

Natural variation and genetic covariance in adult hippocampal neurogenesis

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Adult hippocampal neurogenesis is highly variable and heritable among laboratory strains of mice. Adult neurogenesis is also remarkably plastic and can be modulated by environment and activity. Here, we provide a systematic quantitative analysis of adult hippocampal neurogenesis in two large genetic reference panels of recombinant inbred strains (BXD and AXB/BXA, $n = 52$ strains). We combined data on variation in neurogenesis with a new transcriptome database to extract a set of 190 genes with expression patterns that are also highly variable and that covary with rates of (i) cell proliferation, (ii) cell survival, or the numbers of surviving (iii) new neurons, and (iv) astrocytes. Expression of a subset of these neurogenesis-associated transcripts was controlled in cis across the BXD set. These self-modulating genes are particularly interesting candidates to control neurogenesis. Among these were *musashi* (*Msi1h*) and *prominin1/CD133* (*Prom1*), both of which are linked to stem-cell maintenance and division. Twelve neurogenesis-associated transcripts had significant cis-acting quantitative trait loci, and, of these, six had plausible biological association with adult neurogenesis (*Prom1*, *Ssbp2*, *Kcnq2*, *Ndufs2*, *Camk4*, and *Kcnj9*). Only one cis-acting candidate was linked to both neurogenesis and gliogenesis, *Rapgef6*, a downstream target of ras signaling. The use of genetic reference panels coupled with phenotyping and global transcriptome profiling thus allowed insight into the complexity of the genetic control of adult neurogenesis.

gene array | hippocampus | precursor | quantitative trait loci | stem cell

Neurogenesis in adult mammals is modulated by complex interactions among genetic and environmental factors. Our goal is to use a systems genetics approach to determine how the development of new neurons and glia is modulated by gene polymorphisms, activity, and environmental stimuli. A first step toward this goal is to analyze natural variation and genetic covariance among key neurogenesis parameters such as proliferation, survival, and differentiation of new cells. We have shown that natural variation is substantial among different strains of mice (1–3). The normal range of variation often exceeds the effects of single gene mutations in engineered lines of mice.

In this study, we extended this analysis of normal variation and applied a systems genetics approach to study the covariance structure of four key parameters of adult hippocampal neurogenesis across two large genetic reference populations consisting of a total of 52 recombinant inbred strains. This approach allowed us to exploit covariance of diverse traits to demonstrate biological linkage and pleiotropy (4, 5). The analysis, however, extends beyond a correlative approach. Both of the genetic reference populations used, the BXD and AXB/BXA sets, are standard mapping panels, making it possible to search for gene loci, so-called quantitative trait loci (QTL), that produce common variation and pleiotropy among traits linked to adult hippocampal neurogenesis.

Adult hippocampal neurogenesis originates from proliferating neural-precursor cells in the adult dentate gyrus and proceeds over a number of identifiable stages to the long-term survival of a new granule cell (6–8). In the course of this development, two key phases can be distinguished: an expansion phase, in which the

number of immature postmitotic neurons is increased, followed by a phase of selective, long-lasting survival (9, 10).

Strain differences in adult hippocampal neurogenesis in mice are observed in cell proliferation and survival (3), the distribution of phenotypes among the newly generated cells (1, 2), and how different stages of neuronal development are activated by stimuli that induce adult neurogenesis (11). Strains DBA/2J and A/J show particularly large differences in adult neurogenesis from the canonical mouse strain, C57BL/6J (1). In this study, we explored natural variation in adult hippocampal neurogenesis in two complementary sets of recombinant inbred (RI) strains of mice (BXD and AXB/BXD), which represent inbred progeny of F₂ crosses from C57BL/6J and DBA/2J (BXD), and C57BL/6J and A/J (AXB/BXA) (12–14). On average, and in large panels, each RI strain shows an $\approx 50:50$ pattern of genome inherited from the parental strains and is homologous at every locus. The GeneNetwork (www.genenetwork.org) is an open-access resource that contains genomic and phenotypic information on RI strains (15). Complex traits like neuronal development are likely to show associations with many gene loci. The high number of candidate genes and the lack of sufficiently large breeding panels to increase mapping precision still limit the use of RI strains for the direct identification of quantitative trait genes (16, 17). The combination of expression genetics with classical linkage analysis, however, allows the *in silico* identification of candidate genes controlling polygenic phenotypes as complex as adult neurogenesis and, at the same time, reveals insights into regulatory transcriptional networks underlying such phenotypes (18).

Genetic polymorphisms influence systems-level phenotypes through a network of genes. The small molecular variation is a naturally occurring perturbation of this network that can reveal the genes that comprise it. Discovering this network and the consequences of this variation are facilitated by the use of genetic reference populations. This systems-genetics approach allows one to relate gene-expression data to phenotypes across biological scale. Adult neurogenesis is a phenotype that is particularly amenable to this type of dissection. Fig. 1 outlines the experimental design and the rationale for our study.

Results

Strain Differences in Adult Neurogenesis. We quantified adult hippocampal neurogenesis in 29 BXD and 23 AXB/BXA strains and in the three parental strains, C57BL/6J, DBA/2J, and A/J (see Table 2, which is published as supporting information on the PNAS

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Abbreviations: ASTR, astrocytes; LRS, likelihood ratio statistic; NEUR, neurons; PROL, proliferation; RI, recombinant inbred; SURV, surviving; QTL, quantitative trait loci.

Data deposition: Phenotypes measured in this study were deposited in the GeneNetwork database, www.genenetwork.org (IDs 10795–10798).

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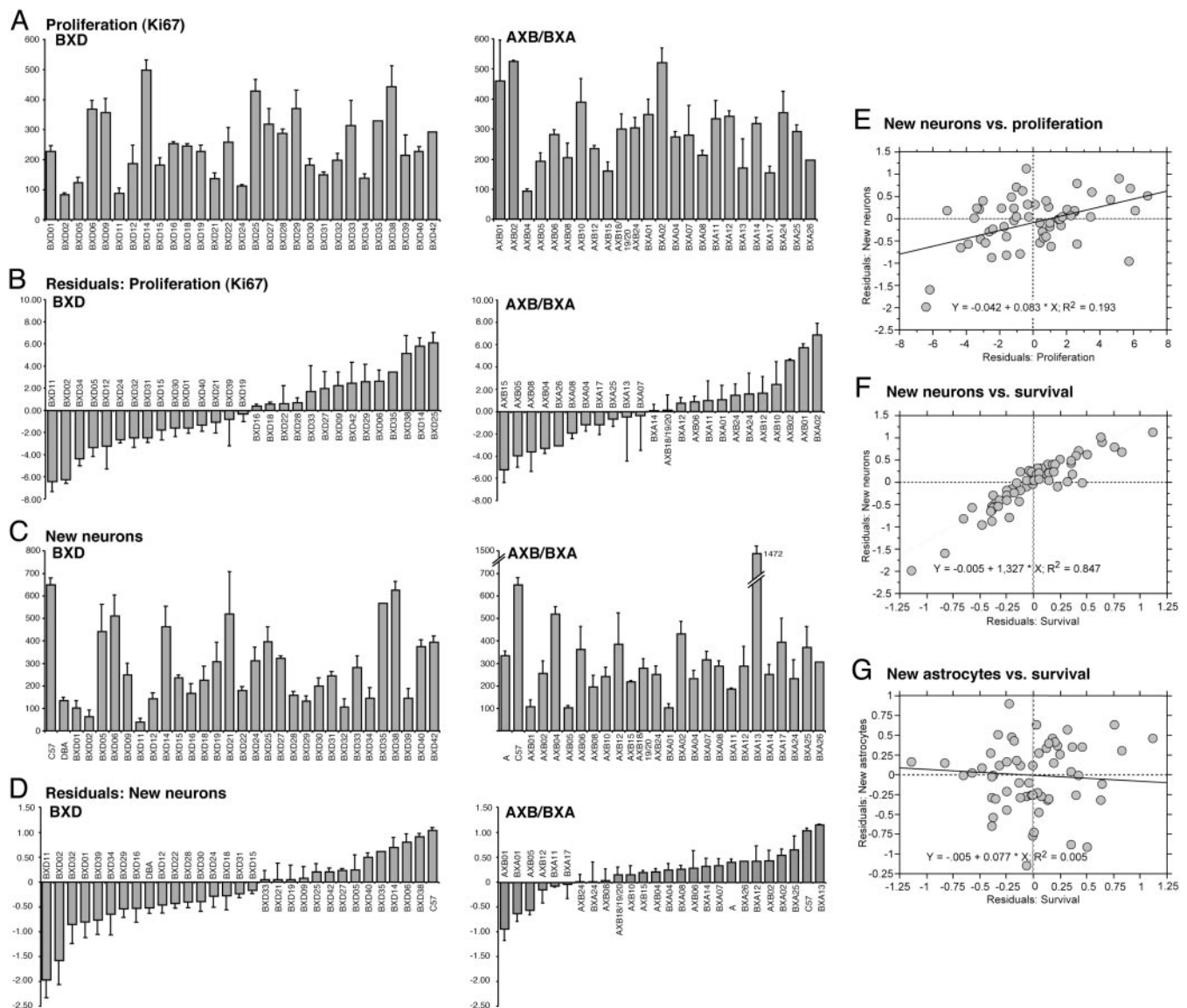


Fig. 2. PROL and number of new NEUR in BXD and AXB/BXA strains of mice. (A) The raw data as assessed by Ki67 immunohistochemistry in the subgranular zone of the dentate gyrus. (B) The residuals after correction for age effects (square transformation). (C) The raw data as assessed by BrdUrd and NeuN immunohistochemistry in the subgranular zone of the dentate gyrus, 4 weeks after the BrdUrd injections. (D) The residuals after correction for age effects (log transformation). The analogous information for SURV and ASTR is found in Fig. 5. (E–G), Predictive power of PROL and SURV for NEUR and ASTR. Regression analysis of the residuals was performed. (E) Cell proliferation was significantly correlated with the number of new NEUR ($P = 0.0006$), but $r^2 = 0.193$. (F) SURV, as assessed by the total number of BrdUrd-labeled cells, irrespective of their phenotype, was also significantly correlated with the number of new NEUR ($P < 0.001$), but, here, $r^2 = 0.847$, indicating a high predictive power of SURV for NEUR. (G) This link was not apparent for ASTR, where SURV was not correlated with NEUR ($P = 0.5628$; $r^2 = 0.005$).

sion in the adult brain. Our study linked the classical analysis of a physiological quantitative trait with the concept of “expression genetics” (21), that is, the genetics of how genes control other genes. The linkage analysis for our four phenotypes (Fig. 3) revealed that a very large number of gene loci are associated with adult neurogenesis, making it unlikely that one QTL with dominating influence on the phenotype will be identifiable. By investigating F1 generations, so-called recombinant intercrosses (22), or congenic lines (23), the search for master regulatory QTL for adult neurogenesis could, in principal, be refined. The need for very large breeding panels to achieve the precision required to confirm single loci makes this approach problematic. This method has been successfully exploited in only relatively few cases (24). Despite the general relevance of direct QTL mapping, the strategy to study associations across physiological and ex-

pression phenotypes adds an entirely different dimension to the genetic analysis of complex traits. The goal is not so much to describe the function of isolated genes (as would be the case in classical QTL mapping) but rather to understand the contribution of regulatory networks to a given phenotype. Irrespective of this, the sensitivity of QTL mapping depends on the precision with which the quantitative trait can be defined and measured. With the identification of further specified subphenotypes in adult neurogenesis (e.g., the dynamics of particular stages of development), the identification of narrow QTLs will become possible.

We show that PROL has a low predictive power ($\approx 19\%$) for NEUR, despite the fact that both traits are correlated and causally related. Similarly, we found that ASTR is quantitatively independent of NEUR. The finding that the genetic determinants of SURV

Table 1. Cis-acting transcripts covarying with adult neurogenesis

Name	Symbol	Description	Ch.	Position	PROL	SURV	NEUR
PROL, SURV, NEUR							
1	1444667_at.B Brdt	bromodomain, testis-specific	5	106.44	-0.6146	-0.7413	-0.8008
11	1444306_at.B Msi1h	Musashi homologue 1(Drosophila)	5	114.56	0.5342	0.6618	0.6387
39	1449435_at.A B4galt3	UDP-Gal:betaGlcNAc β 1,4-galactosyltransferase, polypeptide 3	1	171.21	0.5123	0.5405	0.5667
SURV, NEUR							
2	1434933_at.B 5730557L09Rik	RIKEN cDNA 5730557L09 gene	1	160.88	0.7043	0.6278	
16	1417024_at.A Hars	histidyl-tRNA synthetase	18	36.99	-0.5944	-0.5821	
26	1429951_at.B Ssbp2	single-stranded DNA binding protein 2	13	87.70	0.5576	0.5871	
32	1453170_at.B 2610206C24Rik	RIKEN cDNA 2610206C24 gene	18	32.15	-0.5677	-0.5614	
37	1420800_a.at.A Kcnq2	potassium voltage-gated channel, subfamily Q, 2	2	180.83	0.5795	0.5346	
52	1451096_at.A Ndufs2	NADH dehydrogen, (ubiquinone) Fe-S protei 2	1	171.16	-0.5561	-0.5364	
58	1439843_at.B Camk4	calcium/calmodulin-dependent protein kinase IV	18	33.42	0.5136	0.5708	
70	1459001_at.B Vps33a	vacuolar protein sorting 33A (yeast)	5	122.73	0.5426	0.5328	
73	1417697_at.A Soat1	sterol O-acyltransferase 1	1	156.34	0.5382	0.5341	
78	1450712_at.A Kcnj9	potassium inwardly-rectifying channel, J, 9	1	172.39	-0.5280	-0.5375	
84	1433690_at.A 2210016L21Rik	RIKEN cDNA 2210016L21 gene	5	114.06	-0.5465	-0.5132	
90	1431493_at.B 9530046B11Rik	RIKEN cDNA 9530046B11 gene	5	116.25	-0.5176	-0.5350	
94	1424748_at.A Galnt11	UDP-N-acetyl-α-D-galactosamine	5	23.73	-0.5369	-0.5112	
97	1418839_at.A Glmn	glomulin, FKBP associated protein	5	106.62	-0.5268	-0.5185	
PROL, NEUR							
13	1433646_at.B Mrps27	mitochondrial ribosomal protein S27	13	95.60	0.5569	0.5198	
25	1460175_at.A Rps23	ribosomal protein S23	13	87.06	-0.5041	-0.5029	
PROL, SURV							
2	1419700_a.at.A Prom1	prominin 1	5	42.75	-0.6526	-0.5097	
NEUR, ASTR							
1	1427412_s.at.A Rapgef6	Rap guanine nucleotide exchange factor (GEF) 6	11	54.45	-0.6026	0.6251	

Cis-acting transcripts whose expression is correlated with two or more of the four neurogenesis phenotypes. Transcripts with significant QTL at the site of their physical location are in boldface type.

related to stem-cell activity in the developing and adult brain. Both appear to play a major role in the control of stem-cell maintenance (self renewal) and asymmetric stem-cell divisions (25, 26). *Msi1h* showed the second highest of all correlations and was correlated to PROL, SURV, and NEUR.

Another stem-cell gene not previously related to neural stem cells is *Camk4*, which is a maintenance factor for hematopoietic stem cells (27). *Camk4* is expressed in the adult brain; deletion of *Camk4* causes a cerebellar defect (28). A role of *Camk4* is assumed in the consolidation of long-term memory, a centrally hippocampal function (29). In ganglionic neurons, *Camk4* promoted survival and axonal growth (30). We found an association with SURV and NEUR, which might hint at a similar role in the context of hippocampal neurogenesis.

Ndufs2 mutations are part of complex I deficiency disorders such as Leigh disease and mitochondrial diseases that include encephalopathies (31). A particular hippocampal function has not yet been reported, but in a rat model of depression, NADH dehydrogenase was up-regulated in the hippocampus (32), and a role for NADH

dehydrogenases has been suggested in the context of neurodegenerative disorders (33). *Ndufs2* was inversely related to the adult-neurogenesis phenotypes, consistent with the idea of neurogenesis being down-regulated in depression and neurodegeneration.

Ssbp2 is a tumor-suppressor gene whose overexpression in hematopoietic stem cells is accompanied by C-myc down-regulation (34). *Ssbp2* was positively correlated with SURV and NEUR, suggesting that, as in myeloid cells, *Ssbp2* might be linked to growth arrest and cellular differentiation (35).

Mutation of *Kcnq2* is associated with benign familial neonatal convulsions, a self-remitting developmental disorder. A role in neuronal maturation has been hypothesized, because *Kcnq2* might mediate inhibitory action during that phase of development of glutamatergic neurons in which GABAergic interneurons provide a transient excitatory input to the new cells (36). A transient GABAergic innervation has also been found in hippocampal progenitor cells *in vivo* (37). A short splice variant of *Kcnq2* is detectable in undifferentiated neuronal cells (38).

Kcnj9 has been identified as the likely quantitative trait gene controlling basal locomotor behavior (39). *Kcnj9* is highly expressed in strains with low basal locomotor activity. Physical activity induces and maintains adult neurogenesis (40). We found a negative correlation between *Kcnj9* expression and adult neurogenesis.

Five of the cis-acting transcripts did not suggest any known link to neuronal development. Of all these transcripts, *Brdt* showed the strongest, albeit negative, association with adult neurogenesis ($R = -0.8$). *Brdt* was considered to be specifically expressed in mid- to late spermatocytes (41), but is also expressed during brain development (42). Polymorphisms in *Soat1* (ACAT1) have been identified as risk factors in Alzheimer's disease (43). The function of *Hars* lies in protein biosynthesis; a negative correlation with adult neurogenesis is not very plausible based on the currently available information. Two transcripts were related to the Golgi system. *Galnt11* is a calcium-binding protein of the Golgi membrane.

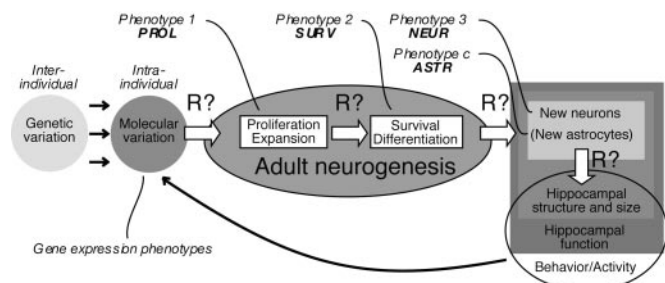


Fig. 4. Conceptual scheme highlighting the relationship between genomic factors, gene expression, and measured phenotypes of adult neurogenesis.

Vps33a is involved in the formation of cellular vacuoles and the trafficking between Golgi and lysosomes (44).

The only cis-acting gene whose expression levels linked neurogenesis and astrogenesis was *Rapgef6*, a downstream target of *m-Ras* and, as such, involved in cytoskeleton reorganization. Increased *Ras* in neural stem cells was found to lead to an increase in gliogenesis (45). *Rapgef6* expression was positively linked with astrogenesis and negatively linked to neurogenesis.

Our study shows that several genes with correlating expression patterns rather than single master switches control complex phenotypes like adult neurogenesis. The structure of the genetic network built by these genes remains unknown at present. These data suggest that scientific approaches studying one gene at a time will not reveal much about the main principles governing the genetic control of complex phenotypes like adult neurogenesis.

Methods

Animals. Twenty-nine BXD/Ty and 23 AXB/BXA RI strains, and the three parental strains C57BL/6J, DBA/2J, and A/J were obtained from The Jackson Laboratory. The BXD strains were generated by crossing C57BL/6J and DBA/2J mice. BXD strains are inbred lines derived from brother–sister matings starting from an F₂ cross (12, 13). AXB/BXA are based on reciprocal intercrosses between C57BL/6J and A/J (14). Animals were kept in conventional laboratory cages with standard food and access to food and water ad libitum. We studied five animals from each of the parental strains, three each of the great majority of recombinant inbred strains, two each of AXB1, AXB2, AXB4, BXA12, BXD2, BXD18, and BXD35, and one BXA26 animal. The mice were between 36 and 106 days old, but most were close to 70 days (Table 2). All were females.

Immunohistochemistry. All mice received one daily i.p. injection of BrdUrd for five consecutive days. Four weeks after the final BrdUrd injection, the mice were killed and their brains processed as described in ref. 10. Immunohistochemistry for proliferation marker Ki67 [polyclonal rabbit antibody, Novocastra (Newcastle Upon Tyne, U.K.) 1:500], BrdUrd [monoclonal rat, Harlan Sera-lab (Loughborough, Leicestershire, U.K.) 1:400], neuronal marker

NeuN (monoclonal mouse, Chemicon, 1:100), and astrocytic marker S100 β (polyclonal rabbit, Swant, 1:2,500) was performed and analyzed quantitatively as described in ref. 10.

Correction for Age Effects. Because age is a strong negative regulator of adult neurogenesis (19), data were corrected for age effects. Modeling was performed by using SAS PROC GLM, v 9.0 (SAS Institute, Cary, NC). Residual diagnostics were performed by using PROC PLOT, PROC UNIVARIATE, and PROC CORR.

Heritability-Estimation Procedures. For each trait, heritability was estimated by use of SAS PROC VARCOMP, v 9.0 (SAS Institute). Variance components were estimated by using the restricted-maximum-likelihood method. The heritability was calculated as the ratio of between-strain variance to total (between-strain plus within-strain) variance.

Correlation Comparisons and QTL Analysis. Assessment of phenotypic covariance and QTL analysis were performed with the GeneNetwork (www.genenetwork.org). Phenotypes measured in our study were deposited in the public GeneNetwork database (record IDs 10795–10798). Pearson product-moment correlations for the corrected numerical values were calculated as implemented in GeneNetwork. Details of the transcript expression data are described in ref. 4. Linkage analysis in WebQTL (the mapping module of the GeneNetwork) is based on a set of >7,482 informative SNPs (Wellcome-CTC SNP data set) plus 1,500 microsatellite markers distributed across all autosomes and the X chromosome. Genome-wide significance levels for assessing confidence in linkage statistics were estimated by comparing the peak LRS of correctly ordered data sets with LRS computed for 1,000 or 2,000 permutations.

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