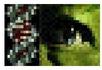 Molecular Vision 2001; **7**:253-260 <http://www.molvis.org/molvis/v7/a36/>
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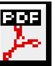
Download Reprint 

Modulation of retinal cell populations and eye size in retinoic acid receptor knockout mice

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Abstract

Purpose: The retinoic acid receptors are expressed from early stages of development in the diverse tissues that make up the vertebrate eye. Their loss has subtle effects on eye development. We adapted sensitive quantitative trait locus (QTL) mapping methods to assess consequences of inactivating alleles of the alpha and beta receptors, *Rara* and *Rarb*, on eye and retinal development. *Rara* is of particular interest because this gene is a candidate for *Nnc1*, a QTL that controls retinal ganglion cell proliferation.

Methods: We studied lines of mice in which expression of the α1 isoform of *Rara* or all isoforms of *Rarb* had been disrupted by gene targeting. We measured eye weight, lens weight, retinal area, and retinal ganglion cell number in each of six genotypes (*Rara* and *Rarb* -/-, +/-, +/+; 10-25 cases/genotype).

Results: Loss of either protein is associated with a small but significant loss of eye weight and retinal area. However, only the *Rarb* knockout has a significant effect on the ganglion cell population and the loss of both wildtype alleles leads to an 8,000 cell deficit. Surprisingly, loss of the *Rara* α1 isoform that is expressed in this cell population from early stages has no effect on number. Null alleles of both genes have little if any effect on lens growth.

Conclusions: Despite its expression in embryonic retina, *Rara* is unlikely to be the *Nnc1* QTL. In contrast, *Rarb*, a gene that maps to Chr 14 and which is not an *Nnc1* candidate gene, has a significant effect on cell number and is therefore a QTL controlling this key population. This raises the intriguing possibility that normal allelic variants of *Rarb* modulate the ganglion cell population in other vertebrates, including humans.

Introduction

Retinoic acid is a potent morphogen and teratogen with widespread effects on limb, eye, and brain development [1-9]. The actions of retinoic acid are mediated by two small families of DNA-binding receptors: the conventional RA receptors (RARs) and the retinoid X receptors (RXRs). These receptors bind to promoter elements of target genes, often as RAR-RXR heterodimers [10,11].

The RAR family consists of three genes: *Rara* on mouse Chr 11 at 58 cM in the *Hoxb-Krt1* interval (17q12 in human), *Rarb* on Chr 14 at 1.5 cM (3p24.3), and *Rarg* on Chr 15 at 58 cM in the *Hoxc-Krt2* interval (12q13) [1,12-16]. All three genes and their proteins are expressed in the embryonic mouse eye, although only *Rara* is expressed heavily in retina [17]. In contrast, *Rarb* and *Rarg* are preferentially expressed in mesenchymal and neural crest derivatives, including the ciliary body, choroid, sclera, and cornea.

Knockouts of these genes and their isoforms, singly and in combination, have been reported previously by Giguere, Chambon and colleagues [10,18,19], and

what is perhaps most striking is the relatively mild, even undetectable phenotypes associated with single knockouts [10,18-20]. In contrast, double knockouts usually have strong or lethal phenotypes. Long ago Sewall Wright made an insightful suggestion, namely that sets of related genes (so-called paralogs), such as the retinoic acid receptors, may be "maintained by selection for quantitative effects, while protected from drastic effects of inactivation or loss" [21,22]. Functional overlap and pleiotropy may be associated with increased trait variability, viability, and plasticity. Normal allelic variants in the retinoic acid receptors may behave like quantitative trait loci (QTLs).

We are interested in determining whether inactivation of these receptors have subtle and unsuspected quantitative effects on eye development for the following reason. *Rara* maps in the same location as *Nnc1*, a QTL that we have shown has a prominent effect on the proliferation of retinal ganglion cells in many common strains of mice [23-25]. The common inbred strain C57BL/6J has the low *Nnc1* variant whereas DBA/2J and C3H/HeJ have the high variant. The difference between these strains is close to 10,000 cells (15-20% of the total ganglion cell population) and *Nnc1* accounts for as much as half the genetic variance in this cell population. The RARA a1 receptor is expressed at an early stage of retinal development in mouse (E10.5), at the initiation of ganglion cell generation [7]. The association between *Rara* gene and *Nnc1* is thus supported by time and place of expression, as well as by chromosomal location. As we show in this paper, however, the effects of inactivation of *Rara* and *Rarb* on the retinal ganglion cell population run precisely counter to expectation; only the inactivation of *Rarb* (a gene that maps to proximal Chr 14) markedly reduced the retinal ganglion cell population.

Methods

Mice

Animals were treated in accordance with the ARVO Statement for the Use of

Animals. The *Rara* line of mice that we used (known formally as the *Rara*^{tm2Ipc} line) have been targeted with a 9 kb insert that disrupts exon 3 of *Rara*. Homozygotes do not express an a1 isoform [26]. The *Rarb* line (the *Rara*^{tm1Ipc} line) contains an inset that disrupts the transcription of all isoforms of the beta receptor [27]. In both knockouts the mutation was introduced into embryonic stem cells derived from 129S2/SvPas that were subsequently injected into C57BL/6 blastocysts. Carriers were backcrossed to C57BL/6 stock for several generations and the C57BL/6-enriched KO stock were subsequently intercrossed to maintain the disrupted alleles. All of the animals that we have studied had the black coat color typical of strain C57BL/6. All animals were bred, raised, and genotyped at McGill University and were shipped as adults to the University of Tennessee for quantitative analysis.

Eyes and optic nerves were taken from 103 mice belonging to each of the three genotypes of *Rara* and *Rarb*. The mix of sexes among the genotypes varied, but the sex ratio across all genotypes was close to 1:1 (49 males, 54 females). The age of mice ranged from 35 to 218 days. The average age of all *Rara* stock was approximately 85 days (range from 50 to 130 days), whereas that of *Rarb* mice was 117 days (range from 35 to 220 days). We also collected data on body weight and fixed brain weight.

Genotyping

DNA was isolated from tails and genotyped by PCR or by Southern blotting. ***Rara***: Mice were genotyped using the method of Li et al. [28]. Genomic DNA was digested with *BglII* and probed using the pSX. ***Rarb***: Tail DNAs were genotyped by PCR using conventional methods. Reaction product (10 µl) was run on 1.4% agarose, stained with ethidium bromide, and photographed. Three primers were used together to identify *Rarb* wildtype and mutant alleles. Primer 1: 5'-GTAGCCATCGAGACACAGAGT-3' anneals to the 5' region of exon 6 was used as a common primer; primer 2: 5'-TGGTAGCCCCGATGACTTGTCC-3' anneals to the 3' region of exon 6 that is deleted in the knockout allele; and primer 3: 5'-CCTCTGAGCCCAGAAAGCGAA-3' anneals to the promoter region of the *Pgk*-neo cassette. Primers 1 and 2 amplify a 121 bp wildtype product whereas primers

1 and 3 amplify a 385 bp knockout product.

Eye weight and lens weight

Animals were deeply anesthetized with Avertin (about 1.0 ml IP). Right eyes were enucleated and weighed immediately. Within one minute the animals were perfused with 0.1 M phosphate buffered saline followed by 1.25% glutaraldehyde and 1.0% paraformaldehyde in 0.1 M phosphate buffer, and then by 2.5% glutaradehyde and 2.0% paraformaldehyde in 0.1 M buffer for electron microscopy [29,30]. Freshly dissected eyes were subsequently placed in the same aldehyde fixative and weighed again 1 h to 5 days later. The weights of fixed eyes, whether perfused (left eye) or immersion fixed (right eye), were generally 6.1% less than those of fresh eyes. The correlation between fresh and fixed eye weights is high (r=0.96). Lens weights were also obtained for these mice.

Retinal wholemounts and sections

The cornea was removed and retina was gently separated from the choroid plexus and the ciliary body. Five radial cuts extending 1-2 mm into the retina toward the optic disc were made, and the retina was flattened onto a slide and coverslipped in Gelvatol [31]. Wholemounts were prepared from the left eyes of a subset of 32 *Rara* cases (about 10 per genotype) and 9 *Rarb* cases (3 per genotype). Low power drawings of retinal wholemounts were traced on a digitizing tablet and retinal areas were determined with a precision of better than 0.5 mm^2. A 1 mm wide strip of retina and pigment epithelium, extending from the head of the optic nerve to the inferior ora serrata was cut and embedded flat in Spurr's resin. A set of 1 μm thick sections were cut along the radial axis, mounted, and stained with hematoxylin. Complete central to peripheral cross-sections of the ventral retina were drawn at low power. The radial depth of cells in the inner and outer nuclear layers was subsequently determined at 7 to 11 evenly spaced sites along sections from three animals belonging to each of the six genotypes at 400x using differential interference contrast optics.

Estimation of retinal ganglion cell number

We used a quantitative electron microscopic assay to estimate the total population of retinal ganglion cells in 47 mice (28 *Rara* and 19 *Rarb* animals). A single nerve was counted in each case. The methods we have used have been described fully [29]. In brief, a series of 22 high magnification electron micrographs were taken using a systematic random protocol across a transverse section of each nerve. A counting frame was traced on the negatives and all axons within the frame and intersecting the upper and right edges were counted using conventional counting rules. To ensure that unmyelinated fibers were recognized, negatives were counted while wearing magnifying glasses. The effective magnification was above 25000x. The average density of axons was multiplied by the area of the nerve cross-section to estimate the total axon population. Data were entered into a relational database (FileMaker Pro, FileMaker, Inc., Santa Clara, CA).

Regression analysis

Eye weight, lens weight and retinal area vary with age, sex, and body weight. This presents a problem in comparing these traits from different genotypes since each genotype may include different numbers of males and females, animals of different ages, etc. Multiple linear regression was performed to investigate relations among these variables, and to reduce unwanted effects on these factors when comparing genotypes. Data on litter size, age, sex, and body weight were available for all mice. Eye weight increases linearly as a function of the logarithm of age and for this reason we used log-transformed age data in the regression analysis. Exploratory analysis and multiple linear and non-linear regressions were carried out using the program DataDesk 6 (Data Description Inc., Ithaca NY). As in our previous studies [30,32] when necessary we have normalized data on eye weight, lens weight, and retina area to those expected of 22 g females at an age of 75 days. Body weights of the three genotypes of each of the two knockout groups did not differ in general. However, for unknown reasons, one set of *Rarb* heterozygotes (three female littermates, 103 day old) was extraordinarily obese (50-60 g). Their eye weighs were normal.

Results

Both eyes from a sample of 103 mice were weighed and averaged. Our main purpose was to quantify effects of inactivation of the *Rara* gene product (a candidate for the *Nnc1* QTL) on retinal development. To ensure adequate power we examined eyes from as many as 25 cases of each of the three genotypes (74 total). In comparison, our analysis of the *Rarb* knockout involved a smaller sample size; approximately 10 cases of each of the three genotypes (29 total, 10 -/-, 7 +/-, and 12 +/+).

Eye weights

The average eye weights (mean±SE in mg) of three *Rara* genotypes (-/-, +/-, and +/+) were 18.3±0.2, 18.7±0.2, and 18.8±0.2, respectively. After adjusting for differences in age, sex, and body weight by multiple linear regressions, we estimate that the loss of each wildtype allele is associated with a drop in eye weight of 0.3 mg (p=0.02, Figure 1). The loss of wildtype *Rara* alleles also has a small effect on body weight of about 0.6 g per allele (p=0.03 when treated as an additive effect and p=0.015 when the wildtype allele is treated as a dominant allele; 1 tailed tests). The same is true of brain weight; the loss of a single wildtype allele is associated with a reduction in brain weight of about 7 mg (mean brain weight is about 450 mg). The effects of the wildtype *Rara* allele on eye weight is dominant and there is no significant difference between heterozygotes and wildtype (p=0.7). This is true for the other major weight traits, body and brain. The *Rara* effect on eye weight, while statistically significant, is modest in size and amounts to only a 0.6 mg reduction from a base of about 19 to 20 mg. *Rara* gene dosage only accounts for 3% of the total phenotypic variance.

Rarb effects on eye size

Eyes of *Rarb* knockout mice are significantly smaller than those of heterozygotes and wildtype mice. The averages of the three genotypes without correction are 17.28±0.32, 18.47±0.36, and 18.83±0.25 mg. Our sample of *Rarb* mice had a wide age range, but even with correction for this factor, the effect of genotype is

still highly significant (p=0.002). In Figure 2 we illustrate our best estimate of the pure gene dosage effect of *Rarb* on eye size after correction for differences in age, sex, and body weight. The loss is about 1.2 mg, which is twice that in the *Rara* knockouts. Approximately 3.5% of the total variance in eye weight is accounted for by dosage of the *Rarb* wildtype allele.

Lens weight

Both fixed lenses from a subset of 57 mice were weighed and averaged (38 *Rara*, 19 *Rarb*). The average lens weights (mean±SE in mg) of three *Rara* genotypes (-/-, +/-, and +/+) were 5.84±0.13, 5.56±0.15, and 5.93±0.12, respectively. The difference of lens weight among three genotypes of *Rara* is very small and insignificant (p=0.6). *Rarb* had a very slight effect on lens weight. However, the significance of the effect is entirely dependent on a single knockout outlier with an unusually small lens. If this case is excluded, then *Rarb* also has no appreciable effect on lens weight (Figure 3). There is however a dramatic qualitative effect of the *Rarb* knockout. In 7 of 9 *Rarb* knockouts, the posterior surface of the lens is darkly pigmented as a result of the retention of a postlenticular membrane as described in the initial analysis of the *Rarb* knockout [33]. Both *Rara* and *Rarb* have an effect on total eye weight, but not on the weight of the lens. Lens makes up 25-30% of total eye weight and the dosage effect discussed in the preceding section is restricted to the non-lenticular part of the eye.

Retinal area

Wholemounts were prepared from eyes of 32 *Rara* animals and 9 *Rarb* animals. Retinal area is also reduced slightly in *Rara* knockouts (*Rara* areas: 19.2 mm^2 (-/-), 19.6 mm^2 (+/-), and 20.1 mm^2 (+/+); p=0.035). The difference between wildtype and knockout groups amounts to about 0.7 mm^2 after correction for sex, age, and body weight. Just over 10% of the total variance in retinal area is accounted for by gene dosage. Unlike eye weight, an additive model fits the data better than a dominance model; heterozygotes tend to have intermediate values. Retinal area is also reduced in the *Rarb* line for both the absolute values (19.4 mm^2, 20.5 mm^2, and 21.0 mm^2 for the three genotypes) and for the residuals

(Figure 4, blue symbols).

Retinal histology

Cell and plexiform layers of retinas of *Rara* and *Rarb* knockout mice have apparently normal structure (Figure 5). Inner and outer nuclear layers are of the appropriate thickness. Cell size and vascular patterns appear normal. Given the generally mild quantitative changes detected in eye weight and retinal area, there may be numerous subtle quantitative distortions in the numbers or characteristics of retinal cells in knockouts and heterozygotes. One example, and a focus of this study, is illustrated by the loss of retinal ganglion cells in the *Rarb* knockout mice, described in the next paragraph.

Retinal ganglion cell population

The ganglion cell population is unaffected by the loss of the *Rara* $\alpha 1$ isoform. Cell counts are 63,000±2,000 (+/+, n=9), 64,000±2,300 (+/-, n=9), and 62,500±1,200 (-/-, n=10), which are not significantly different ($p=0.8$, ANOVA; Figure 5). Multiple regression analysis that controls for differences in body and brain weight differences among groups does not change the neutrality of the *Rara* knockout. In contrast, *Rarb* has a significant effect on RGC number: 64,800±2,300 SE (+/+, n=8), 60,900±4,000 (+/-, n=3), 57,400±2,600 (-/-, n=8; $p<0.05$; Figure 6). The differences among the groups are consistent with an additive model of gene dosage with each wildtype allele contributing approximately 3,700 ganglion cells. The effect of *Rarb* is robust, and a multiple regression analysis that takes into account pleiotropic effects that this gene may have on body weight or brain weight does not materially alter the significance of the effect ($p<0.05$ with control for body and brain weight, 3,500 ganglion cells/allele).

Discussion

Synopsis

Our aim in this report has been to critically assess the effects of two key retinoic acid receptors on the size and structure of the mouse eye, lens, retina, and on the population of the retinal ganglion cells. This work was motivated primarily to test whether *Rara* is a viable candidate gene for the *Nnc1* QTL. Previous studies have looked at qualitative effects of inactivating the major isoforms of *Rara* and *Rarb* on ocular development, but with the exception of an aberration in the development of the hyaloid vessel in the *Rarb* knockouts (persistent hyperplastic primary vitreous) [33,34], no phenotypes have yet been described for knockouts of single receptors in this family. In our work, we have used biometric analysis of the type used in complex trait analysis with the hope of detecting subtle quantitative deviations in knockouts. We have succeeded in detecting several statistically significant and novel changes in both *Rara* and *Rarb* knockouts.

Expression of *Rara* and *Rarb* in relation to effects of the eye

Mori and colleagues [17] have recently described spatial and temporal patterns of retinoic acid receptor expression in eyes of mice from E10.5 through maturity using immunohistochemical techniques. *Rara* and *Rarb* and their proteins are expressed with the characteristics noted below.

> **Retina and PE:** *Rara* is expressed intensely and persistently in the mouse retina starting before the generation of any postmitotic cells (E10.5). Expression is initially widespread but eventually is most intense in the middle of the INL (possibly bipolar cells), and finally in a subset of cells in the ganglion cell layer and the INL. In contrast, *Rarb* expression is weak and is restricted to the INL and the outer neuroblast (ventricular) layer during the period of most intense neurogenesis (E14 to P7). *Rara* is expressed at all ages in the pigment epithelium. In contrast, *Rarb* is expressed in the pigment epithelium only transiently (E14 to P7) and at much lower levels.
>
> **Lens:** *Rara*, but not *Rarb* is expressed at all stages in the lens.

Neural crest/mesenchymal derivatives: *Rarb* is expressed at high levels in sclera and choroid from E10.5 through to about P4. Both *Rara* and *Rarb* are expressed in the iris and ciliary body from E14 through to maturity.

Our finding that *Rarb* has a more pronounced effect on total eye weight than does *Rara* may be consistent with the intense and long lasting expression of *Rarb* and the meager expression of *Rara* in periocular mesenchyme that gives rise to sclera and choroid. Our assumption is that the activation of gene transcription by *Rarb* in a normal molecular context has a cumulative positive trophic effect on eye development. This gene's actual role in transcriptional networks is certainly more complex and at a minimum involves interactions with its main dimerization partners. The weight of the lens is not materially affected by loss of either *Rara* or *Rarb* in isolation, a finding that is surprising given the persistent expression of *Rara* in lens throughout life. The *Rarb* knockout does have a severe and presumably indirect effect on lens development by disrupting the normal course of development of the vitreous and the transient blood supply to the lens.

Nnc1 and *Rara*

The early and intense expression of *Rara* in mouse retina, particularly in the ganglion cell layer and INL, makes it a strong candidate regulator of the development of ganglion cells. When combined with its location in the same 0.2% of the mouse genome as the *Nnc1* locus [24] and in vitro results showing that RA application alters the differentiation of retinal cells [35], the potential of allelic variants of *Rara* to modulate ganglion cell proliferation appears compelling. In contrast, the transient and weak expression of *Rarb* makes it a less compelling candidate for a gene influencing retinal cell populations. However, our results contradict these expectations: the *Rara* α1 knockout mice have normal numbers of ganglion cells whereas the *Rarb* knockout mice have significantly fewer ganglion cells than either wildtype or heterozygous littermates. There are possible explanations for this unexpected finding, but any that tries to preserve *Rara* as a candidate of *Nnc1* will be somewhat less parsimonious than the alternative hypothesis: that *Nnc1* is not an allele of *Rara*. One important caveat is that the α2

isoform of *Rara* may provide a fully functional backup allele in the *Rara* α1 knockout. The α2 is expressed in some CNS regions, particularly the developing spinal cord and striatum [36] and we should not discount the possibility that there is compensatory expression in retina.

Given the expression patterns of *Rarb*, this gene's effects on ganglion cell number is likely to be indirect and could be associated with the persistence of hyaloid vessels and altered vitreal composition. The vitreous normally contains a high concentration of fibroblast growth factor [37], and if the availability of FGF is disturbed this could alter ganglion cell survival. [38]. It would be interesting to determine whether the *Rarb* knockout has an effect on ganglion cell proliferation or the severity of naturally-occurring cell death in mice.

The hitchhiker effect

The interpretation of the effects of gene inactivation in knockouts mice relies on a comparison with the phenotypes of wildtype and heterozygous animals. The use of littermate controls would appear to be an almost ideal control because parental effects and environmental factors will be shared. However, there is an important caveat [39], namely that the segment of the chromosome that carries the knockout gene is derived from strain 129 and this chromosomal interval will carry large numbers of adjacent 129 alleles that may affect the phenotype. For example, the entire *Hoxb-Krt1* interval in the *Rara* α1 knockout mice has a homozygous 129 haplotype, whereas that of the wildtype littermates has a pure C57BL/6 haplotype. This region also happens to have very high gene density; in other words there are hundreds of hitchhiking genes inherited from strain 129. When dealing with complex traits such eye size and neuron number, it is possible that any one of these alleles will exacerbate or neutralize effects of the knockout. In our study there is no exacerbation of a *Rara* effect on retinal ganglion cell number: all three genotypes have the same population. But it is possible that a hitchhiking 129 allele masks a *Rara* effect. To make this possibility more concrete consider the following model: 129X1/SvJ has a larger population of retinal ganglion cells than does C57BL/6J [29] and this difference is probably generated by the *Nnc1*, a locus that overlaps *Rara*. When we transfer high type *Nnc1* alleles from 129 onto the

C57BL/6 background, we expect an increase in ganglion cell number. This increase could amount to 5,000 cells per allele. If at the same time the loss of the functional *alpha 1* isoform has an independent effect that decreases the number of cells by 5,000 per allele, then *Rara* and *Nnc1* will counterbalance and the phenotypes of all three genotypes will be similar. Given this problem, the appropriate control is a B6.129 congenic strain in which the congenic interval covers the particular knockout gene that is been studied. These control lines will be easy to make from the B6.129<Chr 11> and B6.129<Chr 14> that are now being constructed by Nadeau and colleagues [40]. As an interim solution to this problem, we are now studying effects of a second knockout located in the same interval (*Thra*) in which the same 129 alleles have been introgressed onto C57BL/6J. This knockout does appear to have a significant effect on ganglion cell number. Our analysis of single nucleotide polymorphisms (SNPs) in 129X1/SvJ and C57BL/6J indicates that most of the distal half of Chr 11 (from 48 cM to the telomere) has a close common descent. This reduces the risk of a hitchhiker effect associated with the introgressed 129 chromosomal segment.

The QTL approach to the analysis of knockouts

Complex trait analysis is a genetic technique that makes it possible to map gene loci that are responsible for heritable quantitative differences between individuals. These heritable differences can often be relatively small, and sensitive methods and large sample sizes are often used to detect QTLs. In this study of *Rara* and *Rarb* knockouts, we provide an example of how biometric analysis of the type used in QTL mapping [23,30,32] can reveal effects that would be hard to detect using standard qualitative histological methods. Eye weight has proved to be both an easy and remarkably sensitive way to detect differences generated by normal and abnormal alleles. The analysis of the *Rara* and *Rarb* mice provides an interesting reverse genetic approach to complex trait analysis. *Rarb* allelic variants could now be considered "candidate" QTL. It is possible that natural polymorphisms of this gene contribute to variation in ganglion cell number in other populations of mice and possibly even in humans. The combination of standard forward genetic approaches (from phenotype to gene) with this reversed approach could increase the efficiency of complex trait analysis.

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