Genetic modulation of horizontal cell number in the mouse retina

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Neuronal populations display conspicuous variability in their size among individuals, but the genetic sources of this variation are largely undefined. We demonstrate a large and highly heritable variation in neuron number within the mouse retina, affecting a critical population of interneurons, the horizontal cells. Variation in the size of this population maps to the distal end of chromosome (Chr) 13, a region homologous to human Chr 5q11.1–11.2. This region contains two genes known to modulate retinal cell number. Using conditional knock-out mice, we demonstrate that one of these genes, the LIM homeodomain gene *Islet-1* (*Isl1*), plays a role in regulating horizontal cell number. Genetic differences in *Isl1* expression are high during the period of horizontal cell production, and *cis*-regulation of *Isl1* expression within the retina is demonstrated directly. We identify a single nucleotide polymorphism in the 5' UTR of *Isl1* that creates an E-box sequence as a candidate causal variant contributing to this variation in horizontal cell number.

allele-specific expression | follistatin | quantitative trait locus | recombinant inbred strain

The cellular architecture and connectivity of the vertebrate retina is largely conserved across species. What distinguishes these retinas most is their variation in size, in the numbers of each of the different cell types, and in their relative proportions. For instance, old world primates display a two- to threefold variation in the number of cone photoreceptors in the fovea (1, 2), and a twofold variation in the total number of retinal ganglion cells (3). Different strains of inbred mice have also been shown to differ in the size of their cone photoreceptor, amacrine, and ganglion cell populations (4–7). There are multiple potential causes of such variation in retinal nerve cell number, including degenerative changes associated with the accumulated effects of light damage or other processes that affect select types of neurons. In other cases, however, such variation is detected early during development (8), implicating a modulation of the very processes that determine nerve cell number from the outset.

The developmental basis for such variation is not well understood, although polymorphic genes that modulate cellular production and naturally occurring cell death are likely to be major factors. We have previously demonstrated in the mouse retina a nearly twofold variation in the total number of horizontal cells, being a type of retinal interneuron providing feedback inhibition to photoreceptors (9, 10). This large variation across mouse strains is present from the outset of retinal development (11) and is independent of variation in retinal area (9). Furthermore, these horizontal cells modulate their dendritic growth in relation to this variation in cellular density (12), indicating that the determinants of retinal nerve cell number also indirectly impact differentiation.

The present investigation has adopted a forward genetic approach to identify key determinants of this variation in retinal horizontal cell number. We demonstrate a strong heritable dif-

ference in horizontal cell number among 26 recombinant inbred (RI) strains of mice derived from reciprocal crosses between two inbred parental strains, A/J and C57BL/6J (hereafter B6/J). We mapped a quantitative trait locus (QTL) that modulates this neuronal population to the distal end of chromosome (Chr) 13. The QTL overlaps two candidate genes that have been shown to modulate numbers of retinal neurons. Using a conditional gene knock-out strategy, we demonstrate an important role for one of these genes, the LIM homeodomain transcription factor *Islet-1* (*Isl1*), in the suppression of this phenotype. *Isl1* expression is shown to differ between the parental strains during the period of horizontal cell neurogenesis, due to the presence of a *cis*-acting polymorphism. We identified such a potential variant, a single nucleotide polymorphism (SNP) creating a binding site for basic helix–loop–helix (bHLH) transcription factors in the B6/J strain.

Results

Mouse Retina Displays a Large and Highly Heritable Variation in Horizontal Cell Number. Mice of the A/J strain contain $9,884 \pm 168$ horizontal cells, whereas those of the B6/J strain contain $18,471 \pm 288$ cells (mean and SE; Fig. 1, white and black bars). F₁ hybrids (B6AF1) have a value intermediate to the two parental strains, $14,417 \pm 263$ cells (Fig. 1, light gray bar). Members of the AXB/BXA strain family have genomes that are a unique mix of A and B haplotypes across chromosomes. These progeny strains have numbers of horizontal cells that are distributed widely across the range defined by the two parental strains (Fig. 1, dark gray bars). Note, however, that variance within any strain is low, relative to that observed across strains, the coefficient of variation for each strain being less than 4%. Resampling the parental strains with additional mice at the end of the experiment confirmed no procedural or criterion drift contaminating our data collection procedures (Fig. 1, second white and black bars). These data show that horizontal cell number is a highly heritable trait, with a narrow-sense additive heritability (h^2) of 0.89 (13). Such high heritability makes horizontal cell number a particularly attractive trait for genetic dissection.

A QTL for Horizontal Cell Number Resides on Distal Chromosome 13. We mapped the variation in this trait to a narrow locus at the distal tip of Chr 13, peaking at or beyond reference SNP (rs) 4230072 (113.52 Mb) (Fig. 2). This QTL, named “Horizontal cell

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Fig. 1. Estimated total number (mean and SEM) of horizontal cells in the retina of A/J (white bars) and B6/J (black bars) strain mice, in the B6AF1 progeny (light gray bar), and in 26 RI strains of mice derived from the parental strains (the A/B/BXA strain set; dark gray bars). n = number of mice sampled per strain.

number control, Chr 13” (*Hcnc13*) is associated with a significant likelihood ratio statistic (LRS) score of 30.0 ($P < 0.05$), with *B* alleles increasing trait values. The additive effect of two *B* alleles at this locus accounts for ~3,250 cells, being roughly 40% of the difference between the parental strains. Polymorphisms within this QTL must therefore modulate horizontal cell number.

Two Candidate Genes That Modulate Retinal Nerve Cell Number Are Positioned at the QTL. One candidate gene within this QTL is follistatin (*Fst*), positioned at 115.24 Mb. Administration of follistatin during the neurogenetic period has been shown to increase the number of retinal horizontal cells in chicken embryos (14), and so we examined the effect of disrupting *Fst* gene expression by excising a floxed allele of *Fst* in cells expressing the *Cre* gene within the developing mouse retina (15, 16). Effective cre-mediated recombination of *Fst* within the retina was confirmed by qPCR, yet Fig. 3*A* shows, for the opposite retinas of these *Fst*-conditional knock-out (CKO) mice, no significant change in horizontal cell number compared with control littermates, in either the center or the periphery of the retina.

Another candidate gene within this QTL is *Isl1*, positioned at 117.08 Mb. Recent studies have shown its conditional deletion in the mouse retina to lower the number of cholinergic amacrine cells, bipolar cells, and retinal ganglion cells (17, 18). We consequently examined horizontal cell number in the *Isl1*-CKO retina and in littermate controls. Horizontal cell density was significantly elevated in the central parts of the *Isl1*-CKO retina (Fig. 3*B* and *C*, *Left*), being increased by 41%. Densities in the

periphery were only marginally increased, by 8% (Fig. 3*B* and *C*, *Right*), likely due to the mosaicism in cre-recombinase activity: *Six3-cre* drives *LacZ* reporter expression in the *Rosa26* reporter line more thoroughly in the central than in the peripheral parts of the mouse retina (e.g., figure 1e in ref. 19). Conditional deletion of *Isl1* therefore increases the number of horizontal cells.

***Isl1* Is Not Expressed in Horizontal Cell Precursors.** The transcription factor genes *Foxn4*, *Ptf1A*, and *Prox1*, each of which is thought to play a hierarchical role in the specification of retinal cell fate, have all been shown to produce profound deficits in horizontal cell number when knocked out (20–22). How *Isl1* interacts with this hierarchy is unknown. *Isl1* is not normally expressed by horizontal cells in the mature mouse retina (23, 24), and we have confirmed that it is not expressed in *Prox1*-expressing precursors, a subset of which will become horizontal cells (20) (Fig. 4): Crossing mice with tamoxifen-dependent cre-recombinase driven by the *Isl1* locus (25) with the *Rosa26* reporter line expressing *LacZ* (26), and administering tamoxifen on embryonic (E) days E12.5–E16.5, yielded no *Prox1*⁺ cells (magenta) on E18.5 that also expressed the *LacZ* gene product, β -galactosidase (green).

Isl1 is, however, expressed by one type of horizontal cell in the chick retina, the axonless horizontal cell, whereas another LIM homeodomain transcription factor gene, *Lim1*, is expressed exclusively by the axon-bearing horizontal cells (14, 27), the only type present in the mouse retina (28). Overexpression of *Isl1* in postmitotic horizontal cells in the chick retina results in a switching to the axonless fate, presumably due to a repression of

Fig. 2. Genomewide QTL mapping of horizontal cell number. Each chromosome, from centromere to telomere, is represented left to right along the x axis. The *Right* y axis plots the additive effect, being the extent to which the presence of an *A* (green line) or *B* (red line) allele contributes to an increase in horizontal cell number. The *Left* y axis plots the strength of the linkage of horizontal cell number to different intervals along the genome (blue line). Pink and gray horizontal lines indicate genomewide likelihood ratio statistics (LRS) for significant (<0.05) and suggestive (<0.67) QTL, respectively. A single significant QTL, *Hcnc10*, at the distal end of chromosome 13 is present.

Fig. 3. (A) Conditional deletion of *Fst* does not alter horizontal cell density, in either the central or peripheral parts of the retina. (B) Conditional deletion of *Isl1* yields an increase in horizontal cell density, relative to littermate control mice, which is most pronounced in the central parts of the retina relative to the periphery. *n* = number of mice sampled. (C) Retinal fields from the center and periphery of *Isl1*-CKO mice and control littermates immunolabeled for calbindin. (Scale bar, 100 μ m.)

endogenous *Lim1* by *Isl1* (27). Conversely, the increase in horizontal cells, shown here in the *Isl1* conditional knockout, suggests a role for *Isl1* that is incompatible with *Lim1* expression and horizontal cell differentiation. These two LIM homeodomain transcription factor genes, *Isl1* and *Lim1*, are therefore likely to be mutually inhibitory within the mouse retina, as they are in other systems (29, 30), participating in the production of horizontal cells (27). The phenotypic difference in horizontal cell number between the parental strains, therefore, may arise from allelic variation within a regulatory sequence for *Isl1*.

Cis-Acting Regulatory Variants Control Parental Differences in *Isl1* Expression. We sequenced the *Isl1* gene and confirmed the presence of four previously documented synonymous sequence differences between A/J and B6/J, identifying no additional SNPs within the coding region. We next examined potential regulatory regions of this gene, including the putative promoter (~1 kb upstream of the transcriptional start site), 5' UTR, the first two introns, and the 3' UTR, using the more complete sequencing of A/J as part of the Mouse Genome Project by the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/resources/mouse/genomes/>). Sequence analysis revealed numerous possible regulatory SNPs between these two strains, including previously undocumented SNPs (Table S1). To ascertain whether the parental strains differ in their expression of *Isl1*, we measured transcript levels using qPCR during the period of horizontal cell genesis as well as in maturity, finding significantly greater levels of expression in A/J, the strain with fewer horizontal cells (Fig. 5A). We also compared the relationship between *Isl1* expression and horizontal cell number across the two parental and 26 RI strains of the AXB/BXA strain set from our previous microarray analysis of adult whole eye mRNA (6), available on GeneNetwork for public access (accession no. GN210). We found a significant negative correlation between *Isl1* expression and horizontal cell number across these 28 strains ($r = -0.525$, P value = 0.00357).

To test directly whether differences in *Isl1* expression between the parental strains are at least partially controlled by a *cis*-regulatory variant, we examined the allele-specific expression (ASE) of *Isl1* in the retinas of reciprocal F₁ offspring of the A/J and B6/J strains. Significant differences between the parental alleles in the F₁ animals would indicate *cis*-modulation, whereas *trans*-acting regulation of *Isl1* expression would be reflected by the lack of difference between parental allelic expression, because both *A* and *B* alleles of *Isl1* will be equally affected by *trans*-factors (31). The significance of the difference in allelic expression was evaluated by comparing the deviation of the ratio of allelic expression in the mRNA from retina taking into account the difference in amplification efficiency of the alleles using genomic DNA (gDNA) as a control. In the genome of F₁ hybrids, there is a copy of each of the alleles in every somatic cell; the ratio in allelic abundance following amplification of the gDNA is therefore expected to be 1. Deviation from this ratio, however, is frequent, being a result of various factors such as secondary DNA structure, presence of unaccounted DNA polymorphisms that can influence the efficiency of allelic amplification, and the ratio between alleles, which should affect gDNA as well as the mRNA/cDNA equally. Any difference in allelic expression due to *cis*-elements alone should be expressed as a deviation in the ratio of allelic abundance profiled in the mRNA of reciprocal F₁ hybrids relative to the gDNA. We found the expression of the *A* allele in *Isl1* to be significantly greater, in both reciprocal hybrids (Fig. 5B), validating the mechanism of *cis*-regulation of this gene.

An SNP Creates an E-Box in the *Isl1* 5' UTR in B6/J Mice. We used two predictive programs of transcription factor binding sites, TESS and MATCH, to identify potentially functional consequences of sequence differences between A/J and B6/J in the promoter re-

Fig. 4. (A) Cells of the *Isl1* lineage born between E12.5 and E16.5, positive for the *LacZ* reporter gene product β -galactosidase (green), are all positioned in the inner retina on E18.5. (B) Horizontal cell precursors, identified by their expression of Prox1 (magenta), are known to migrate beyond the future horizontal cell stratum in the outer retina, raising the possibility that they too are derived from the *Isl1*-expressing cells in the inner retina at these early stages. (C) When both channels are viewed together, however, not a single Prox1⁺ precursor is found to coexpress β -gal. Samples are from the central retina, but identical results were obtained at the far retinal periphery. (Scale bar, 50 μ m.)

gion and 5' UTR of *Isl1* (32, 33). One difference stood out, in the 5' UTR where multiple bHLH transcription factors were predicted by both programs to bind to the B6/J but not A/J sequence (rs545658554). This group of transcriptional regulators targets a palindromic hexanucleotide DNA sequence known as an E-box, CANNTG (34). The inner two nucleotides of the E-box may vary, but the outer bases are conserved (35). The E-box is not present in A/J due to the G nucleotide located at position 5 (CAGCT/GG). The A allele with the G nucleotide is more highly conserved (Fig. 5C), being shared with five other vertebrates, including dog (XM_848628), horse (XM_001916545), cow (NM_001099130), chimpanzee (XM_001150577), and human (NM_002202). This is also true of wild-derived subspecies and species of *Mus*, including *Mus musculus musculus*, *Mus musculus castaneus*, and *Mus spretus*, which have also been sequenced as part of the Mouse Genome Project. In marked contrast, 14 other strains of mice have the less conserved B allele. This suggests that the E-box is a regulatory element now widely propagated in a subset of laboratory mice, including B6/J.

Discussion

Horizontal cells display a conspicuous variation in their number within the mouse retina that is independent of variation in the overall size of the retina itself. This variation is highly heritable, with surprisingly low within-strain differences—a coefficient of variation of less than 4% for every RI strain examined. Although sets of identified neurons in invertebrates often display this level of tight genetic control (36), there is no precedent for such precision in the mammalian central nervous system. The minimal residual nongenetic variation is presumably due to a combination of intrinsic developmental noise and sampling error (7).

The heritable variation in horizontal cell number is independent of the variation in other types of retinal neurons in these same strains. Cone photoreceptors, for instance, are 70% more abundant in the B6/J strain relative to A/J (6), whereas dopaminergic amacrine cells exhibit a fourfold variation in total number across this same family of RI strains (4). The former trait mapped to a locus on Chr 10 (6), whereas the latter mapped to a locus on Chr 7, where at least three genes modulating dopaminergic amacrine cell number were shown to be present (4). The variation in horizontal cell number mapped to a locus on Chr 13, where two promising prospective candidates were identified, *Fst* and *Isl1*. Despite the documented role for *Fst* in controlling horizontal cell number in the chick retina (14, 37), no comparable effect was detected in *Fst*-CKO retinas. Conditional knock-out of *Isl1*, by contrast, produced a substantial increase in horizontal cells in a retina with drastically reduced numbers of ganglion cells, cholinergic amacrine cells, and bipolar cells (17, 18), indicating a suppressive role for *Isl1* upon horizontal cell number. Indeed, *Isl1* mRNA levels were greater in the A/J strain with lower numbers of horizontal cells during the period of horizontal cell neurogenesis (38). We found only synonymous

SNPs in the coding region of the *Isl1* gene, but detected an SNP in the 5' UTR, which creates a bHLH binding motif in the B6/J strain that may participate in the regulation of *Isl1* expression. Consistent with this, we have shown that *Isl1* transcript levels are controlled by a *cis*-acting variant in retinal tissue.

Exactly how the modulation of *Isl1* expression affects horizontal cell number remains to be determined. Previously, we had shown that all horizontal cells arise from the Math5⁺ precursors that also generate the Isl1⁺ amacrine and ganglion cells as well as certain photoreceptor cells (39, 40). Given that *Isl1*-lineage cells do not produce horizontal cells (Fig. 4), that Lim1 protein is found only in horizontal cells (24), and the inhibitory relationship between these two genes (29, 30), a mutually exclusive expression pattern of *Isl1* and *Lim1* among common precursors could participate in determining horizontal cell number. The conditional knockout of *Isl1* may alleviate additional precursors of such repression of *Lim1*, allowing them to differentiate as horizontal cells. In the B6/J retina, the presence of the E-box may ensure further repression of *Isl1* to allow more cells to express *Lim1*, becoming horizontal cells. This E-box-mediated repression of *Isl1* expression is likely regulated by a bHLH transcriptional repressor specific to horizontal cell precursors. In other precursors that give rise to Isl1⁺ cells in the B6/J retina (23), *Isl1* expression may not be suppressed, due to the absence of this bHLH transcriptional repressor and/or to the presence of other transcriptional activators.

bHLH transcription factors have been shown to be integral during embryonic development, playing roles in myogenesis, heart development, hematopoiesis, and neurogenesis (34). Of the various bHLH transcription factors predicted to bind the E-box, *E2A* and *NeuroD1* are expressed in developing vertebrate retina (41), and have been reported to play a role in retinal neurogenesis and differentiation (42, 43). In these instances, *E2A* and *NeuroD1* are considered to be proneural bHLH proteins, acting as transcriptional activators. However, they have been described in other tissues to function as transcriptional repressors during development (44, 45). In the present instance, their proposed action upon *Isl1* is predicted to be repressive, given the greater number of horizontal cells in B6/J and the greater *Isl1* mRNA expression observed in the A/J strain. The creation of a novel E-box due to an SNP, leading to altered gene expression, is not unprecedented (46), including an instance when this resulted in the suppression of gene expression (47). This polymorphism in the *Isl1* 5' UTR may therefore contribute to the variation in horizontal cell number documented herein.

Prominent variation in nerve cell number is predicted to have functional consequences for neural organization and circuitry (36). In the case of the horizontal cells, dendritic field area is modulated in these parental strains of mice to maintain an average dendritic overlap of around 6.0 (12). Because variation in the number of their afferents, the cone photoreceptors (6), is

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