brain weight.

A secondary QTL was mapped to the X Chr in the BXD set. I looked for evidence of a sex-difference in brain weight among reciprocal C57BL/6J and DBA/2J F₁ males, since they inherit their X Chr from different strains. There was no difference in brain weight between reciprocal hybrid males. However, this comparison was not paired; there were 6 B6D2F₁ males and 18 D2B6F₁ males. Regardless the similarity of reciprocal F₁ males does not preclude a sex association because detection could be confounded by autosomal variation if the effect size is too small.

A study by [Belknap et al. \(1992\)](#) mapped suggestive QTLs controlling brain weight in BXD strains to Chrs 2, 6, 7, 11, and 13. The locus Belknap identified on Chr 7 was proximal around 14 cM, while my AXB/BXA analysis detected a QTL on distal Chr

7. The loci identified on Chr 11 by Belknap are proximal, including *Pmv-22*, *Glns*, and *Hba*, which map between 8 cM and 16 cM. In my BXD mapping analysis, the best linkage to brain weight variance was between 11 cM and 16 cM, at *Glns-ps1* and *D11Mit53*, respectively, with the peak LOD score at 12 cM. Therefore, I have confirmed the Chr 11 QTL reported by Belknap et al. (1992). There are several explanations why I did not confirm more of the QTLs detected in the Belknap et al. (1992) study. First, the Belknap study used only 20 strains, whereas I use 26. Second, in the Belknap study the brain weights were regressed with respect to body weights by computing brain to body weight ratios, rather than a regression analysis. And third, the significance of Belknap's QTLs are only suggestive since they are nominal p values, whereas mine are genome-wide significant.

Effect size

Hundreds of genes are probably involved in controlling neuron number, however, only a dozen or so contribute to the variation in cell number. Many of these QTLs will have small effects on global neuron number while some will have large effects. It is the genes with large effects that will be detected in a linkage analysis, and thus, the four brain weight QTLs are expected to have relatively large effect size. The two QTLs mapped on Chr 14 in the ABXD5F₂ cross, account for 5.9% and 5.1% of the phenotypic variance in brain weight. In addition, the suggestive QTL mapped in the ABXD5F₂ cross on Chr 18, accounts for 3.5% of the phenotypic variance in brain weight. The explained variance estimates are not likely to be overestimates since the sample size of the ABXD5F₂ cross ($n = 522$) is large enough for reliable estimates of effect size. Thus, in the ABXD5F₂ cross, three major effect QTLs account for 60% of the genetic variance and contribute to ~15% of the phenotypic variance in brain weight.

Surprisingly, significant QTLs were not identified in multiple crosses. Thus, each cross has unique major effect brain weight QTLs segregating, despite some common

origins of the parental strains. In addition to the two major brain weight QTLs mapped to Chr 14 in the ABXD5F₂ cross, a secondary QTL on proximal Chr 14 was mapped in the AXB/BXA strains. The detection of three QTLs for brain weight on Chr 14 indicates that brain weight QTLs cluster on Chr 14.

Candidate genes

The complexity of a quantitative trait can limit the precision to which a QTL can be mapped, making the direct cloning of the QTL intractable. However, due to the large number of genes that are already cloned, in many cases, positional cloning will not be necessary. One can simply search for candidate genes that map within the QTL interval. Candidates would have a compatible function or expression pattern, or, when mutated, disturb the phenotype under study. For instance, the *tubby* gene was a candidate for a QTL controlling diet-induced obesity susceptibility in mice because a mutation in *tubby*, which mapped to the same region as the QTL, caused a severe mouse obesity phenotype (Kleyn et al., 1996). Most QTLs have been identified fortuitously in this way, whereby a gene is recognized by a major morphological mutation and genetic variants among strains are subsequently found.

A search for brain weight QTL candidates was performed using the Portable Dictionary of the Mouse Genome developed by Robert Williams and attainable through the web <https://genenetwork.org/>, and from the Mouse Genome Informatics (MGI) database, located at the Jackson Laboratory (<http://www.informatics.jax.org/>). The human genome was also inspected for candidate genes in mouse conserved-syteny regions using a homology search in the MGI database. QTL candidates were selected if they mapped within the QTL interval, are expressed in the brain, and are known to be involved in cell proliferation, or when absent or mutated disrupt brain development. Two candidates for the secondary QTL that mapped to the proximal end of Chr 11, between 0 and 1 cM, are the mouse homolog of the human gene neurofibromatosis type

2 (*NF2*) and the beta subunit of brain-specific Ca^{2+} /calmodulin-dependent protein kinase II (*Camk2b*). The human *NF2* gene encodes a tumor suppressor protein called schwannomin. A mutation in *NF2* causes an autosomal dominant disorder that increases the chance of developing benign tumors in the nervous system. The expression of *NF2* is found on embryonic days 15 and 16 in cells that are migrating from the ventricular zone to the cortical plate. *NF2* is likely to play a role in controlling cell proliferation, perhaps in onset of differentiation. The candidate *Camk2b* is first expressed in the brain on E12.5 and continues to be expressed in adult brain. The specific role that *Camk2b* performs in the brain is unknown.

A number of potential *Bsc1* candidates map within the 2 LOD CI between 11 cM and 16 cM on Chr 11. These include *RAB1*, a member of the RAS oncogene family, *Krct*, a member of a novel subfamily of serine/threonine kinases, *Otx1*, *creatine kinase brain, related sequence 5* (*Ckb-rs5*), *epidermal growth factor receptor* (*Egfr*), and *epidermal growth factor receptor-related sequence*, (*Egfr-rs*). All of these genes are expressed in the brain. *Otx1* is an attractive candidate because of its role in cortical neurogenesis. *Otx1* expression in mouse begins at E10 in the rostral brain and continues until late gestation. Mice homozygous null for *Otx1* have defective sense-organ development and neurogenesis (Suda et al., 1999). *Egfr* maps to 9.0 cM, a little too proximally for *Bsc2*, but was thought to be an attractive candidate because of EGF's effect on cell proliferation (see Introduction—*developmental control of neuron number*). However, *EGFR* has been dismissed as a candidate because the average brain weights from mice carrying a mutation in *EGFR* called waved-2 (*wv2*) are the same as wildtype brains (*wv2*— 488 ± 20.7 , $n=4$ and *wt*— 485 ± 9.0 , $n=12$). The mutant *wv2* has a single point mutation in *EGFR* that produces a 5–10 fold decrease in autophosphorylation and over a 90% reduction in substrate phosphorylation (Leutke et al., 1994).

The best *Bsc2* candidate is *Msx3*. The gene *Msx3* maps to 69 cM on Chr 7 within the 2 LOD CI for *Bsc2* and is expressed in the ventricular zone of the dorsal neural tube

during early development (Wang et al., 1996). The best *Bsc3* candidate is orthodenticle, *Otx2*. *Otx2* maps to 20.5 cM on Chr 14, is expressed in the developing central nervous system, and plays an important role in regional morphogenesis of the anterior brain structures (Suda et al., 1999). Unfortunately, I did not find any candidate genes for *Bsc4* on distal Chr 14.

This brain weight mapping study has identified the location of four brain weight QTLs. In addition a survey of candidate has suggested some exciting candidates for brain weight QTLs. In particular three homeobox containing genes, *Msx*, *Otx1* and *Otx2*. Homeobox genes have been suggested to function in some instances, as a regional proliferation index. Future studies could involve testing some of these candidate genes. However, narrowing the QTL interval will probably be necessary to determine the best candidate and proceed with cloning.

It will be exciting to discover the identity of the genetic variants and the mechanism by which they influence brain weight. In addition, the influence of these genetic variants in the variation of brain weight in other species can be tested to determine if there are common evolutionary mechanisms, as expected.

Chapter 4: Genetic and Environmental Control of Retinal Ganglion Cell Variation

Introduction

Evidence of global genetic control over cell number is found from the linear scaling of brain structures with brain size across species (Finlay and Darlington, 1995). There is also evidence of localized genetic control over cell number within particular brain structures. The genetic control within brain structures is necessary for the conservation of neuron number in the event of species-specific adaptations. Some examples of species-specific adaptations associated with neuron number are the capacity for bird song and foraging memory in birds, and the lateralization of language in humans. With each species adaptation, selective pressures for behavior must have acted upon the natural variation in the neuron population within the species. Examining the genetic bases of variation in discrete cell population within a species will shed light on the genetics of species-specific adaptations in neuron number. In this study, I examine the genetic and environmental control of variation in a single well-defined neuron population, the retinal ganglion cell population, among different inbred strains of mice.

Retinal ganglion cells are the sole projection neurons relaying visual information from the eye to the brain via their axons in the optic nerve. The morphological and physiological attributes of retinal ganglion cells have been described in many different species (Rodieck and Brening, 1983). The relative simplicity of the retinal circuitry and its accessibility make the retina an attractive model system for studying CNS development. Consequently, a lot is known about the development of the retina, and in particular ganglion cell differentiation. The differentiation of a large portion of ganglion cell has been shown to require a POU-domain transcription factor, Brn-3b (Gan, 1996).

Other factors that may be involved in ganglion cell differentiation are fibroblast growth factor (Guillemot and Cepko, 1992) and the transcription factor RPF-1 (Zhou et al., 1996).

Estimating numbers of distinct neuron populations can be tedious and error prone. However, retinal ganglion cell number can be estimated precisely and quickly by estimating the number of axons in the optic nerve. This is important because the precision of heritability estimates and gene mapping rely on the ability to obtain accurate phenotype data. In order to map genes that control ganglion cell number, specifically, the variance attributed to cofactors, such as brain weight, body weight, sex, and age, needs to be minimized. In this chapter, I estimate the variance in ganglion cell number associated with cofactors using linear regression. I also examine the precision with which ganglion cell numbers can be estimated. Finally, as a prelude to mapping QTLs responsible for variation in ganglion cell number between mouse strains I determine the heritability of ganglion cell number and the minimum number of effective genes.

Materials and methods

Mice

Optic nerves and brains were taken from mice, belonging to 18 standard inbred strains. All of these strains were obtained from the Jackson Laboratory (Bar Harbor, ME). Optic nerves and brains were taken from 26 BXD recombinant inbred strains and two groups of intercross progeny generated from crosses between BALB/cJ and CAST/Ei (CCASF₂) and between BXD32 and CAST/Ei (32CASF₂). I also obtained optic nerves and brains from five isogenic F₁ hybrids, (32CASTF₁, CCASF₁, CAF₁/J, B6AxCFA₁/J, PLSJF₁). The hybrids CAF₁/J and PLSJF₁ were obtained from the Jackson Laboratory and the remaining were generated in the University of Tennessee animal colony. Data for

the F₁ hybrids and their parents can be found in the [Table 4.3](#). In three of the five hybrids, the parents have large differences in ganglion cell number. For example, 32CASF₁ progeny were generated from parents BXD32 and CAST/Ei. The BXD32 strain has the highest ganglion cell number measured among the strains ($75,800 \pm 1,980$) and CAST/Ei has the lowest ganglion cell number ($45,000 \pm 1,000$).

Some of the strains studied carried mutations affecting the retina. Six strains carried a mutation in the β -phosphodiesterase gene (C3H/HeJ, C3H/HeSnJ, CD-1, PL/J, SJL/J, and MOLD/Rk). A mutation in the β -phosphodiesterase gene leads to retinal degeneration (*rd*). Retinal degeneration in the most extreme cases results in the complete loss of rod photoreceptors by 2 months of age. However, it is not evident that *rd* affects ganglion cell number, since all the strains carrying *rd*, with the exception of MOLD/Rk, have average ganglion numbers and one strain even has a high ganglion cell number. The following strains carry the albino allele of the gene tyrosinase (A/J, AKR/J, BALB/cJ, CD-1, 129/J, 129/SvJ, NZW/LacJ, SJL/J). Albino mice typically have a reduction in the proportion of retinal ganglion cells with uncrossed projections, but there is no association between the albino allele and adult ganglion cell number ([Rice et al., 1995](#)).

Tissue fixation

Optic nerves were dissected from mice transcardially perfused with EM grade fixative. The optic nerves were processed in 96 well microtiter plates. The procedure involved gradually dehydrating the nerves with increasing concentrations of alcohol, staining with 2% osmium, and infiltration with Spurr's resin. Embedded nerves were thin-sectioned, placed on formvar-coated grids, and stained with lead citrate and uranyl acetate.

Estimating ganglion cell number

Ganglion cell number was estimated by counting their axons within the optic nerve. Previous studies have demonstrated that axon counts are reliable estimates of ganglion cell number, (Chalupa et al., 1984; Lia et al., 1986; Perry et al., 1983; Rice et al., 1995; Williams et al., 1986), see table 2). Nerves were photographed in a grid pattern on a JEOL EX2000II electron microscope typically at a magnification of ~ x12,000. The nerve area was determined by photographing the entire nerve at low magnification, usually at 200x or 250x. Immediately after sampling the nerve at high magnification and photographing the entire nerve at low magnification, the high and low magnifications were calibrated by photographing a grid replica (2160 lines/mm, obtained from EMS, Fort Washington, PA). Axons were counted within a 63 x 86 mm counting frame drawn on the micrograph. To give unbiased estimates only those axons intersecting the top and right edge of the frame were included in the count. Total axon estimates were calculated by multiplying the mean axon density (per micrograph area) by the total optic nerve area.

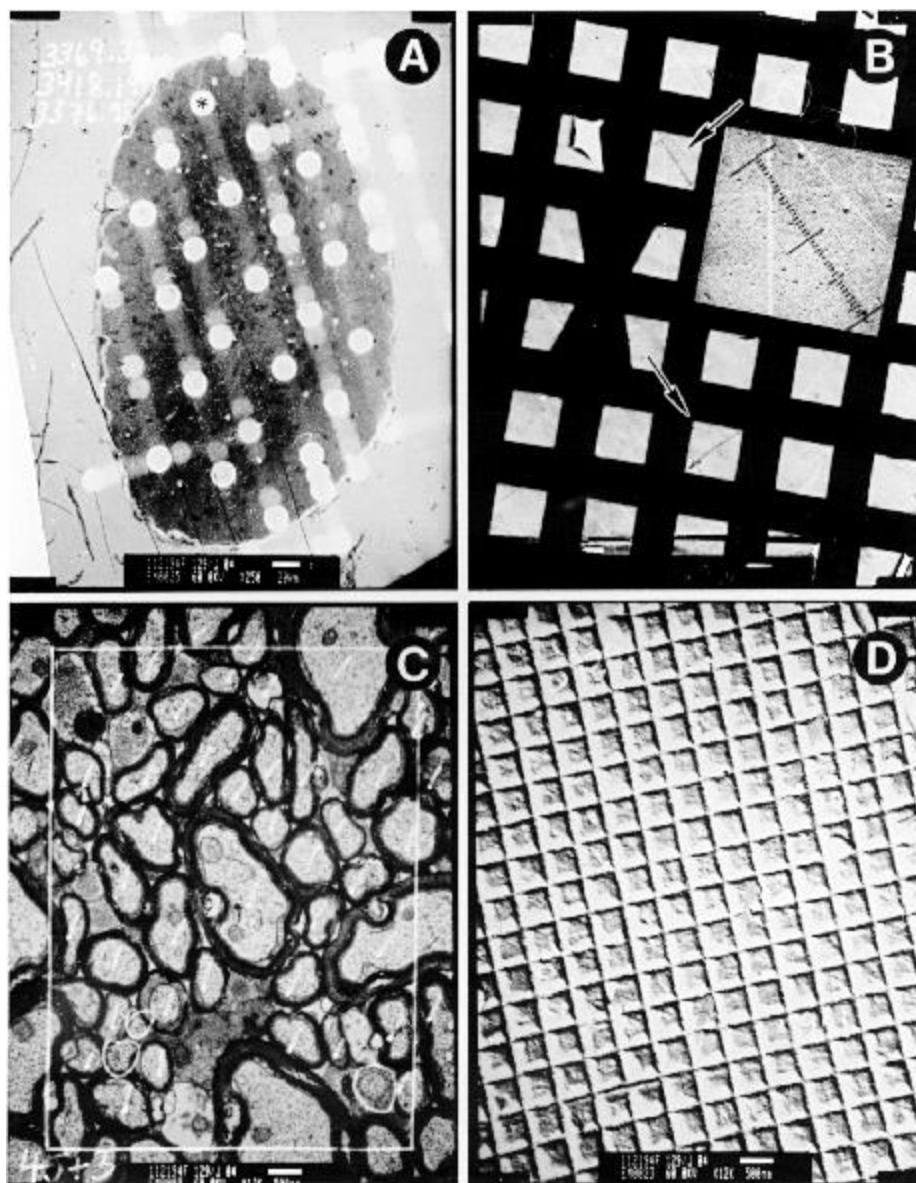


Figure 4.1. Method for estimating ganglion cell numbers. Ganglion cell number for strains, species, and subspecies of mice. The major subdivisions of mice are indicated in the left margin and are described in the Materials and Methods. Numbers for each strain denote average ganglion cell number, standard error of the mean (SEM) and the number of cases. These four prints were made from negatives used to estimate ganglion cell number. A and B are low-power electron micrographs of an optic nerve cross-section (A) and calibration grid (B). C and D high-power electron micrographs of an optic nerve sample site (C) and calibration grid (D). The white spots on the nerve in A are areas that were bleached by the electron beam during sampling at high-power. The asterisk in A marks the site that is shown at high magnification in C. The axon margin was outlined with a sharpie marker using the aid of a light microscope. The area of the nerve was calculated by tracing the outlined nerve on a digitizing tablet. The two

arrows in B mark the sites where delimits spanning 80 grid units was etched into the negative with a small scalpel and this distance measured under a dissecting microscope with a digital caliper. Axons were counted within a counting frame drawn in C. The axon count in this sample is 45 myelinated and 3 unmyelinated fibers (circled). The area of the counting frame was calibrated using calibration grating in D. The dimensions of the calibration grid units are 0.463×0.463 .

Heritability

The broad-sense heritability of ganglion cell number was calculated from the ratio of the genetic variance (V_G) over the total phenotypic variance (V_P) found in the outbred strains. V_G was calculated by subtracting the average variance in ganglion cell number, found within the two outbred strains (CD-1 and CARL/ChGo), by the average environmental variance found within the inbred strains. The environmental variance was obtained by averaging the variance within 18 inbred strains and 6 F_1 hybrids. The average variance served to reduce the effects of variance deviation resulting from unique genotype–environment interactions. A close approximation to heritability in the narrow-sense was made using the Hegmann and Possidente (1981) method. The number of effective loci influencing ganglion cell number in the F_2 intercross mice was calculated using the Castle-Wright equation.

Results

Variation among strains, species and subspecies

The ganglion cell number among standard inbred strains of mice range from 50,600 in A/J to 69,000 in BXD32 ([Table 4.1](#)). The average ganglion cell number for the 18 standard inbred strains is $59,300 \pm 1130$ ($n = 150$). The average coefficient of variation (CV) for inbred strains is 6.9%. The variance among strains is much greater than within strains ($F_{(16,119)} = 15.0$, $p < 0.0001$).

There are large differences in ganglion cell number between closely related inbred strains. For example, the strains 129/J and LP/J originated from a common ancestor in the mid-1920s, but their average ganglion cell population differs by ~17,000. A difference of 11,000 ganglion cells was discovered between shipments of C57BL/6J mice from the Jackson Laboratory. The different shipments of C57BL/6J originated from different breeding rooms at the Jackson Laboratory. No non-genetic factors were identified that could explain the differences in ganglion cell number of the mice residing in the two rooms. Thus, the difference in ganglion cell number is possibly due to a new genetic mutation arising in the separate breeding colony.

Table 4.1. Average ganglion cell number for 18 standard inbred strains

Strain	RGC	SE	SD	CV	<i>n</i>
129/J	63,772	±1,771	±4,339	6.8%	7
129/SvJ*	69,094	±5,391	±7,625	11.0%	3
A/J	50,615	±1,319	±3,490	6.9%	8
AKR/J	62,788	±935	±2,091	3.3%	6
BALB/cBy	55,859	±1,178	±3,331	6.0%	9
BALB/cJ	63,393	±2,290	±6,058	9.6%	8
C3H/HeJ	66,262	±3,857	±5,455	8.2%	3
C3H/HeSnJ	67,796	±1,448	±2,048	3.0%	3
C57BL/6JAx1	66,082	±1,656	±4,684	7.1%	9
C57BL/6J*	55,413	±814	±4,071	7.3%	26
C57BLKS/J	65,667	±1,886	±4,217	6.4%	6
C57L/J	53,174	±2,654	±5,935	11.1%	6
CBA/CaJ	56,028	±1,203	±2,691	4.8%	6
CE/J	63,593	±2,536	±5,072	8.0%	5
DBA/2J	63,351	±1,208	±4,186	6.6%	13
LP/J	52,225	±1,989	±5,262	10.1%	8
NZB/BinJ	61,063	±1,600	±3,579	5.9%	6
NZW/LacJ	63,711	±727	±1,259	2.0%	4
PL/J	55,976	±1,309	±3,462	6.2%	8
SJL/J	52,473	±1,770	±3,958	7.5%	6
Average	60,417	±1,877	±4,141	6.9%	7.5

*Pooled data from pigmented and coisogenic albino mice. The statistics SD, SE, and CV were corrected for bias because of small sample size (Sokal and Rohlf, 1981). Data for the strain 129/SvJ is new and is not published in Williams et al. (1996).

Ganglion cell number among species and subspecies ranges from 45,000 to 64,300. The average CV for these strains is 7.4% ([Table 4.2](#)). Note that the strain CARL/RpGo is outbred and as expected has a higher degree of variation, CV = 14%.

Table 4.2. Average ganglion cell number for inbred representatives of wild strains

Strain	Species	RGC	SEM	SD	CV	<i>n</i>
CASA/Rk	<i>M. m. castaneus</i>	47,205	±1,380	±2,390	5.1%	4
CAST/Ei	<i>M. m. castaneus</i>	45,047	±1,040	±3,451	7.7%	12
CZECHII/Ei	<i>M. m. musculus</i>	59,207	±3,482	±8,530	14.4%	7
MOLD/Rk*	<i>M. m. molossinus</i>	43,758	±4,476	±8,952	20.5%	5
WSB/Ei	<i>M. m. domesticus</i>	57,380	±1,759	±4,655	8.1%	8
PANCEVO/Ei	<i>M. spicilegus</i>	64,300	±1,383	±3,093	4.8%	6
SPRET/Ei	<i>M. spretus</i>	59,049	±1,896	±4,240	7.2%	6
CARL/ChGo**	<i>M. caroli</i>	51,263	±2,270	±7,177	14.0%	11
Average		53,401	±2,211	±5,311	10.2%	7.4

*MOLD/Rk carries the *rd* allele and there is a high incidence of necrotic axons in the optic nerve of older mice. Estimates from the two youngest MOLD/Rk cases (49,200 and 47,200) are probably better representatives. **CARL/ChGo is an outcrossed strain.

The ganglion cell probability distribution of the 57 inbred strains, including 19 inbred laboratory strains and 38 recombinant inbred strains ([Fig 4.2](#)). The probability distribution has three modes at 55,500, 63,500, and 75,500 and indicates that there are three distinct phenotypes. The third mode consists of the strains BXD5 and BXD32, which have exceptionally high ganglion cell numbers that are 20% above their high parental strain—DBA/2J. A Kolmogorov–Smirnov goodness-of-fit test confirms that the inbred strain distribution is not normally distributed ($D = 0.245$, $p < 0.01$)

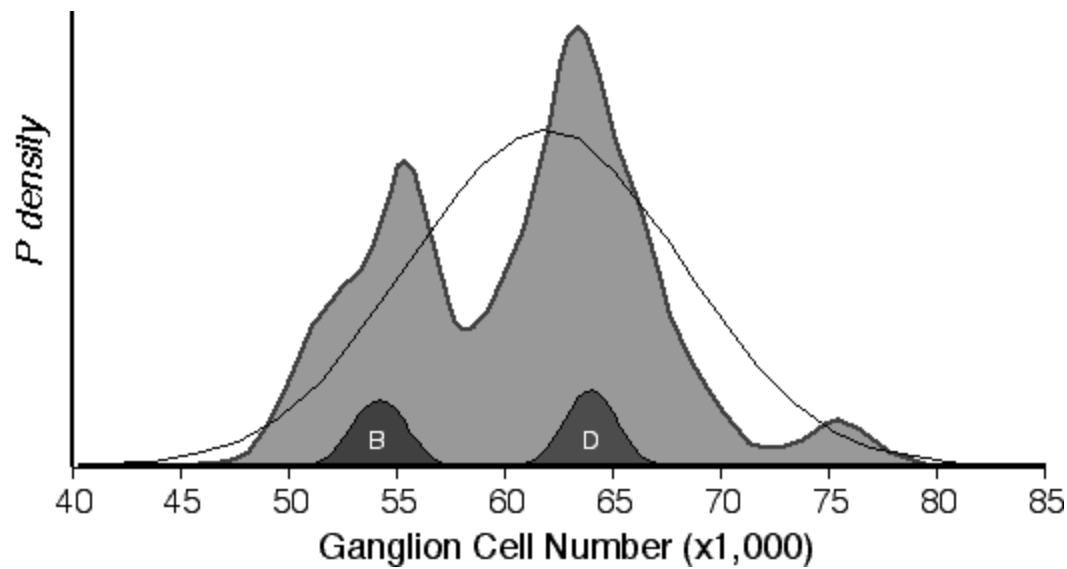


Figure 4.2. Probability density of retinal ganglion cell number for 57 inbred strains. The large filled-in function is a summation of individual normalized probability densities of the ganglion cell averages from 57 strains. The two small filled-in functions labeled B and D are examples of the individual probability densities computed from C57BL/6J (B) and DBA/2J (D) strain averages. The black line is the expected Gaussian distribution based on the mean of all strains ($61,730 \pm 6,310$).

The variance in ganglion cell number within the heterogeneous crosses is higher than the variance within the inbred strains ([Table 4.3](#)). The average of corrected ganglion cell number in the CCAS F₂ progeny is $56,000 \pm 6000$ (SD). The distribution of raw ganglion cell numbers in the CCASF₂ is broad, ranging from a low of 42,000 to a high of 70,000 ([Fig 4.3](#)). The broad range in ganglion cell numbers indicates that multiple genetic factors have segregated that can modify ganglion cell number. The number of genes modulating ganglion cell number was estimated to be one in CCASF₂ cross and three in the 32CASF₂ cross.

Table 4.3 Average ganglion cell population in heterogeneous mice.

Strain	RGC	SEM	SD	CV	<i>n</i>
CARL/RpGo	51,263	±2,270	±7,177	14.0%	11
CCASF ₂	56,501	±679	±7,156	12.7%	112
32CASF ₂	62,020	±819	±9,656	15.6%	140
*CD1/Go	68,338	±2,183	±7,869	11.5%	14
Average	58,701	±1,710	±7,401	12.7%	

*CD1/Go is an outbred laboratory strain.

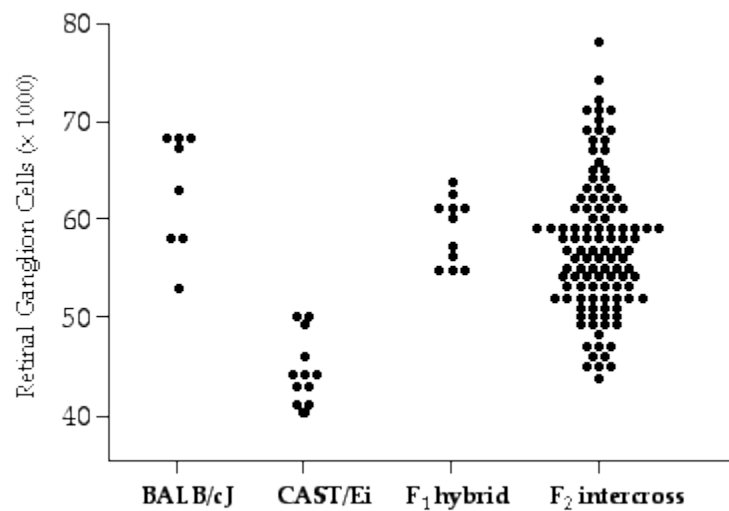


Figure 4.3. Scatterplot of retinal ganglion cell number for CCASF₂, CCASF₁, and parental strains.

Technical variation

Reliability was estimated by sampling a set of 69 nerves two to four times. Usually an adjacent nerve section was photographed and this was done months to several years after the first count. The average of the first count was $58,967 \pm 1145$, whereas that of the second count was $58,667 \pm 1,361$. The test-retest correlation coefficient was 0.83. The mean difference between pairs of estimates was 4,080, and the average SD of sets of replicates was 3,116. The technical coefficient of variation between repeated samples of ~25 micrographs averaged $6.3 \pm 0.5\%$ SE. I calculated the sampling variance across repeated samples and estimated that 60–70% of the technical variance is

associated with sampling error. Variance in calibration errors and axon discrimination can account for the remaining variance.

The coefficient of variation for the standard inbred strain averaged 6.9%. Thus, after taking into consideration the technical coefficient of variation the corrected environmental coefficient of variation is 2.8%.

Correlation between neuron number, ages, sex, body weight, and brain weight

The effects of age, sex, body weight and brain weight on ganglion cell number within-strains were examined by calculating within strain z-scores for retinal ganglion cell number and comparing this value to age, sex, body and brain across strains. The correlation between age, sex, body weight, brain weight and within-strain ganglion cell number within the 18 standard inbred strains were approximately zero, ($n = 124$). In these strains, the sex ratio was 50:50 and ages ranged from 30 to 200 days. The correlation between brain weight and ganglion cell number across strains is +0.38, which is highly significant, ($t = 6.54$, $n = 184$, $p < 0.001$). The correlation between brain weight strain averages and ganglion cell strain averages is +0.46, which is also significant at $p < 0.02$. The corresponding coefficient of determination (r^2) is 21.5 %. The correlation between ganglion cell numbers and brain weights regressed with respect to sex, age, and body weight is roughly the same, $r = 0.42$.

Within BXD recombinant strains, ganglion cell number was not correlated with brain weight, body weight, sex, or age. However, across strains variation in ganglion cell number was significantly correlated with brain weight, $r = 0.42$. The correlation coefficient was reduced to 0.36 when brain weight was corrected for variation in sex, age, and body weight.

In the CCASF₂ cross variation in ganglion cell number was significantly correlated with brain weight explaining 26.8% of the variation in ganglion cell number ($p = 0.0001$). Body weight was also significantly correlated with ganglion cell number ($r^2 = 11.5\%$, $n = 92$, $p = 0.001$), a product of the high correlation between body and brain in

CCASF₂. However, adding brain weight to the regression with body weight abolishes any correlation with body weight. There was no correlation between ganglion cell number and sex, age or parity in the CCASF₂ cross. To reduce the effect of brain weight variation on ganglion cell number, the ganglion cell numbers of CCASF₂ mice were standardized to that of a 437 mg brain, the average brain weight of the F₂ progeny. Correcting for differences in brain weight resulted in a 25% reduction in variance of ganglion cell number among CCASF₂ progeny. Variation in ganglion cell number was also significantly associated with brain weight in the 32CASTF₂ cross, but explained only 15.9% of the variation ($p < 0.0001$). There was also no correlation between ganglion cell number and sex, age or parity in the 32CASF₂ cross.

Heritability and gene dominance

Broad-sense heritability calculated in outbred strains CD-1 and CARL/ChGo is 0.73 and 0.68, respectively. Narrow-sense heritability of retinal ganglion cell number in the standard inbred mouse strains is 0.48. Narrow-sense heritability of ganglion cell number in the BXD strains is 0.47. Heritability for ganglion cell number in the CCASF₂ cross is 0.90 and was reduced to 0.80 with corrected numbers. Heritability of ganglion cell number in the 32CASTF₂ cross is 0.50. Estimates of the effective gene number modulating ganglion cell number are one in the CCASF₂ cross, 2.5 in the 32CASF₂ cross, and 2.4 in the BXD strains. Due to the required assumptions for this calculation, these estimates are likely to be underestimates of the true gene number.

Three hybrids, CCASF₁, 32CASF₁, and PLSJF₁/J, exhibit dominance for high ganglion cell number alleles ([Table 4.4](#)). For example, the average ganglion cell number for CCAS F₁ hybrids is $59,500 \pm 3,400$ (SD) and is roughly 5,000 cells higher than the parental midpoint. The CV of variation within the hybrids in [Table 4.4](#) averages 5.7%. This CV average is not significantly different from the CV of 6.9% found in the inbred strains (one tailed t -test, $p = 0.15$). The heterozygous state in some hybrids increases

fitness that provides a buffer against developmental and environmental effects. However, there is no evidence that the ganglion cell number in these hybrids is more stable than in the homozygous strains.

Table 4.4. Average ganglion cell number in F₁ hybrids and their parental strains

F ₁ Strains	RGC $\pm$ SE	<i>n</i>	Maternal strain	RGC	Paternal strain	RGC	Mid-Parental	d
CCASF ₁	59,457 $\pm$ 1117	32	BALB/cJ	63,393	CAST/Ei	45,047	54,439	5234
32CASF ₁	63,253 $\pm$ 916	7	BXD32	76,219	CAST/Ei	45,047	60,633	2620
CAF ₁ /J	56,426 $\pm$ 1736	8	BALB/cJ	63,393	A/J	50,516	57,004	-578
B6AxC ₁ F ₁	63,731 $\pm$ 1944	18	C57BL/6Jax1	66,082	BALB/J	63,393	64,738	-1007
PLSJF ₁ /J	56,096 $\pm$ 1333	25	PL/J	55,976	SJL/J	54,225	54,225	1871

Discussion

The significantly lower variance in ganglion cell number within strains compared to across strains demonstrates that genetic differences are involved in the variation in ganglion cell number among strains. Heritability estimates for variation in ganglion cell number are high, ranging from 0.80 in CCASF₂ to 0.47 in the BXD strains. Estimates of heritability within crosses generated from parental strains that are widely divergent, such as BALB/cJ, and CAST/Ei parentals for CCASF₂, are typically inflated (Lynch and Lynch, 1992). In addition, the estimates of broad sense heritability in the intercrosses include the effects of gene dominance effects and epistatic interactions among loci are in the neighborhood of 80%. Nevertheless, these estimates are near the 80% estimate of genetic control over granule cell number in the dentate gyrus of mice

(Wimer and Wimer, 1989). Heritability estimates such as these are based on ratios between genetic and nongenetic variance. Consequently, minimizing environmental variance increases measured heritability. In this study, all mice were reared in a pathogen-free laboratory environment—a situation that eliminates numerous sources of environmental differences, and that almost certainly increases estimates of genetic control compared to dispersed wild populations of mice.

The ganglion cell numbers of CCASF₁ and 32CASF₁ exhibit dominance with respect to the high parental. This dominance could result from hybrid vigor (see discussion in [Chapter 2](#)). In addition, the dams that rear the F₁ hybrids (BALB/cJ and BXD32) have a larger body size compared to that of CAST/Ei. The BALB/J and BXD32 neonates are larger in size compared to CAST/Ei neonates. Case in point, when reciprocal matings were performed, the CAST/Ei females were dying in labor trying to birth these larger size hybrid neonates untypical of their species. The genetic factors producing a small body size in CAST/Ei could have pleiotropic effects on brain weight and neuron numbers. Without the body size restriction imposed, the CAST/Ei contribution the CCASF₁ ganglion cell number might raise above the midparental range. The dominance of the CCASF₁ is also reflected in the distribution of the CCASF₂ ([Fig. 4.3](#)).

An analysis of isogenic animals makes it possible to assess the consistency with which the genome guides the generation of traits such as neuron number. When environmental differences are minimized, the residual variance is due to microenvironmental effects and developmental noise. In a systematic analysis of the grasshopper nervous system a remarkable level of variation in neuron number was found both within and between isogenic groups that had been reared in a tightly controlled environment. In one exceptional group, half of the animals had the standard set of six ocellar interneurons whereas the other half had seven to nine interneurons ([Goodman, 1979](#)). In contrast, in a survey of optic nerves from a set of more than 100

isogenic crustaceans (Macagno, 1980) found only a single exception to the rule of 176 axons. Thus, the developing nervous system is capable of excruciating control. However, it is not known how stringently large populations are developmentally regulated in the vertebrate CNS. The average corrected coefficient of variation in ganglion cell number is 2.8%. Thus, the developmental control of ganglion cell number is stringent. Although, coefficients of variation do range from 2.0% in NZW/LacJ to 11% in C57L/J indicating that the degree of developmental stringency is under genetic control. In addition, these differences could result from some of sampling artifacts discussed in the methods

Variation in ganglion cell number was significantly correlated with brain weight across strains and within the intercrosses. However, there was no correlation between within strain z-scores and brain weight. The absence of a correlation between ganglion cell number and brain weight within strains suggests that the significant correlation found across strains is not due to common non-genetic factors, such as the maternal environment. Thus, the association of ganglion cell number and brain weight is due to the global variation in cell number that originates early during development before the divergence of specific cell lineages. Furthermore, the variation in brain weight is a summation of the correlated variation of individual populations as distinct as the retinal ganglion cells.

There was no decline in ganglion cell number with age in any of the mice studied. This finding is corroborated by a study reporting no age-related decline in ganglion number in the retina of young and old rhesus monkeys (Kim et al., 1996). There are many reports of age-related decrease of neurons in the cerebral cortex and cerebellum neuron populations, but there are also many reports of no age-related decrease in neuron number within distinct nuclei (Jacobson, 1991). In summary, a decline in neuron number is not an inevitable result of aging and may depend on the endogenous level of oxidative stress in a particular neuron type.

The bimodality of strain averages is a surprising and important finding that provides evidence that there are single polymorphic genes that have comparatively large effects on neuron number. This conclusion is strengthened by the estimate from the Castle-Wright formula of one to three genes with large effect size modulating ganglion cell in the F2 intercrosses. A speculation based on these findings is that variation in other neuron populations may also be controlled by relatively small numbers of quantitative trait loci that have major effects. The low gene number and high heritability of ganglion cell number suggests that it should be possible to map one or more genes with a quantitative effect on ganglion cell number.

Chapter 5: Mapping Genes Controlling Variation in Retinal Ganglion Cell Number Using Recombinant Inbred Strains and F₂ Intercross Progeny

Introduction

The high heritability of retinal ganglion cell number among mouse strains indicates that the variation in ganglion cell number results primarily from genetic differences. Furthermore, the prominent bimodality in ganglion cell number among 57 inbred strain averages demonstrates that only a few genes with major effects are responsible for variation in ganglion cell number. Together, these findings suggest that it should be possible to map QTLs that are responsible for variation in ganglion cell number between strains. Only one other study has mapped QTLs controlling neuron number in mice. This study, by Dains et al. (1994), examined variation in the number of striatal cholinergic neurons among inbred mouse strains. A greater than 50% variation was found between BXD strains. The narrow-sense heritability in the BXD strains was found to be around 0.30 and the number of effective factors was from 3 to 4. Using BXD strains, QTLs associated with variation in cholinergic neuron number were mapped to chromosomes 1, 6, 9, 11 and 12.

In this study, I use BXD recombinant inbred strains and two F₂ intercrosses in a linkage analysis to map QTLs that produce variation in retinal ganglion cell number. In order to map genes that modulate ganglion cell number directly, rather than genes that modulate global neuron number, I have mapped using ganglion cell number residuals regressed with respect to brain weight. Thus, the QTLs that are detected are expected to have specific effects on the ganglion cell population.

Variation in neuron number

After detecting significant linkage between a QTL interval and variation in ganglion cell number, the next step is to look for candidate genes within the QTL

interval. There are two criteria for selecting candidate genes that map within the QTL interval. The first is that it is expressed during prenatal or early postnatal eye development, and the second is that it has an assigned function consistent with a role in cell production or cell death. In this study, I examine two candidate genes that meet these criteria. The candidate genes were examined by comparing ganglion cell numbers in wild type mice to those in mice carrying a nonfunctional transgene at the candidate locus.

Materials and methods

The BXD recombinant inbred strains were used to map genes modulating ganglion cell number. Two F₂ intercrosses were then used to confirm QTLs mapped in the BXD RI strains and to map additional QTLs. I generated two groups of intercross progeny from matings between inbred strains BALB/cJ and CAST/Ei (CCASF₂, *n* =112), and BXD32 and CAST/Ei (32CASF₂, *n* =140). The parental strains BALB/cJ, BXD32 and CAST/Ei have ganglion cell numbers of 63,400, 75,800, and 45,000, respectively. To perform a genome-wide linkage analysis DNA was genotyped from CCASF₂ and 32CASF₂ individuals at 110 microsatellites.

Candidate genes were tested by comparing ganglion cell counts from mice containing a nonfunctional transgene at the candidate locus to ganglion cell counts from mice carrying a normal functional candidate. Two candidate genes were tested, *retinoic acid receptor alpha 1* (*Rara1*) and *thyroid receptor alpha* (*Thra*). The *Rara1* null mice were obtained from Vincent Giguere at Royal Victoria Hospital in Montreal, Canada, while the *Thra* null mice were obtained from Douglas Forrest at Mount Sinai in New York. The homozygous *Rara1* null mice appeared completely normal, whereas the *Rara* null mice had high postnatal lethality and testis degeneration (Lufkin et al., 1993). The

homozygous *Thra* null mice have a 20% lower heart rate and a 0.5 °C lower body temperature, but otherwise are healthy and fertile ([Wikstrom et al., 1998](#)).

The examination of the *Rara1* and *Thra* knock-out mice described here is preliminary work carried out as part of a collaboration with Drs. Vincent Giguere and Douglas Forrest. The preliminary work on *Rara1* has been published ([Zhou et al. 1998](#)). Final publications are in progress (Zhou G, Strom R, Giguere V, Williams RW).

Results

The average ganglion cell number in the BXD strains ranges from 52,500 in BXD27 to 76,200 in BXD32. There are 8 strains with low ganglion cell means and 14 strains with high ganglion cell means. In [Table 5.1](#), the low strains are assigned L and the high strains are assigned H. There are five strains with intermediate ganglion cell numbers that fall within $\pm 2,000$ of the mid-parental value of 59,000 and these strains are assigned an I in [Table 5.1](#).

Table 5.1 Corrected retinal ganglion cell number for BXD strains and parents, C57BL/6J and DBA/2J.

Strain	RGC	± SE	Type	<i>n</i>	Brain weight
C57BL/6J	55,413	± 800	L	26	475
DBA/2J	63,351	± 1,200	H	13	412
BXD1	58,714	± 1,058	I	11	465
BXD2	65,790	± 1,562	H	6	432
BXD5	71,228	± 1,162	H	6	526
BXD6	64,275	± 805	H	6	388
BXD8	60,913	± 2,092	I	8	412
BXD9	65,449	± 1,883	H	3	422
BXD11	60,734	± 1,013	I	9	437
BXD12	56,256	± 1,421	L	2	434
BXD13	54,869	± 1,491	L	7	427
BXD14	63,507	± 1,435	H	7	442
BXD15	63,246	± 973	H	6	443
BXD16	62,294	± 1,124	I	6	469
BXD18	55,151	± 1,192	L	4	427
BXD19	67,009	± 1,200	H	9	431
BXD20	61,312	± 1,728	I	10	398
BXD21	59,383	± 1,330	I	8	443
BXD22	63,244	± 944	H	5	457
BXD23	52,797	± 911	L	6	434
BXD24	64,150	± 918	H	7	391
BXD25	53,778	± 1,314	L	7	431
BXD27	52,483	± 973	L	6	393
BXD28	53,430	± 1,644	L	7	407
BXD29	64,350	± 1,182	H	6	413
BXD30	67,429	± 1,137	H	6	399
BXD31	66,733	± 1,053	H	6	426
BXD32	76,219	± 1,914	H	10	434

A comparison of ganglion cell number in the 26 BXD strains and the genotypic strain distribution at 529 loci resulted in the best match at *Tstap91A* located on Chr 11 at 75 cM. A correlation between alleles at *Tstap91A* and ganglion cell number resulted in a coefficient of +0.72. The LOD score for the linkage between ganglion cell number and *Tstap91A* is 4.5. [Table 5.2](#) shows the concordance between the genotypic strain

distribution of loci on Chr 11 and the putative genotype at the RGC locus based on its parental resemblance. There are 20 strains with RGC phenotypes concordant with alleles at *Tstap91A*, while only 1 strain is discordant and 5 strains are intermediate.

Table 5.2. BXD strain distribution pattern for retinal ganglion cell number and genotypes at five loci on Chr 11.

Locus	c M	BXD strains																							
		1	2	5	6	8	9	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	3
								1	2	3	4	5	6	8	9	0	1	2	3	4	5	7	8	9	0
<i>D11Ncvs58</i>	56	B	D	D	B	D	D	B	D	B	D	D	D	B	D	D	D	D	B	B	B	D	B	D	B
<i>Hoxb</i>	56	B	D	D	D	D	D	B	B	B	D	D	D	B	D	D	D	D	B	D	B	D	B	D	D
RGC number*		I	D	D	D	I	D	I	B	B	D	D	I	B	D	I	I	D	B	D	B	B	B	D	D
<i>Tstap91A</i>	57	B	D	D	D	D	D	B	B	B	D	D	D	B	D	D	D	D	B	D	B	B	B	D	D
<i>Krt1</i>	58	B	D	D	D	D	D	B	B	B	D	D	D	B	D	D	D	D	B	D	B	B	B	B	D
<i>Mpmv8</i>	62	B	D	D	D	D	D	B	B	B	D	D	B	D	D	D	D	D	B	D	B	B	B	B	D

*The alleles for RGC number are based on the strain's resemblance to the C57Bl/6J (B) or DBA/2J (D) phenotype. The B allele was assigned if ganglion cell number was <57,000 and the D allele was assigned if the ganglion cell number was >61,000. Strains with ganglion cell numbers >57,000 but <61,000 were assigned an intermediate (I) allele type.

Controlling for the *Tstap91A* indicated secondary loci on distal Chr 1 at *D1Ncvs60* and on proximal Chr 7 at *Iapls3-4;1-11*. Controlling for these two secondary loci increased the LOD of *Tstap91A* to 6.7 at 57 cM, with a 2 LOD CI between *Hoxb* and *Krt1*, at 56 and 58 cM, respectively ([Fig 5.1](#)). The second close match with ganglion cell number was found at *D14Pas1* on Chr 14 at 12 cM, with a correlation coefficient of +0.60 and an LOD score of 3.5. A linkage test while controlling for the variance at *D14Pas1* detected secondary loci on Chr 2 at *D2Byu2* and on Chr 6 at *D6Mit86*. Interval mapping

while controlling for these two secondary loci increased the LOD at *D14Pas1* to 5.9 at 15 cM, with a 2 LOD confidence interval from 11 to 17 cM (Fig 5.2).

A permutation test in the BXD data set while controlling for secondary loci defined the appropriate genome-wide conditional significance thresholds. The LOD scores required for a suggestive, significant and highly significant linkage are 3.0, 4.7, and 6.0, respectively. The QTLs detected on Chr 11 with peak LOD score of 6.7 exceeds the highly significant threshold. The locus on proximal Chr 14 with a peak LOD score of 5.9, almost reaches the highly significant threshold. The QTL mapped to Chr 11 has been named *Neuron number control 1* (*Nnc1*) (Williams et al., 1998), and the QTL mapped to Chr 14 has been named *Neuron number control 2* (*Nnc2*).

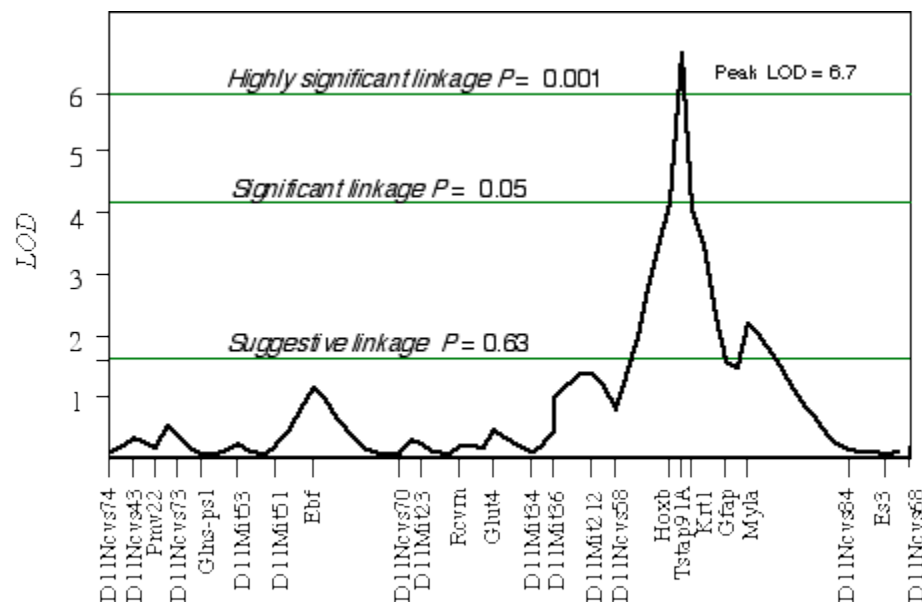


Figure 5.1. Linkage map demonstrates the QTL *Nnc1* on Chr 11 in the BXD data set. LOD scores were computed at 1 cM intervals while controlling for *Iapls3-4; 1-11* on Chr 7 and *D1Ncv860* on Chr 1.

My analysis detected a significant linkage on Chr 14 that was not reported in Williams et al., (1998). A possible explanation for the additional linkage is a difference in the regression procedure. In this proceeding, I truncated the ganglion cell numbers in

the BXD5 and BXD32 strains before the regression of ganglion cell number and brain weight. I did this because the ganglion cell numbers for BXD5 and BXD32 exhibited overdominance and in the case of BXD5 also an overdominance in brain weight. In a regression analysis, a large deviation in just a few data points can distort the true association. The overdominance in these strains may result from unique non-additive epistatic interactions or a combination of genes with increaser alleles, which could produce a global increase in cell number, and may interfere with linkage associations.

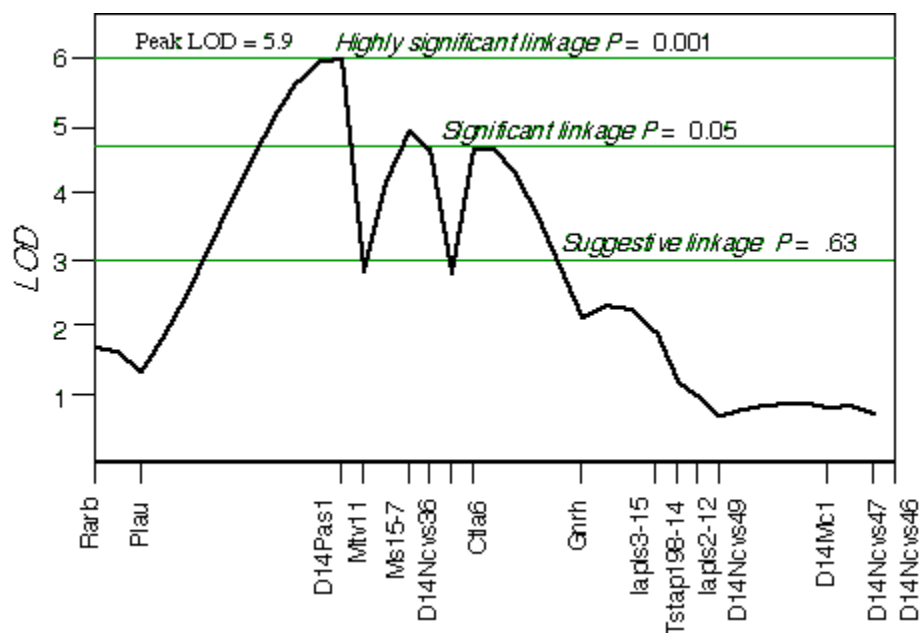


Figure 5.2. Linkage map demonstrates the QTL *Nnc2* on Chr 14 in the BXD data set. LOD scores were computed at 1 cM intervals while controlling for *D2Byu2* on Chr 2 and *D6Mit86* on Chr 6.

Testing Nnc1 candidate genes by examining knock out mice

Three excellent candidates for *Nnc1* that map within the *Hoxb*—*Krt1* interval, *retinoic acid receptor alpha 1* (*Rara1*), *thyroid receptor alpha* (*Thra*), and the neuregulin receptor *ErbB2*. All three of these receptors are expressed in the developing retina and the ligands for these receptors are known to influence retinal cell number (Bermingham-McDonogh et al., 1996; Ruberte et al., 1993; Sjöberg et al., 1992). The

addition of neuregulin, the ligand that activates the receptor *ErbB2*, promotes ganglion cell survival in culture ([Bermingham-McDonogh et al., 1996](#)). The addition of retinoic acid and thyroid hormone to retinal cultures increases the differentiation of photoreceptors ([Kelley et al., 1995](#)). A reciprocal relationship in differentiation potential is thought to occur between the early- and late-generated retinal cell types. Thus, an increased bias for the generation of ganglion cells could have repercussions on the number later-generated photoreceptors.

The average ganglion cell number in the transgenic mice carrying a null *Rara1* is $62,494 \pm 1160$ ($n = 10$) compared to the average for normal *Rara1* mice of $62,998 \pm 1930$ ($n = 9$) ([Fig. 5.3A](#)). There are no significant differences in ganglion cell number between the homozygous mutant *Rara1* and the normal *Rara1* mouse, $p = 0.82$. In contrast, the average number of ganglion cells in the transgenic mice carrying a null *Thra1* is $63,645 \pm 1884$ ($n = 9$), compared to an average of $74,030 \pm 1137$ ($n = 8$) in mice carrying a normal *Thra1* ([Fig. 5.3B](#)). The difference in ganglion cell number between the homozygous null and normal *Thra* mice is highly significant at $p = 0.0005$.

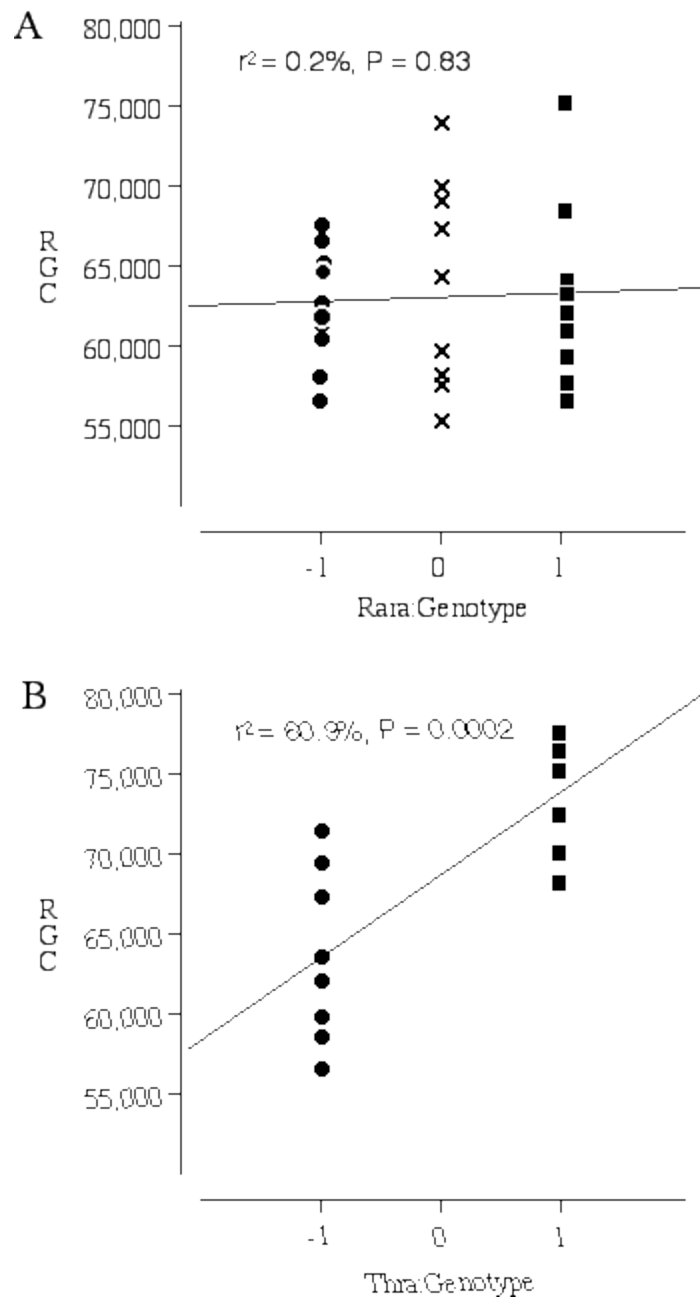


Figure 5.3. Ganglion cell numbers for transgenic mice carrying homozygous null *Nnc1* candidate genes (-1), heterozygous null (0) and wildtype genes (1). (A) *Rara1* (-/-)(-/+)(+/+) comparison; (B) *Thra* (-/-)(+/+) comparison.

In CCASF₂ mice, ganglion cell number corrected with respect to brain weight ranged from 41,000 to 70,000. I performed a linkage analysis between ganglion cell number and the genotypes of 30 extreme CCASF₂ progeny. The best association with

ganglion cell number was on distal Chr 1 at *D1Mit105* with a LOD score of 3.5. The second best linkages were on proximal Chr 15 at *D15Mit12* with a LOD score of 3.2 and on mid-distal Chr 7 at *D7Mit238* with a LOD score of 2.3. Linkage testing while controlling for the Chr 1 locus detected a linkage on Chr 16 near *D16Mit29*. Composite interval mapping on Chr 1 while controlling for the QTL intervals on Chrs 7, 15, and 16 resulted in an LOD score of 9.3 between *D1Mit502* and *D1Mit105*. Linkage testing while controlling for *D15Mit12* detected a linkage on Chr 10 near *D10Mit282* with a LOD score of 2.7. Interval mapping while controlling for the QTLs on Chrs 1, 7, and 10 increased the LOD at *D15Mit12* to 4.0.

Increasing the number of progeny in a linkage analysis can improve the estimate of the QTL location. Interval mapping on Chr 1 with 100 CCASF₂ while controlling for *D15Mit12* and *D13Mit18* resulted in an LOD of 6.0 between *D1Mit105* and *D1Mit57*, with the peak score at 82 cM, 2 cM distal of *D1Mit105* ([Fig. 5.4](#)). The 2 LOD CI is from 77 cM to 88 cM. Variation at *D1Mit105* is estimated to explain 15% of variance in cell number, however, estimates in small sample sizes such as this one, tend to overestimate the effect size. Interval mapping on Chr 15 with 100 CCASF₂ and controlling for *D1Mit105* resulted in an LOD of 2.0 at *D15Mit12*, the most proximal marker.

A permutation test with the complete data set while controlling for secondary loci defined the genome-wide significance thresholds. The LOD scores required for a suggestive, significant and highly significant linkage are 2.0, 3.5, and 5.4, respectively. The LOD score reached 2.0 at *D15Mit12* and is therefore only a suggestive linkage. The LOD score in the interval between *D1Mit105* and *D1Mit57* reaches 6.0, and is a highly significant linkage. I have named the QTL on distal Chr 1 *neuron number control 3* (*Nnc3*).

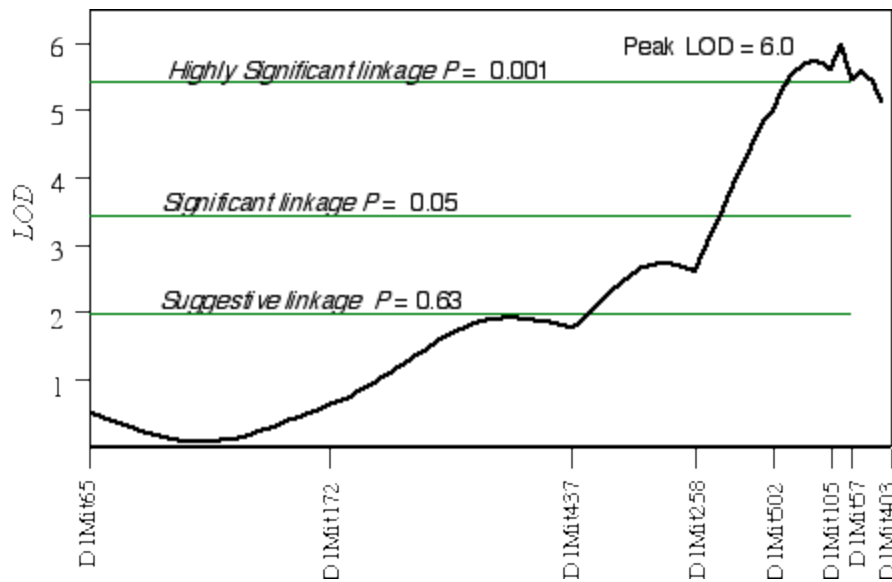


Figure 5.4. Linkage map demonstrates the QTL *Nnc3* on distal Chr 1 in the CCASF₂ data set. LOD scores were computed at 1 cM intervals while controlling for *D15Mit12* and *D13Mit18*.

In 32CASF₂ mice, ganglion cell number corrected with respect to brain weight ranged from 41,000 to 70,000. A comparison between ganglion cell number and genotypes in the 45 extreme 32CASF₂ progeny detected a linkage on mid-Chr 16 at *D16Mit190* with a LOD score of 2.6. A linkage test while controlling for the *D16Mit190* resulted in detecting secondary loci *D13Mit203* and *D17Mit10*. Composite interval mapping on Chr 16 while controlling for *D13Mit203* and *D17Mit10* increased the LOD score to 6.0 at *D16Mit190*, which maps at 41.5 cM ([Fig. 5.5](#)). The 2 LOD CI is from 22 cM to 50 cM. A permutation test defined the appropriate genome-wide significance thresholds. The LOD scores required for a suggestive, significant and highly significant linkage are 2.0, 3.6, and 5.9, respectively. The linkage at *D16Mit190* with a LOD score of 6.0 is therefore a highly significant linkage. I have named the QTL on Chr 16 *neuron number control 4* (*Nnc4*).

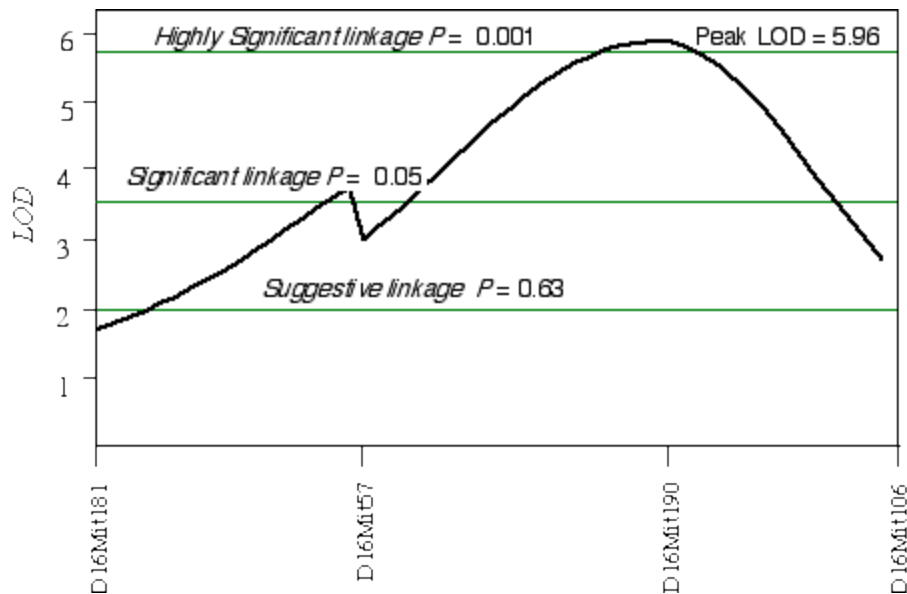


Figure 5.5. Linkage map demonstrates *Nnc4* on Chr 16 in the 32CASTF₂ data set. LOD scores were computed at 1 cM intervals while controlling for *D13Mit203* and *D17Mit10*.

Discussion

Synopsis

I have mapped four significant QTLs that modulate ganglion cell number in mice to Chr 1, 11, 14, and 16. These QTLs have been named *Nnc1*, 2, 3, and 4. *Nnc1* maps to 57 cM on Chr 11, *Nnc2* maps to 15 cM on Chr 14, *Nnc3* maps to 82 cM on Chr 1, and *Nnc4* maps to 41.5 cM on Chr 16.

Candidates

The significantly reduced ganglion cell number in mice carrying a null *Thra* transgene compared to the wildtype supports the candidate gene *Thra* for *Nnc1*. *Thra* maps within 1–2 cM of *Nnc1* on chromosome 11 ([Montgomery et al., 1997](#)), and is expressed within the developing chick retina ([Sjoberg et al., 1992](#)). The ligand of *Thra*, triiodothyronine, is known to influence retinal ganglion cell fate determination ([Hoskins, 1985](#)), retinal maturation rate ([Macaione et al., 1984](#)). Hypothyroidism during retinal development results in decreased cell density in the ganglion cell layer ([Hoskins,](#)

1985; Navagantes et al., 1996). Interestingly, the addition of the *Thra* ligand, triiodothyronine, to cultured fetal rat hypothalamus cells stimulates the release IGF-1, a known mitogen in retinal cultures (Binoux et al., 1985). A candidate gene for *Nnc2* would map on Chr 14 between 11 cM and 17 cM, preferably near the peak linkage statistic at 15 cM. Two fantastic candidates for *Nnc2* are *bone morphogenic protein receptor-2/4* (*BMPR*) and *bone morphogenic protein* (*BMP*). *BMPR* and *BMP* map to 13 cM and 14 cM (Beppu et al., 1997). *BMPR-2* is a serine-threonine kinase receptor that is strongly expressed in the developing optic vesicle of the rat (Obata et al., 1999), and in differentiating ganglion cells in the chick (Carri et al., 1998). The function of *BMPR* in the neural retina is not known, however, in the brain *BMPRs* are involved in proliferation and differentiation (Zhang et al., 1998).

The candidate genes for *Nnc3* would map on Chr 1 between 77 cM and 88 cM, preferably close to the peak linkage statistic at 82 cM. The best *Nnc3* candidate is *retinoid X receptor gamma* (*Rxrg*). *Rxrg* maps just within the QTL interval at 88 cM and is expressed in the developing mouse retina (Dolle et al., 1994). *Rxrg* null mice appear normal with respect to growth, behavior, fertility and viability (Krezel et al., 1996). Interestingly, *Rxrg* forms a heterodimer with *Thra*, the *Nnc1* candidate, and together the pair binds to thyroid hormone response elements to activate gene transcription (Force et al., 1994).

The candidate genes for *Nnc4* would map on Chr 16 between 22 cM and 50 cM with the peak association at 41.5 cM. The 2 LOD confidence interval for this QTL interval is very broad and searching for a candidate gene within this broad an interval is essentially an exercise in optimism. However, an attractive candidate does map within the interval at 27 cM, and this gene is the *enhancer of split homolog-1* (*HES1*). *HES1* is basic helix-loop-helix (bHLH) transcription factor expressed in progenitor cells of the developing neural retina (Kageyama et al., 1997). Interestingly, *HES1* is involved in the Notch pathway, where it acts downstream of Notch to inhibit neural

differentiation. In mice carrying a null *HES1* transgene the retina differentiates prematurely and forms abnormal rosette structures (Tomita et al., 1996). Genetic variants in *HES1* could alter the expression level or timing of *HES1* and this would affect the number of retinal progenitors produced.

Chapter 6: Developmental Mechanisms Controlling Retinal Ganglion Cell Variation Among Inbred Mouse Strains

Introduction

The distribution of ganglion cell strain averages for 57 inbred mouse strains is bimodal, with distinct modes centered at 55,000 and 64,000. Most of the bimodality in strain averages is controlled by a major effect QTL, neuron number control 1 (*Nnc1*). This QTL is located on Chr 11 and is closely linked to three genes known to influence retinal development—the retinoic acid receptor alpha, neuregulin, and the thyroid hormone receptor. *Nnc1* could influence ganglion cell number by modulating either cell production or the severity of natural cell death.

My principal objectives in this study were to determine the developmental mechanism by which allelic variants at *Nnc1* control neuron number, and to assess the relative contributions of cell production and cell death in the consistent and pronounced differences in ganglion cell among strains of mice. I examined ganglion cell production in ten strains—four selected from the low mode, four from the high mode, and one each from the high and low extreme ([Fig. 1](#)). I included the strains C57BL/6J and DBA/2J—the parental strains of the BXD RI set in which *Nnc1* was mapped. Mechanisms generating neuron number differences between these two strains can be confidently assigned to *Nnc1*. Total ganglion cell production was estimated by counting retinal ganglion cell axons at birth, a time at which ganglion cell generation in mice is complete ([Dräger, 1985](#)), but before significant ganglion cell death has begun ([Linden and Pinto, 1985](#)). The results of this study have been published ([Strom and Williams, 1998](#)).

Materials and methods

Mice

As illustrated in [fig. 6.1](#), strains of mice were chosen primarily to represent the two major modes in ganglion cell number. Three standard inbred strains were selected from the low mode (C57BL/6J, A/J, and LP/J) and four standard inbred strains were selected from the high mode (BALB/cJ, C3H/HeSnJ, CE/J, and DBA/2J). All of these strains were obtained from the

Jackson Laboratory, (Bar Harbor, ME). In addition, I selected two strains—CAST/Ei and BXD32—that have exceptionally low and high ganglion cell numbers, respectively. BXD32 was obtained from Dr. Benjamin Taylor at the Jackson Laboratory. CAST/Ei is an inbred strain derived from *M. musculus* castaneus that I obtained from Dr. Eva Eicher at the Jackson Laboratory. Finally, I studied an outbred sample of *Mus caroli* that I refer to as CARL/ChGo, a strain that falls into the low ganglion cell mode. CARL/ChGo is a partially inbred strain of *M. caroli* given to us by Dr. Dan Goldowitz at the University of Tennessee. Both CARL/ChGo and CAST/Ei are representatives of wild species endemic to Southeast Asia. All mice were mated at the University of Tennessee mouse colony to produce neonates. The day of birth was designated postnatal day zero (P0).

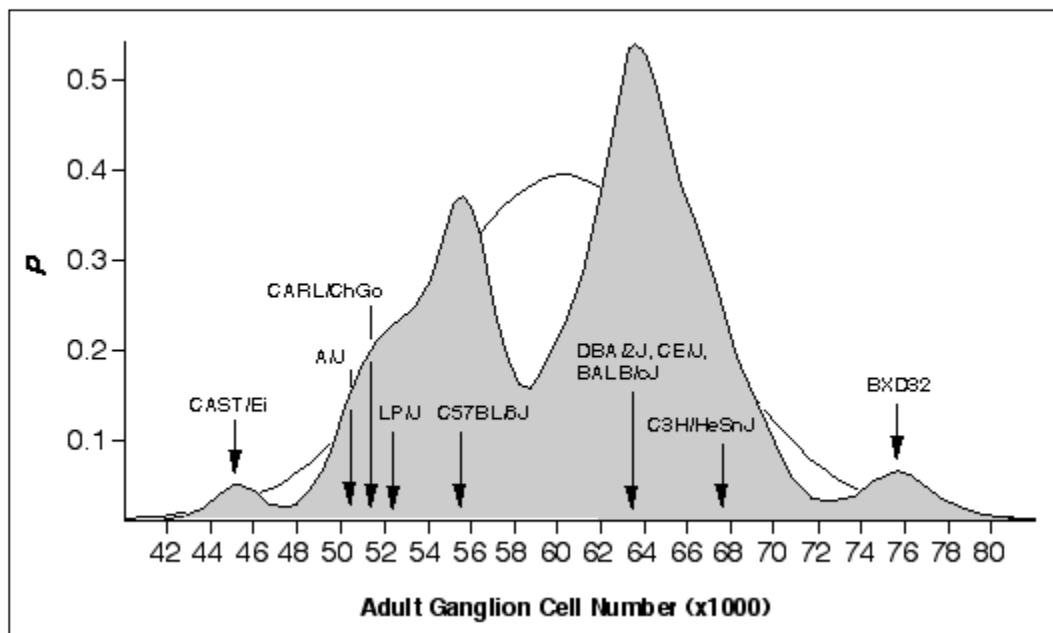


Figure 6.1. Bimodal distribution of adult ganglion cell averages for 60 inbred strains. The strains include 38 recombinant inbred strains and 17 standard inbred strains listed in [Chapter 4](#), and 5 additional strains. A Gaussian probability distribution was computed for each strain and summed to obtain a probability density plot (see [Chapter 2](#), (Williams et al., 1996). The figure shows that most strains fall into two main modes. The Gaussian function drawn in the background has a mean of 60.6 ± 6.3 (x1000), the average and SD of the 60 strains. The arrows designate the strain averages for the ten strains examined in this study.

Tissue fixation

The neonates were anesthetized by placing them on ice for several minutes. Neonates were then perfused transcardially with 0.1 M phosphate buffered saline (0.9%), followed by fixative (2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M phosphate buffer). Midorbital segments of optic nerves were dissected from the neonates, osmicated, and embedded in Spurr's resin. Nerves were thin-sectioned, placed on formvar-coated grids, and stained with lead citrate and uranyl acetate.

Estimating ganglion cell number

I estimated ganglion cell numbers by counting axons in P0 optic nerve cross-sections. Axons were counted as described in [Chapter 4](#), Methods section, with the following exceptions. Nerves were photographed in a grid pattern at ~X30,000 using a JEOL EX2000II electron microscope. Unmyelinated axons were easily identified and counted ([Fig. 6.2](#))

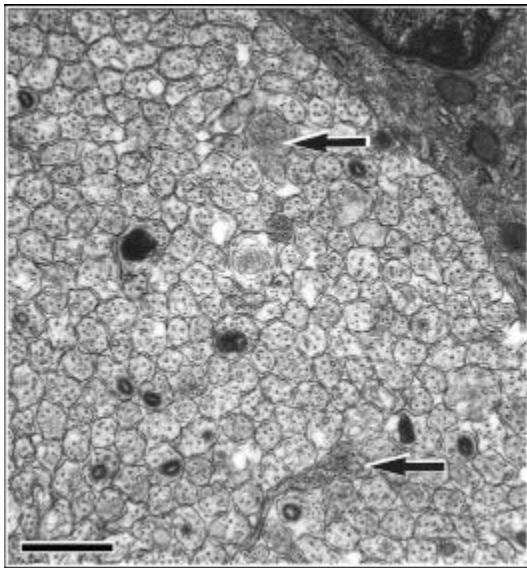


Figure 6.2. Cross-section of a neonatal optic nerve. Magnification is 15,000. The scale bar in the bottom left corner represents 1 μm . Axons at this stage have relatively uniform diameters, with a mean fiber diameter of $\sim 0.4 \mu\text{m}$. Axons can be recognized unambiguously in well-fixed tissue. The two structures marked by arrows are astrocyte processes and were not counted.

I counted necrotic axons in neonatal optic nerves from two strains belonging to the high mode and two strains belonging to the low mode. I did this by systematically scanning the entire optic nerve cross-section for necrotic axons at X15,000. The criteria for distinguishing necrotic axons are those described in Williams et al., (1986). I also searched for growth cones in the sample of photographs used for counting axons and by scanning several optic nerves at high magnification (>X40,000).

Results

The retinal ganglion cell population at birth ranged from 131,000 to 224,000 ([Table 6.1](#)). The mean for all 46 cases is $182,500 \pm 4,400$ SE. This value is almost three times higher than the average for an equally diverse sample of adult mice (see [Chapter 4](#), (Williams et al., 1996)). I counted an average of five neonates per strain. The coefficient of variation within strains averaged 8.2%—only slightly higher than the 7.2% value obtained for adult mice (see [Chapter 4](#), (Williams et al., 1996)). The small increase is probably due to the technical difficulty of counting axons before they are myelinated. Given the anticipated variation in the stage of maturation of sets of neonatal mice sacrificed at birth, this CV is low and suggests that the ganglion cell population within a strain is comparatively stable at this stage of development. The average coefficient of error (the standard error divided by the sample mean) averaged 4.5% in neonates and 2.5% in adults. These values provide an assessment of the reliability of adult and neonatal ganglion cell counts.

Table 6.1. Ganglion cell number and percentage cell loss

Strain	Phenotype	Adult mean $\pm$ SE	<i>n</i>	P0 mean $\pm$ SE	<i>n</i>	Absolute Cell Loss	Percent Loss
BXD32	very high	75,800 $\pm$ 1,900	11	199,500 $\pm$ 5,500	6	123,700	62%
C3H/HeSnJ	high	67,800 $\pm$ 1,400	3	224,000 $\pm$ 8,100	5	156,200	70%
CE/J	high	63,600 $\pm$ 2,400	5	207,000 $\pm$ 8,900	3	143,400	69%
BALB/cJ	high	63,400 $\pm$ 2,200	8	180,800 $\pm$ 7,700	4	117,400	65%
DBA/2J	high	63,400 $\pm$ 1,100	13	202,100 $\pm$ 7,900	4	138,700	69%
C57BL/6J	low	55,500 $\pm$ 1,300	13	185,700 $\pm$ 6,400	7	130,200	70%
LP/J	low	52,200 $\pm$ 1,900	8	171,400 $\pm$ 8,000	5	119,200	70%
CARL/ChGo	low	51,300 $\pm$ 2,200	11	131,200 $\pm$ 5,500	3	79,900	61%
A/J	low	50,600 $\pm$ 1,300	8	155,600 $\pm$ 6,000	6	105,000	67%
CAST/Ei	very low	45,000 $\pm$ 1,000	12	146,600 $\pm$ 6,600	3	101,600	69%

Cell production

If strain differences in adult ganglion cell numbers result from differences in the number of neurons that are generated, then at birth each strain should have a population that is approximately three-fold higher than its adult mean. The slope of the regression should be close to 1:3 and the correlation should be high. This is almost exactly what I found. The slope of a free regression for the ten strains is 0.26 ± 0.07 ([Fig. 6.3](#)). Forcing the regression line through the origin produces the expected slope of 1:3 with an excellent fit (inset to [Fig. 6.3](#)). The positive y-intercept (11,600 adult cells) in the free regression may result from sampling error, non-linearity

of cell death, or may indicate a basal level of cell production. The correlation coefficient of the free regression in [Fig. 6.3](#) is 0.81, and the corresponding coefficient of determination (r^2) is 0.66. Thus, two-thirds of the variance in adult cell number can be readily explained by strain differences in cell genesis.

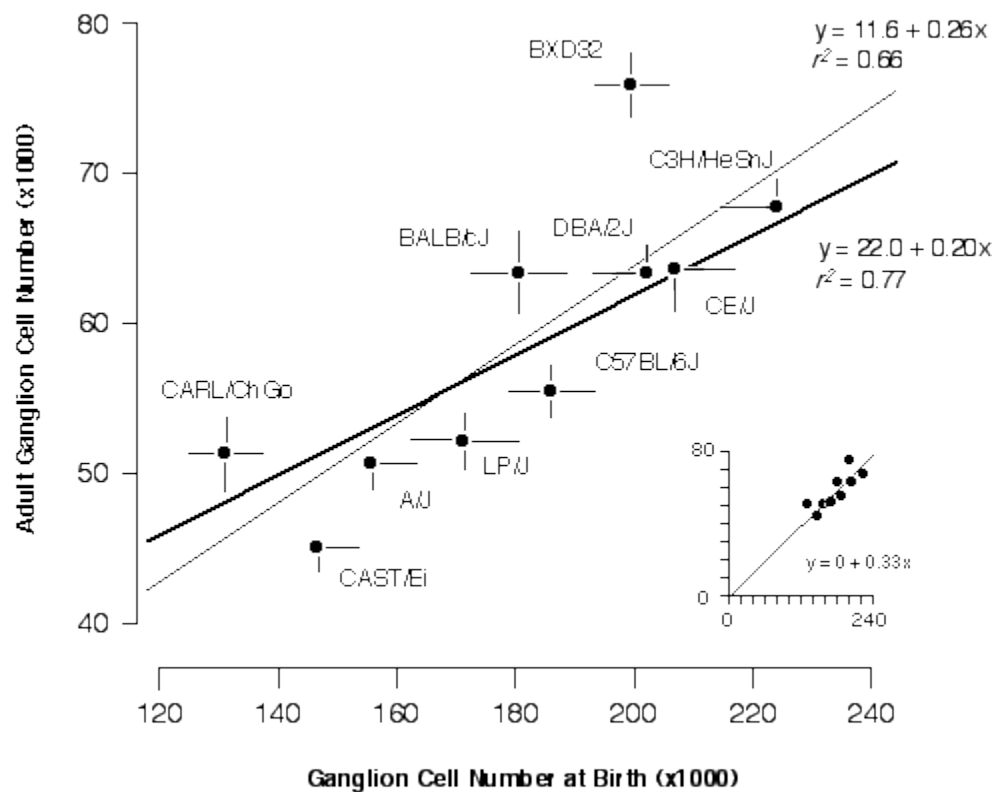


Figure 6.3. Regression of P0 and adult ganglion cell number averages for ten strains. The error bars represent one standard error. The thin regression line includes all strains and the coefficient of determination for these data is 0.66, whereas the dark regression line excludes strains CAST/Ei and BXD32 and the coefficient of determination is 0.77. The inset plots the same data but with the regression line forced through the origin. A regression through the origin is expected because when cell production is zero adult cell number must be zero.

I was particularly interested in understanding the process that produces bimodality in adult strain averages and for this reason I also restricted the analysis to the eight strains belonging to high and low modes ([Fig. 6.3](#), bold line). The coefficient of determination for this subset of points is 0.77, indicating that the bimodality is generated primarily by differences in

ganglion cell production. The remaining "unexplained" variance must result from strain differences in the severity of cell death, developmental noise, and technical error.

This statistical analysis is complicated by two factors. First, the parameters plotted in [Fig. 6.3](#) are dependent—total cell production cannot be less than the adult population. Second, the distribution of adult values is far from normal ([Fig. 6.1](#)). Conventional statistical estimates are therefore difficult to interpret. To address these problems I carried out Monte Carlo simulations to test cell production and cell death models using seed parameters taken from the adult distribution ([Fig. 6.4](#)). I subtracted the adult population from the neonatal population to insure independence between the parameters ([Fig. 6.4A](#)). [Figures 6.4B](#) and [6.4C](#) show the outcomes of two typical Monte Carlo simulations in which I plotted adult cell number against the number of lost cells. The first model ([Fig. 6.4B](#)) assumes that all differences in adult cell number are caused by matched differences in cell production and that cell death is strictly proportional to cell production. The second model ([Fig. 6.4C](#)) assumes that all differences among adult strains are caused by variation in the severity of cell death and that at birth all strains have roughly the same cell population ($\sim 180,400 \pm 18,400$ cells). In the cell production simulation ([Fig. 6.4B](#)), the regression slope is +1.2, whereas in the cell death simulation ([4C](#)) the slope is -1.1. My actual data set ([Fig. 6.4A](#)) with its slope of +1.5 strongly supports a cell production model.

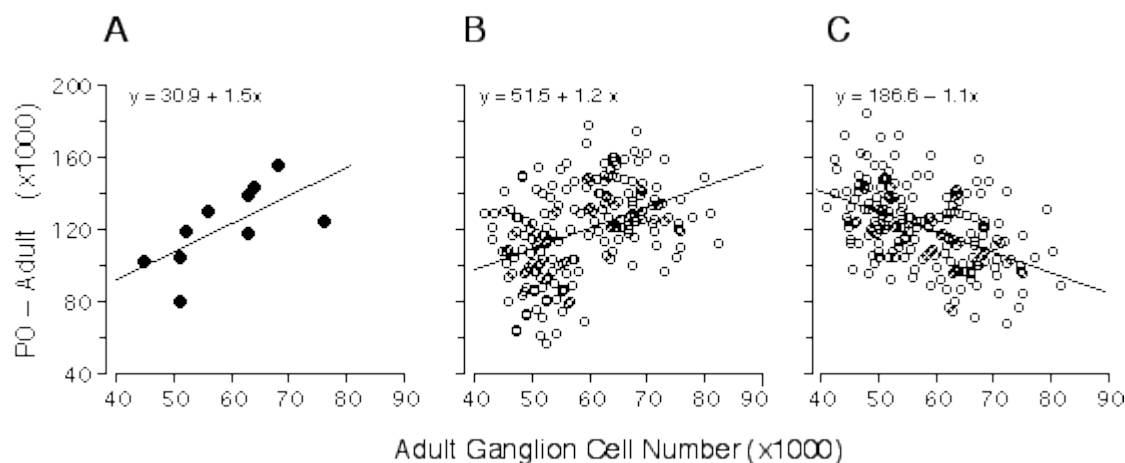


Figure 6.4. Regression of numbers of cells that are lost (number at P0 minus the number at maturity) and adult ganglion cell number from our data (A), and two alternative Monte Carlo simulations (B and C). The first model (B) assumes that all differences in ganglion cell number

are caused by cell production differences, whereas the second model (C) assumes that all differences are caused by variation in the severity cell death. Monte Carlo data sets consisted of 200 numbers randomly selected from normal distributions. In both models, high and low adult ganglion cell groups, ($n = 100$ each), were selected from two normal distributions with seed parameters (mean and standard deviation) from the 5 high ($66,800 \pm 5,400$) and 5 low ($50,920 \pm 3,800$) strains that I studied. In the production model (B), means were obtained from two normal distributions with seed parameters (mean and standard deviation) from the 5 high ($202,680 \pm 15,500$) and 5 low ($158,100 \pm 21,200$) strains. In the case of the cell death model (C), where no production differences are assumed, the neonatal means were obtained from a single distribution, with a mean and standard deviation of all 10 strains combined ($180,390 \pm 18,400$). The slope obtained with our real data is +1.5 (A), while the cell production (B) and cell death (C) models are +1.2 and -1.1, respectively. The positive slope from our data is close to that of the simulated cell production model, demonstrating that differences in adult ganglion cell number is predominantly due to differences in cell production. I used Model I linear least-square regression for this analysis because the measurement error term is without bias. Adult ganglion cell number was subtracted from P0 ganglion cell number to make the y-axis formally independent of adult ganglion cell number plotted on the x-axis.

The 10 inbred strains were divided into high (BALB/cJ, C3H/HeSnJ, CE/J, BXD32, and DBA/2J) and low groups (C57BL/6J, A/J, CAST/Ei, CARL/ChGo, and LP/J). Mean adult ganglion cell numbers for these groups are $66,800 \pm 2,700$ and $50,900 \pm 1,900$, respectively. There are highly significant differences in ganglion cell production between these groups, with means of $202,700 \pm 7,800$ and $158,100 \pm 10,600$, respectively (t test, $p < 0.001$). In contrast, there is no significant difference in the percentage of ganglion cell loss between high and low groups, with mean percentage cell loss relative to neonatal values of 66.9% and 67.5%, respectively ($p = 0.42$).

Nnc1 was mapped using recombinant inbred strains generated from the parental strains, DBA/2J and C57BL/6J. For this reason a comparison between these two strains is especially germane in discovering how *Nnc1* modulates ganglion cell number. The severity of percentage cell death was closely matched between DBA/2J and C57BL/6J — 69% and 70%, respectively. In contrast, DBA/2J produces ~16,400 more cells than C57BL/6J. This result, together with our previous finding of additive gene action (see [Chapter 5](#), and , indicates that the substitution of a single allele at *Nnc1* is associated with a production difference of approximately 8,000 cells.

Cell death

With the exception of strains BXD32, CARL/ChGo, and BALB/cJ, the average percentage of cell death among strains is relatively uniform — $69\% \pm 1.2\%$ ([Table 6.1](#)). While the percentage of cell death is relatively uniform, the absolute magnitude of ganglion cell death is variable among strains and is highly correlated with production values ([Table 6.1](#)). There are some notable exceptions to this generality. The percent cell death in BXD32 and CARL/ChGo is significantly lower than in other strains (t test, $p < 0.05$, with Bonferroni correction). Estimates of ganglion cell production are similar in BALB/cJ and C57BL/6J, yet these strains have adult populations that differ by about 8,000 cells ([Table 6.1](#)). A slight reduction in the severity of cell death in BALB/cJ (65% loss) appears to account for this strain's relatively high cell number at maturity. In this instance, the marked strain difference in adult population size results primarily from variation in the severity of cell death. Differences in cell death can also compensate for differences in cell production. For example, CARL/ChGo produces an average of 131,000 ganglion cells — 20,000 to 40,000 fewer cells than A/J and LP/J, respectively — yet all three strains have very closely matched adult populations ([Table 6.1](#)).

Necrotic axons and growth cones

The validity of our quantitative analysis depends on the assurance with which I can estimate total ganglion cell production in mice. If much cell loss occurs before birth or much cell addition occurs after birth, then production estimates based on axon counts in the optic nerve at P0 will be too low. To rule out the possibility that significant cell death occurs prenatally, I counted necrotic axons in neonatal optic nerves from strains belonging to the high and low modes using criteria described by Williams and colleagues (1986). Necrotic axons are relatively easy to see, and it was practical to count all sites of necrosis in a single optic nerve cross-section. Necrotic axons at P0 make up 0.02% and 0.05% of the fiber population in cases selected from the low strains, A/J and C57BL/6J, respectively, whereas they make up 0.07% and 0.09% of the population in cases selected from the high strains, BXD32 and C3H/HeSnJ, respectively. The fact that a somewhat higher incidence of necrosis was noted in nerves taken from the high strains makes it very unlikely that variation in early axon loss accounts for differences between adult

values. Growth cones were exceeding rare in all material and fewer than 5 profiles among all cases met even a relatively lax criteria for these structures (Colello and Guillery, 1992; Williams et al., 1986).

Specificity of strain differences

Do strain differences in retinal ganglion cell number correspond to differences in total brain weight or are differences among strains specific to the ganglion cell population? The correlation of ganglion cell number and brain weight across individual mice is 0.37, but when strain averages are used the correlation rises to 0.75. This suggests that about half the variance in neonatal ganglion cell number can be explained directly or indirectly by differences in brain weight. As assessed by quantitative DNA analysis, brain weight differences among neonatal mice are primarily due to differences in total cell number (Zamenhof and van Marthens, 1978). Thus, mechanisms modulating ganglion cell number may have common effects on cell number in the other parts of the CNS. Alternatively, the correlation between ganglion cell number and brain weight could suggest that variation of ganglion cell number is the result of differences occurring very early in a neural progenitor pool, perhaps during neurulation. The correlation between strain averages of adult brain weight and ganglion cell number for the same strains is only 0.51. Given the wide confidence intervals of correlations computed with low numbers of cases, the difference between the adult and neonatal correlations (0.51 and 0.75, respectively) may be due to sampling error. However, it is also conceivable that strain variation in cell death decreases an initially high correlation between brain weight and retinal ganglion cell number. In any case, the cellular specificity of the strain differences is likely to be low, and I expect differences in numerous other neuronal cell populations to be closely matched with the differences I find in ganglion cell number.

Discussion

The analysis demonstrates that most of the variation in adult ganglion cell number among strains of mice can be traced to differences in cell production. Allelic variants at the *Nnc1*

locus on Chr 11 generate the pronounced bimodality in ganglion cell population size by modulating ganglion cell production.

Generation and death of retinal ganglion cells Generation of retinal ganglion cells in mice begins on E11 and lasts until just before birth (Dräger, 1985). There is a short delay between neurogenesis and the time at which ganglion cell axons extend into the optic nerve (Colello and Guillery, 1992). This delay could deflate estimates of total cell production. However, very few ganglion cells are produced after E18 and as anticipated from the work of Colello and Guillery (1992), I did not find growth cones in neonatal optic nerves. It is therefore unlikely that the estimates of total production are biased downward by late ganglion cell generation.

In contrast, ganglion cell death begins at, or just before, birth, peaks between postnatal days 4–6, and is essentially complete by P12 (Linden and Pinto, 1985). At the peak of cell loss, between 5,000 and 10,000 ganglion cells are eliminated per (Williams et al., 1990). However, fewer than 2,000 cells are lost on the day of birth in mice, consistent with my observation of very few necrotic axons—less than 300 per nerve. At this rate it is improbable that more than a total of 10,000 ganglion cells are lost prenatally. In chickens there is as much as a three day delay between the onset of ganglion cell degeneration in the retina and the elimination of axons in the optic nerve (Rager et al., 1978). If there is a similar delay in mice then axon counts should preserve our production estimates. Nonetheless, the estimates of total production maybe biased downward slightly by the early loss of ganglion cell axons. However, the magnitude of this error is sufficiently small (~10,000 cells) that I did not think this loss warranted correction.

Variation in retinal ganglion cell death

The severity of cell death is close to 68–70% in most strains of mice. However, there are three exceptional strains with less severe loss. Three to nine percent fewer cells are lost in BXD32, CARL/ChGo, and BALB/cJ. BXD32 is particularly interesting because it has the highest adult population ($75,800 \pm 1,900$) among the 60 strains I have now examined. Yet at birth BXD32 has an unexceptional number—199,500—that is lower than three other strains. Clearly, one or more genes controlling rates of ganglion cell death are responsible for the high adult cell

number in this strain. It would be feasible to map a cell death gene by crossing BXD32 to a strain with similar ganglion cell production but higher cell death.

Variation in the severity of cell death may result from differences in titers of neurotrophic factors. The neurotrophins—BDNF and neurotrophin-3/4—have been found to increase survival of retinal ganglion cells in chicken and rat ([Ma et al., 1998](#); [Rosa et al., 1993](#)). Neuregulin, found on the cell surface and as a secreted protein, can also increase survival of neonatal rat retinal ganglion cells in culture ([Bermingham-McDonogh et al., 1996](#)). Differences in the concentration or expression time of these neurotrophic factors, their receptors, or components within their signaling pathways could produce variation in the severity of naturally occurring ganglion cell death.

Nnc1 controls cell production

Approximately 77% of the variation among adult strains result from differences in the production of ganglion cells. The percentage of cell death in high and low groups does not differ significantly (66.9% and 67.5%, respectively). I conclude that variation in adult ganglion cell number among inbred mouse strains results predominantly from differences in cell production. Comparison of our data with the Monte Carlo simulations ([Fig. 6.4](#)) corroborates this conclusion. A major motivation for undertaking the present study was to determine how and when allelic variants at *Nnc1* influence the size of the ganglion cell population. Collectively, the results strongly indicate that *Nnc1* modulates ganglion cell number by influencing cell production, and because ganglion cell production occurs before birth, this result provides a time frame for the action of *Nnc1*.

Chapter 7: Discussion

I have mapped eight major effect QTLs that modulate neuron number in mice. These QTLs are the first loci known to control normal variation in cell number in the vertebrate CNS. Identifying the specific genes that these QTLs reside in awaits candidate gene analysis. However, even if the QTLs are not cloned in the near future, once the mouse and human genome is completely sequenced, the identification of QTLs mapped in mouse will help to assign the function of genes. Today, with the advent of expressed site sequence tag mapping, genes are being mapped at a breakneck speed. Thus, the bottleneck now is identifying gene function and QTL mapping will certainly aid in this task.

In chapter 5, I mapped a QTL, *Nnc1*, that is responsible for more than half of the genetic variance in ganglion cell number in mice and generates the pronounced bimodality found among strain averages (see [Chapter 5](#), (Williams et al., 1998). *Thyroid hormone receptor alpha* is a superb candidate gene for *Nnc1* and the reduced ganglion cell number in the *Thra* null mice provides further support for *Thra*'s influence on ganglion cell number. Further support for *Thra* can be acquired by first identifying the genetic variant at the *Thra* locus and determining whether the variant is associated with variation in ganglion cell number in other strains.

The genetic variants can be found by sequencing the gene from the high and low strains. Sequencing has become so efficient that entire intervals have been sequenced to identify a genetic mutation ([Yu et al., 1996](#)). Another way to locate the genetic variants is to run short segments of the gene on a neutral 5% polyacrylamide gel and look for shifts in the band migration between the DNA from high and low strains. This technique is called single strand conformation polymorphism (SSCP) and can detect even a single point mutation ([Orita et al., 1988](#)).

A candidate gene must be proved definitively. Proof for a candidate gene can be obtained if the quantitative phenotype changes in a transgenic animal expressing the allelic variant. In the same concept, gene candidates can also be tested by transferring the QTL interval onto another background strain with the generation of a congenic mouse strain (Smithies and Maeda, 1995). For polygenic traits, such as epilepsy, congenics have been used to refine the location of putative QTLs and measure QTL effects (Frankel, 1995).

Nature of the QTL

Substantial trait variation within a population in the wild result from the absence of selective pressures and indicates that the trait is not important for survival. However, the magnitude of the variation in brain weight and ganglion cell among inbred strains is probably not representative of the wild populations from which they were derived. During the inbreeding process inbred strains may have encountered new selection pressures that could have resulted in a non-random selection of alleles. Thus, the allele frequency among inbred strains may not represent the wild populations from which they were derived. Surprisingly, the genetic divergence found among the inbred strains averages 40%, which is larger than that expected from mixing the progenitor subspecies (Crusio, 1992). The wide divergence between strains could result from a selection of heterozygosity during the early generations of inbreeding and then the subsequent fixation of one allele (Fitch and Atchley, 1985). Alternatively, the large genetic variation among mice could result from the absence of selective pressures in the sustained life of the laboratory mouse, allowing over generations the accumulation of mutations that would be deleterious in the wild. However, it is worth emphasizing that an extensive variety of mice were sampled from a wide range of ages, both sexes, and taken from different litters and different mothers within strains. The environmental range that we

have sampled is appreciable and is typical of most research colonies, perhaps even stable wild populations. Nevertheless, the specificity of the genetic variation should still be representative and the increased genetic diversity between inbred strains of mice is advantageous for the detection of quantitative trait loci with small effects.

Human brain size is roughly twice as big as that of a chimpanzee's yet their genetic code differs by only 1.6%. How is it that this small genetic difference can account for the substantial morphological differences found between chimpanzees and humans? It is possible if the genetic variation affects the expression parameters of key genes during development, such as growth hormones, which have widespread growth effects (Slack and Ruvkun, 1997). Genetic variation within key genes could produce effects by changing their level or timing of gene expression. For example, a higher level of IGF-I expressed under the control of the metallothioneine promoter in transgenic mice results in brains weighing 50% more and containing 21% more DNA compared to normal mice (D'ercole, 1993). Differences in the timing of gene expression can affect the timing of developmental events resulting in heterochronic differences between species. An example of heterochrony is the difference in duration of brain growth between chimpanzees and humans. Brain growth ceases at birth in chimpanzees, but continues to grow for another two years after birth in humans (Raff, 1996). Evidence that heterochrony exists in developing brain processes between species of *Mus* was found from like-genotype cells clustering in the brains of interspecies chimeric mice when they are typically intermixed within intraspecies chimeras (Goldowitz, 1989). There is no doubt that heterochrony plays a significant role in the generation of evolutionary differences.

Genetic variation has been found in two key regulatory genes that function in the somatotrophic growth axis. Size variants of *growth hormone* and *insulin growth factor 2*, are found to segregate in large and small body sized mice (Elliott et al., 1990; Winkelman and Hodgetts, 1992). How the genetic variants produce differences in body size are not

known. Alternatively, some genes may function only to create quantitative variation by tweaking the efficacy of key genes. Many genes are redundant, since knocking out some genes result in no obvious phenotype. This genetic redundancy could serve to maintain quantitative variation in the species. A species with an abundance of quantitative variation would be better equipped to adapt through natural selection in the presence of environmental pressure. However, it remains to be shown whether these "disposable" genes actually contribute to genetic variation.

Recently, much progress has been made characterizing the molecular basis of natural variation in bristle number in *Drosophila* (Long, 1995). Five QTLs that associate with variation in bristle number have been mapped to Chr 3. Genes that map nearby and are known to be involved in bristle formation were selected as candidate genes. One of the candidate genes is *Delta*. *Delta* is a transmembrane protein involved in the lateral inhibition process of bristle formation. *Delta* serves as the ligand for Notch and can activate the Notch pathway and suppress the neurogenic fate. A lower level of *Delta* expression in a cell would down regulate Notch leading to a neurogenic cell fate such as the bristle, an external sensory organ of the peripheral nervous system. Two sites that associate with variation in abdominal and sternopleural bristle number have been identified in the introns of the candidate gene *Delta*. How the sequence variants in *Delta*'s introns change *Delta*'s function and modify bristle formation is not yet known. However, if the two sites in *Delta* are located within enhancer sequences, which are known to reside within introns, the expression level of *Delta* could be altered. The two genetic variants in *Delta* are individually responsible for 12% and 6% of the total genetic variation in bristle number on Chr 3. This large effect size demonstrates that natural quantitative variation in vertebrates can result from a small number of loci with large effects.

Macromutations in genes that affect neuron number can provide insight into the genetic control of neuron number. For example, in *Drosophila* a mutation called gene

minibrain (*mnb*) results in a marked reduction in the optic lobes and central brain hemispheres. The *mnb* gene encodes a cell type-specific serine-threonine protein kinase involved in the regulation of cell division (Tejedor et al., 1995). The mouse and human homologs of *mnb* have been mapped to Chr 16 and 21, respectively (Shindoh et al., 1996). Transgenic mice carrying extra dosages of the gene *mnb* have learning deficits, implicating *mnb* as the critical gene that leads to the learning problems and smaller brain size characteristic of trisomy 21, or Down syndrome (Smith et al., 1997). Thus, the critical role *mnb* plays in cortical neurogenesis is conserved from *Drosophila* to humans. A mouse mutation called megencephaly causes hypertrophy of the brain resulting in a 25% larger brain size (Donahue et al., 1996). This mutation was mapped to mid-distal Chr 6 in the mouse. It is not known how the megencephaly mutation increases cell number or whether a human homolog is responsible for megencephaly-related syndromes in humans, e.g., Sotos syndrome, Robinow syndrome, Canavan's disease, and Alexander disease. Natural allelic variants in genes such as *mnb* and *megencephaly* could produce natural variation in brain weight.

The work presented here on the natural variation of neuron number in mice has provided a glimpse into the genetic bases of variation in neuron number. Our understanding of the genetic bases of natural variation in CNS structure both within and between species is still in its infancy. In light of the recent advances in mapping tools and genomic databases, the future holds much promise for rapid advances in our knowledge of the genetic bases of natural variation in the brain.

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