Martin Dunitz Taylor & Francis healthsciences

Genetic sources of individual differences in the cerebellum

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The highly regular anatomy of the cerebellum that results from myriad genetic, environmental, and stochastic events during pre- and postnatal development is nonetheless quantitatively very different among individuals. Understanding the sources of these individual differences represents an immense challenge to those interested in the cerebellum. Here we highlight the use of new methods to dissect individual differences to their genetic sources by reviewing quantitative trait locus mapping efforts in the mouse model system. We further suggest and illustrate how to combine these methods with other modern genetic techniques to accelerate our understanding. Finally, we embed these methods in a hypothetical line of cerebellar research to illustrate the vast potential of combining complex trait analysis with a systems neuroscience perspective.

Keywords:
quantitative trait locus – QTL mapping – complete trait analysis – individual differences – cerebellar size and form – foliation – gene microarray

Airey DC, Lu L, Shou S, Williams RW.
Genetic sources of individual differences in the cerebellum.
Cerebellum 2002; 1: 233–240

Introduction

Understanding the causes of individual differences in cerebellar structure and function will require a battery of techniques. These will include modern and classical genetic methods such as quantitative trait locus (QTL) mapping, targeted or random mutagenesis, gene expression microarrays, or the analysis of spontaneous mutants, inbred or artificially selected lines.[1] In this mini-review, we focus primarily on the use of quantitative trait locus mapping as applied to dissecting the sources of variation in the cerebellum among mice. QTL mapping defines the chromosomal locations of polymorphic gene loci (genes with multiple alleles segregating in a population) that generate variation in quantitative traits. We discuss QTL analysis of the adult mouse cerebellum, and review: (i) the importance of studying variation; (ii) the mechanics of QTL mapping; (iii) the heritability of variation in cerebellar structure; (iv) QTL studies of the mouse cerebellum, and (v) recent data from a new experimental design for QTL mapping. We provide supporting gene expression microarray data to show how the combined use of genetic methods can accelerate the analysis of complex traits. We end by suggesting a direction for future studies of the mouse cerebellum, in which the goal is to bridge genetic and behavioral differences with systems neuroscience.

Received 7 December 2001; Revised 21 March 2002; Accepted 22 March 2002

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The importance of studying individual differences

A quantitative trait such as cerebellar size is complex in origin, affected by many genes and the environment. The familiar bell curve typifies the continuous distribution of most complex traits. Quantitative trait locus mapping discovers a set of polymorphic gene loci that are collectively responsible for this variation by statistically associating phenotypic variation with genetic variation at defined genetic markers. Consequently, genes that have only a single allele cannot be mapped. This is not a flaw since only polymorphic genes are causal agents of trait variation. An important point is that the study of transgenic or knockout mice may be of little etiological importance to differences between individuals.[2] Spectacular failures in cerebellar development in various genetically engineered mice may point to genes critical to correct formation of the cerebellum (and as such are important; see ref. 3), but over-, under-, or null-expression of a gene that naturally has no allelic variants will not be informative as to why the trait varies in the population. The study of spontaneous or induced mutations can be a complementary approach to understanding individual differences when an allelic series is engineered or present, and duplicates or mirrors extant normal varations.[4] If the goal is to understand genes that

give rise to individual variation in normal traits, or disease traits that are complex in origin, QTL mapping is an appropriate beginning.[1]

The basic mechanics of QTL mapping

Statistically savvy geneticists are constantly devising new ways to map quantitative trait loci, and technical innovations are as much the norm in this rapidly moving field as in molecular genetics. We cannot present a full exposition of these methods, but for those interested in comprehensive introductions to QTL mapping methods, see Lynch and Walsh[5] or Liu.[6] For those with less interest in the statistical underpinnings of QTL mapping, we recommend a lucid review by Broman[7] and an online version of a short course in QTL mapping for neuroscientists at http://www.nervenet.org/papers/ShortCourse98.html. QTL mapping methods using mice differ in either or both the organization of the genomes used (e.g., intercross, backcross, recombinant inbred, advanced intercross, consomic, congenic, or outbred lines of mice) and the form of the statistical association evaluated (e.g., t-test, ANOVA, correlation, regression, linear model, nonparametric approaches, Bayesian approaches, etc.). However, all of these methods are fundamentally similar in that they relate phenotypic variation to genetic variation at defined chromosomal locations. For didactic purposes we will illustrate the simplest approach to QTL mapping for the reader; other methods can be considered refinements.

We will 'map' two QTLs controlling cerebellum structure in a set of recombinant inbred lines, the BXD lines. Recombinant inbred lines are derived from many generations of brother-sister matings from an initial cross of two inbred progenitor strains (in this case, C57BL/6J and DBA/2J). Being inbred, they are homozygous at all loci. They are called recombinant because, in the F2 and subsequent generations, chromosomes from the progenitor strains recombine and exchange parts. Thus each strain is a composite mix of the progenitor strains' alleles, and at any particular locus, any BXD strain will have a genotype of two C57BL/6J alleles (B/B) or two DBA/2J alleles (D/D). We gather 30 or so of these lines together and measure the mean weight of the cerebellum in each line. We might pause to consider the variation in this measure before proceeding. In particular, we might consider how the data are distributed, and we might ask if some of the variation is due to genetic effects. We can perform a test to check for normality and we can compare within-line variation (environmental and technical) to between-line variation (genetic) to guage genetic influence. Satisfied with our phenotype data (see Table 1 in ref. 8), we can proceed to gather genetic data. We need to know the genotype of each mouse line at multiple sites on all chromosomes. We evaluate each site for each line using a genetic marker, usually a simple sequence length polymorphim (SSLP), of which thousands have now been accurately mapped. Genotyping is performed using PCR and gel electrophoresis (e.g., http://www.nervenet.org/papers/PCR.html). Fortunately, because recombinant inbred lines are inbred and isogenic, their genotypes, already determined by other researchers, are archival and public domain;[9] in practice the strains do not need to be re-genotyped. We are now ready to associate the distribution of cerebellar weights and that of marker genotypes. The simplest method to evaluate potential associations uses analysis of variance (ANOVA) to compare the means of the phenotype split by genotype.

A statistically supported difference in the trait of comparison, split by marker class, is evidence that there is a QTL near the marker. Performing ANOVAs at over 300 markers, we discover two very significant (multiple-test corrected) results, on distal chromosome 1 with the marker "D1Mit150" and mid-distal chromosome 8 with the marker "D8Mit312". By displaying cerebellar weight by genotype graphically, the effect of the QTL is obvious (e.g., see Figure 4 in ref. 8).

Individual differences in mouse cerebella size and form

The basis of any QTL mapping study is heritable genetic variation. To establish heritability it is not good enough to know that single gene mutations affect the cerebellum. Rather, heritable variation can be easily demonstrated using isogenic lines of mice, where between-line (genetic) variation can be compared to within-line (environmental) variation. Inouye and Oda[10] first made a comprehensive but qualitative assessment of the foliation patterns of 13 inbred strains and noted appreciable between-strain variation but much less within-strain variation. Cooper and colleagues[11] conducted a diallele cross between four inbred strains (DBA/2J, C57BL/10J, BALB/cJ, SJL/J), examining the presence or absence of a single fissure. These authors convincingly demonstrated additive and dominance genetic variation, as well as maternal effects, for the presence or absence of the intraculminate fissure. Wahlsten and Andison[12] reported careful measurements of 12 cerebellar fissures (count and depth) of two inbred strains (C57BL/6ByJ, BALB/cByJ) and 7 derived recombinant inbred strains (the CXB). Their measurements showed continuity rather than dichotomy in the depth and number of cerebellar fissures, suggesting these traits are determined by many genes rather than by simple Mendelian inheritance patterns. The authors also showed that larger cerebella are moderately associated ($r = 0.45$) with deeper fissures and more sulci. Extending this work, Garretson and Neumann[13] examined the variance in fissure number in intercross mice

derived from the same inbred strains used by Wahlsten and Andison. By comparing F2 and F1 mice they estimated genetic variance to account for approximately 50% of the cerebellar variation they measured. In a companion study, Neumann et al.[14] examined total fissure number in a different set of 26 recombinant inbred strains—the BXD strains derived from the progenitors C57BL/6J and DBA/2J. Between-strain variance accounted for about two-thirds of the variance in fissure number. Analysis of cerebellar weight in the BXD strains by Airey et al.[8] showed genetic factors to account for roughly 50% of the variance. Collectively, these biometric studies of the mouse cerebellum show that its size and form are a complex product of multiple genetic and environmental effects. This suggests the cerebellum is amenable to QTL mapping with the goal of identifying the genetic sources of normal variation in its size and form.

QTL mapping of the mouse cerebellum: published results

In one of the earliest examples of QTL mapping in the CNS, Neumann, Garretson et al.[14] explored the genetic variation in folial patterning in BXD mice. Using t-tests, the authors compared fissure number from the cerebella of 26 BXD recombinant inbred strains with published strain distribution patterns (SDP) for 664 genetic markers. At two loci, the results were highly significant: *Cfp2 (Cerebellar folial pattern*) on Chromosome (Chr) 5 and *Cfp3* on Chr 7; that is, at each locus, the mean number of fissures for strains with C57BL/6J alleles differed from strains with DBA/2J alleles. According to Neumann (personal communication), these loci *Cfp2* and *Cfp3* act generally in that they affect multiple fissures (total number). Neumann, Mueller et al.[15] and Garretson and Neumann[13] detected one other locus on Chr 4, *Cfp*1, that apparently affects the presence or absence of three specific fissures, but not total fissure number.

As part of a research program targeting genetic influences on regional differences in brain structure in mice[8,16–18] and eventually cell number, we also examined the BXD recombinant inbred strains in a QTL mapping study, though our analysis was primarily directed at total cerebellar size ($N = 34$ strains), and the size of the cell-rich internal granule layer (IGL; $N = 31$ strains), rather than foliation. The results demonstrated QTLs on Chr 8 *(Cerebellar size 8*a, or *Cbs8*a) and Chr 1 *(Cbs1*a) with substantial additive effects. *Cbs8a* is particularly intriguing because this QTL is also associated with the volume of the cell-rich IGL and with fissure number, using data from.[14] Alleles from DBA/2J mice at *Cbs8a* are associated with increased cerebellar weight, volume, IGL volume, and fissure number. We hope the reader has noticed one of the key advantages of using recombinant inbred strains for QTL mapping: data are cumulative over time and across laboratories. Data gathered one year for the BXD strains can be compared or combined with that gathered the next, or multiple labs can tackle a difficult phenotyping effort collaboratively. One key disadvantage is that recombinant inbred strains are relatively few in number, but as discussed in the next section, solutions are already at hand.

The formation of folial patterns is thought to be related to the patterns of granule cell genesis in the cerebellum.[15,19] Wahlsten and Andison noted a moderate correlation between cerebellar weight and extra fissures and sulci, as stated above. Our finding that DBA/2J alleles at *Cbs8a* increase IGL volume and the number of fissures is also consistent with this hypothesis. In another tantalizing result based on observations that thyroxine is negatively associated with cerebellar growth and fissure number,[20,21] Neumann, Garretson et al.[14] determined from published data on serum T4 levels in 7 BXD strains[22] that the *Cfp2* SDP was significantly associated with both fissure number and T4 concentration. Although none of the QTLs for size or folia currently overlap the chromosomal location of thyroid hormone receptor alpha *(Thra* on Chr 11), it is easy to see from such an analysis that QTL mapping strategies can play an important role in functional, hypothesis-driven research programs.

Research by Le Roy-Duflos[23] best exemplifies a role for QTL mapping in the functional (behavioral) neurogenetics of the mouse cerebellum. Le Roy-Duflos conducted a QTL mapping study of F2 mice from NZB/BINJ and C57BL/6By progenitors, mapping not only the presence or absence of cerebellar folia, but also a measure of hindlimb motor coordination. She discovered two QTLs linked to the declival fissure and five QTLs linked to the intraculminate fissure, with no common genetic control of the two fissures. Of six QTLs mapped for hindlimb coordination, the confidence interval for *Tne-1q* (Chr 1, 87–100.2 cM) overlapped two QTLs affecting the declival fissure, *Cpfd-1* (51.1–94.1 cM) and *Cpfd-2* (83.6–107.6 cM). The co-localization of QTLs on Chr 1 suggests the impairment of locomotion is due to differences in cerebellar morphology generated by these loci.[23] It is noteworthy that *Cbs1a* also overlaps these loci.[8]

QTL mapping of the mouse cerebellum: recent data

Defining the locations of QTLs that affect cerebellum structure and function is feasible; we predict that in the next five years methods will become available to directly and routinely identify the underlying genes.[24] Currently this remains a challenging enterprise that will benefit from new methods and mapping resources. In this section we present recent unpublished data from a new

type of experimental cross. Full exposition of the uses and potential of this cross will be presented in another paper.[25]

A disadvantage of mapping QTLs using recombinant inbred (RI) strains is the small number of strains available. We have tested a diallele cross of one of the RI sets, the CXB. There are only 13 of these strains available, too few to map all but the QTLs with the largest effects. By crossing each strain with every other strain, this set expands to 78 nonreciprocal RIX strains. Mapping cerebellar weight in the 13 strains identifies no significant associations. In contrast, mapping cerebellar weight in the 78 RIX strains identifies up to 9 QTLs (Figure 1). Again, a QTL is found on distal Chr 1 that overlaps those determined by Airey et al.[8] and Le Roy-Duflos.[23] Currently, it is unknown what gene or genes on distal Chr 1 might cause these phenotypic differences. A number of candidate genes known to affect the cerebellum reside in this region (for discussion see refs 8 & 23). To create a more comprehensive list of candidate genes we have begun to build expression databases for the cerebellum using Affymetrix gene arrays.

A database of mRNA expression differences in cerebellar tissue can suggest candidate genes for QTLs.[26] Such a database would allow recovery of candidate genes based on the search criteria of co-localization with a given QTL. This approach can be refined by databasing gene array results from multiple lines of mice, perhaps a complete RI set. Genes near a QTL that express different amounts of transcript in a way that reflects the strain distribution pattern of alleles could be quickly recovered to provide strong candidate genes.

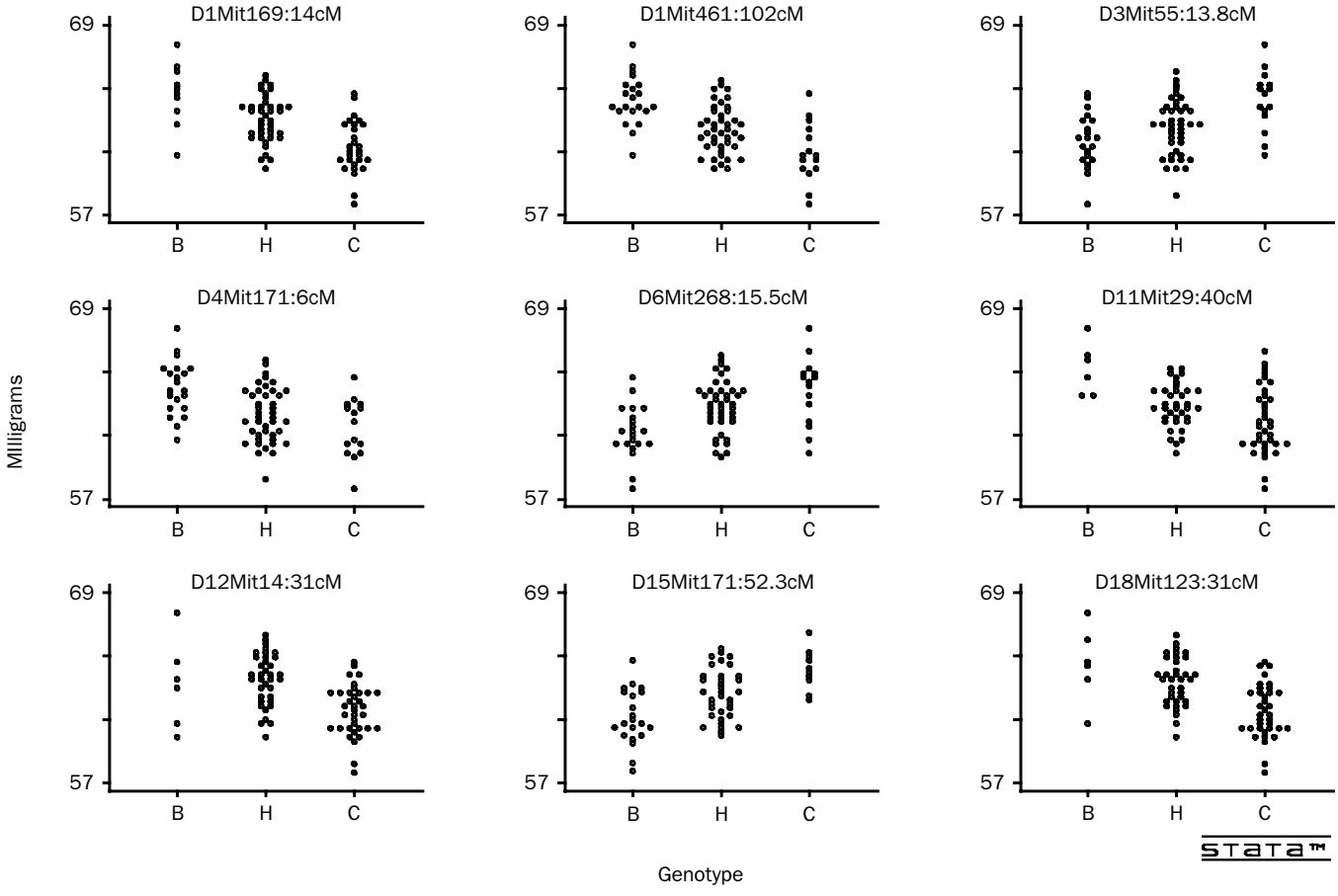

Figure 1
QTLs affecting cerebellar weight in CXB RIX strains. Dotplots of 78 RIX strain means for weight of the cerebellum by genotype at nine microsatellite markers. B is two BALB/cByJ alleles, C is two C57BL/6ByJ alleles, H is heterozygote. For each marker name, the number after the "D" indicates the chromosome number. Each dot is the mean value for about 10 mice. Cerebellar weight is post fixation and adjusted by linear regression to remove covariation with brain weight. The distribution of adjusted cerebellar weights is normally distributed with a mean ± 1 standard deviation of 62.6 ± 1.93 mg and a range of 10 mg. Overall differences by genotype at each locus are significant at $p < 0.05$, after correction for multiple tests across ~400 markers. The amounts of variance explained, additivity (per C allele) and dominance for these loci as determined by free model single marker association using Map Manager QTX software are D1Mit169:32%:-1.66 mg:+0.08 mg, D1Mit461:33%:-1.64 mg:-018 mg, D3Mit55:18%:+1.27 mg:-0.42mg, D4Mit171:20%:-1.25 mg:-0.45 mg, D6Mit268:20%:+1.30 mg:+0.31 mg, D11Mit29:19%:-1.65mg:-0.72 mg, D12Mit14:22%:-0.83 mg:+1.08 mg, D15Mit171:23%:+1.50 mg:+0.27 mg, D18Mit123:27%:-1.50 mg:+0.32 mg. The variance explained for the set of markers is 47%; this is less than the sum of the individual markers, because the markers share variance, otherwise known as non-syntenic association. Composite interval mapping, which tests the incremental effect of a marker above a background set, retains the distal Chr 1 locus and the proximal Chr 4 locus. Conservatively, evidence for two QTLs is strong, but it is imprudent to discount the other seven QTLs, because these data are correlational.

Integration of gene array methods with QTL analysis reaches its zenith if we use the expression results for a set of RI lines as phenotypes for QTL mapping. There is no academic reason not to pool cerebella from many isogenic individuals and run as many as 10 arrays per line to gain hundreds or thousands of expression phenotypes. This would allow us to parse variation in expression levels into a large and extremely interesting set of QTLs. We have begun integrating our QTL analysis of the mouse cerebellum with array technology by looking at expression differences in the CXB recombinant inbred mice (Figure 2, Table 1).

QTL mapping and cerebellar function: eyeblink classical conditioning

We have shown that QTLs for global and local cerebellar traits can be readily detected. Furthermore, it is likely that these QTLs may bias individual differences in some of the diverse motor or cognitive processes of the cerebellum.[27] Le Roy-Duflos[23] has already provided an example tying structure to function with a set of QTLs. Given the manifold and indirect relationships between gene, brain and behavior, we think QTL analysis of cerebellar function can bear fruit if care is taken in selecting the behavior and controlling neural system.

As neuroscientists interested in the cerebellar function, how can we leverage QTL analysis to make the leap from genes to brain differences and then to individual differences in behavior? In order to construct a truly synthetic and hierarchical analysis of the cerebellum, we ideally need to start with a well-described, non-trivial behavior in which the controlling neural circuitry is established, amenable to quantitative stereological methods and one which shows some kind of parametric relation to heritable behavioral variation. A high degree of experimental control, conservation across species, particularly mice and humans, and the chance to integrate across other neural systems would also be beneficial. A particularly rich literature describes the role of the cerebellum in learning and memory and the formation of classically conditioned eyeblink reflexes.[28,29] Microstimulation, cluster and unit recording, and lesion data indicate that the anterior interpositus nucleus

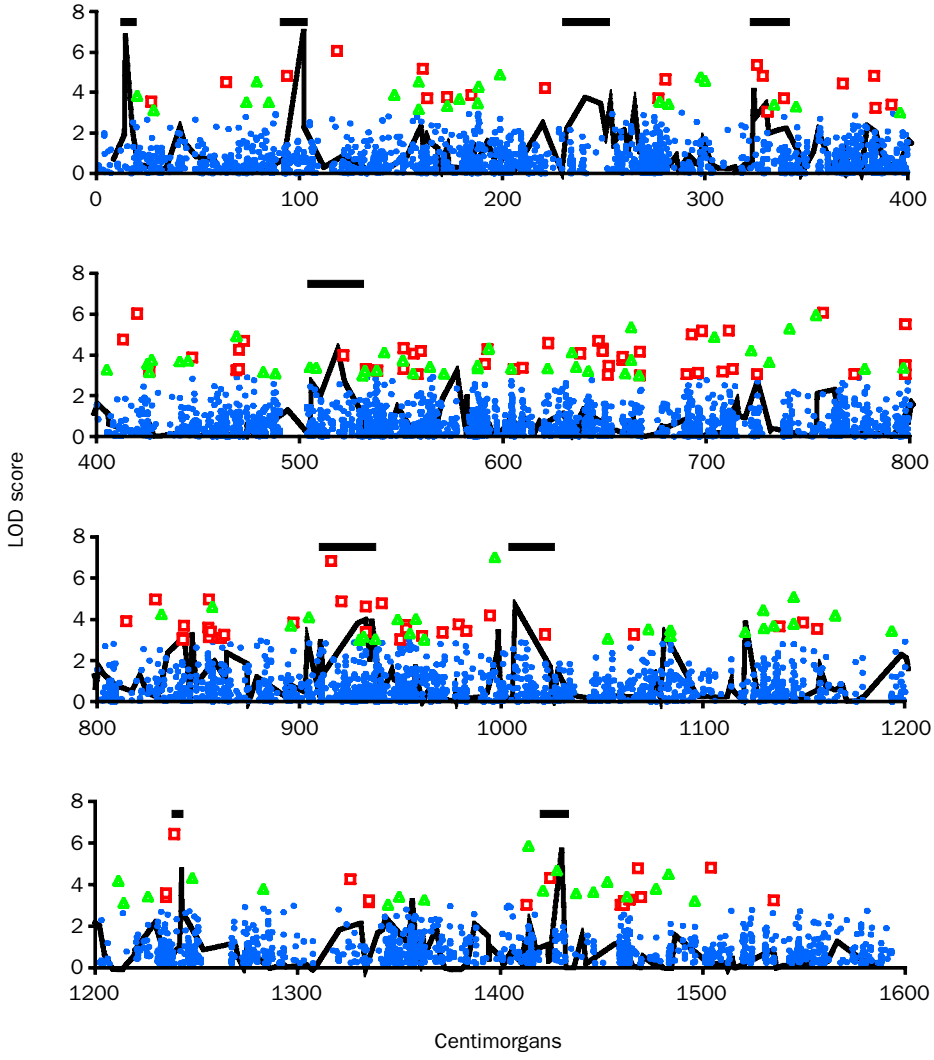

Figure 2
Combining QTL and gene array results to discover candidate genes for RIX cerebellar loci. This plot shows the connected LOD scores (black line) computed for the association of the distribution of alleles from 78 RIX strains with cerebellar weight at ~400 genetic markers spanning the genome. LOD = 4 represents significance after correction for multiple tests. Black bars at the top of the graph indicate the 2-LOD support interval containing the QTLs reported in the text and shown in Figure 1. Plotted in colored symbols are the LOD scores for differences in expression levels of 6909 mapped genes between adult mouse cerebella and forebrain. Differences were determined using Affymetrix U74Av2 GeneChips. Twenty-three chips were used. Values were averaged across chip for adult mouse forebrain tissue and cerebellum tissue. Paired t-tests were performed between forebrain and cerebellum expression levels for the 6 strains C57BL/6ByJ, CXB5, CXB6, CXB7, CXB10, and CXB12. Probabilities from the resulting t-statistics were transformed to LOD scores by –log(t prob). Blue circles represent expression levels below a LOD of 3 regardless of the direction of difference, red squares represent higher expression levels in the cerebellum, green triangles lower expression levels in the cerebellum. LOD = 3 represents a reasonable exploratory stringency given the number of t-tests performed; not all genes on the U74Av2 chip are different. Candidate genes from this analysis are tabulated in Table 1.

Table 1
Candidate genes for cerebellar weight QTLs

Chr:cM	Genbank	Symbol	Name	Paired t-test	LOD	Level in Cerebellum
1:94	AF016697	Dfy	Duffy blood group	2.00E-05	4.8106	high
2:106	AF004294	Mytl	Myelin transcription factor 1	6.00E-05	4.2065	high
4:7.7	X61397	Calsl	Carbonic anhydrase-like sequence 1	4.00E-06	5.3551	high
4:10.5	D26352	Calbl	Calbindin 1, 28 kD	1.00E-05	4.8288	high
4:13	AI847837	Casp8ap2	Caspase 8 associated protein 2	0.0009	3.0594	high
4:21	D17546	Col15a1	Procollagen type 15, alpha 1	0.0002	3.6917	high
6:18.5	D28492	Casp2	Caspase 2, see Nedd2	0.0001	3.988	high
11:23	X51986	Gabra6	Gamma aminobutyric acid receptor, alpha 6	1.00E-07	6.825	high
11:28	AB016257	Slc22a4	Solute carrier family 22 (organic cation transporter), member 4	1.00E-05	4.8699	high
11:40	AI504474	Atp2a3	ATPase, Ca++ transporting, ubiquitous	2.00E-05	4.6201	high
12:46	D37801	Ptpn21	Protein tyrosine phosphatase, non-receptor type 21	0.0006	3.2551	high
15:50	Y13439	Mapk12	Mitogen-activated protein kinase 12	4.00E-07	6.4161	high
18:26.5	D45203	D0H4S114	P311 protein	5.00E-05	4.3147	high
4:16.1	AB015200	Hpca	Hippocalcin	0.0004	3.398	low
6:2	AA733372	D6Wsu176e	DNA segment, Chr 6, Wayne State University 176, expressed	0.0004	3.3865	low
6:5	D17584	Tac1	Tachykinin 1	0.0004	3.3614	low
11:39	X68273	Cd68	CD68 antigen	0.0007	3.1391	low
18:30	X06368	Csflr	Colony stimulating factor 1 receptor	2.00E-05	4.6643	low

which receives Purkinje neuron input from the lobulus simplex (HVI) is critical to conditioned eyeblink reflexes. Research by Woodruff-Pak and colleagues[30–33] shows that Purkinje cell number (in rabbits) and cerebellar volume (in humans) predicts the rate of conditioning. More Purkinje cells or more cerebellar volume results in more rapid conditioning. In mice, there is already evidence of strain differences in conditioning rates.[34] Finally, different forms of eyeblink classical conditioning engage hippocampal function as well (e.g., trace conditioning), providing the potential for an analysis of multiple memory systems.[35] These observations immediately suggest a line of research that we briefly delineate for the reader.

As stated, in the only inbred strain comparison of eyeblink conditioning, Bao et al.[34] convincingly demonstrated differences in learning capacity between the two common inbred strains C57BL/6 and BALB/c. The BALB/c mice showed more eyeblink conditioned responses over an extensive 10 day period of delay conditioning. BALB/c mice also showed significantly greater conditioned response amplitudes in the EMG eyeblink measures. C57BL/6 and BALB/c are closely related to the parental strains of the CXB recombinant inbred strains (C57BL/6ByJ and BALB/cByJ). We suggest that eyeblink conditioning in the CXB RI hybrids above and stereological examination of their cerebella would provide the basis for important QTLs controlling Purkinje cell number and learning capacity. A few fascinating twists can be added.

First, Nowakowski[36] demonstrated a CA3 hippocampal lamination defect (Hld) in BALB/c. In experimental crosses with C57BL/6J, transmission was consistent with a single autosomal dominant gene. This leads to an exciting opportunity to investigate the effect of this mutation in hippocampal-dependent trace conditioning procedures in the CXB RIX mapping panel, and potential interactions with QTLs controlling delay conditioning, which does not require hippocampal function.

Second, research by Woodruff-Pak and colleagues[30–33] has demonstrated an age-related decline in Purkinje cell number that is associated with decline in eyeblink acquisition performance. Adding an aged cohort could provide insight into genetic mechanisms mediating degeneration, or conversely, robust aging.

Third, the environmental manipulations of caloric restriction, enriched environments, and exercise could be employed to understand important norms of reaction of eyeblink conditioning QTLs. Caloric restriction has been shown to have antiaging effects[37,38] and is therefore a complementary environmental manipulation to the aging component above. Two inbred 13 strains are available from the National Institute on Aging that have been calorically restricted: C57BL/6 and BALB/c. Enriched environmental manipulations have a long history of published effects on neural development,[39,40] most recently highlighted by the work of Gage and colleagues.[41] Neurobehavioral genetic studies of eyeblink conditioning in mice in impoverished and enriched environments could differentiate QTLs that are dependent on these environments. Lastly, exercise has been shown to modulate neurogenesis in the hippocampus[42] and angiogenesis in the cerebellum.[43] Exercise could thus also be used to investigate environmental modulation of eyeblink conditioning QTLs.

Eyeblink classical conditioning in mice meets all of our requirements above (and more) and should therefore be a focus of future neurogenetic analyses of cerebellar structure and function. A seminal gene array study has already been reported that highlights the set of mRNA expression differences between conditioned and unconditioned rabbits.[44] Presumably, by using pooled tissue from isogenic RIX lines, a similar array study in mice could point to the subsets of genes responsible for individual differences in acquisition performance, rather than conditioned versus unconditioned states.

Conclusions

This review has focused on the use of quantitative trait locus mapping as a tool to accelerate our understanding of the causes of individual differences in cerebellar structure and function. Our intention has been to highlight the as yet sparse literature of cerebellar QTLs and to illustrate how this approach can be combined with other modern genetic tools. Finally, by illustrating the hypothetical use of QTL methods in a well-studied neural system, we hope to have mainstreamed its potential for the researcher interested in cerebellar structure and function.

Acknowledgments

The authors thank Dr Gu, Dr Kulkarni, Shu Hua Qi, Emily Carp, and Allison Wilkes for assistance in the laboratory. We thank Divyen Patel (www.genome-explorations.com) for his support of the Affymetrix experiments. We thank Dr Christine Collins for critical reading of the manuscript. This study was supported by a Human Brain Project/Neuroinformatics program funded jointly by the National Institute of Mental Health, National Institute on Drug Abuse, and the National Science Foundation (P20-MH 62009). Animal use permission: IACUC UTHSC approval number 680. This mini-review has been solicited by Mario-Ubaldo Manto, MD, PhD.

Note

[1] A diallele cross, and the relatively sophisticated ANOVA used to analyze it, compares several strains and all F1 crosses between them, and allows decomposition of the trait variance into several parts.

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