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***Math5 (Atoh7)* gene dosage limits retinal ganglion cell genesis**

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Abstract

The basic helix–loop–helix factor *Math5 (Atoh7)* is critical for the determination of retinal ganglion cell (RGC) fate in mice. Recently, genome-wide association studies have identified the *ATOH7* locus as a major determinant of variation in the human optic disc area, which is directly correlated with the RGC number. These studies suggest that the level of *Math5* expression may determine the ultimate number of RGCs. To test this hypothesis, we systematically compared optic nerve area and RGC axon number in C57BL/6J congenic *Math5*^{+/-} and *+/+* mice at young adult and neonatal ages by transmission electron microscopy. Optic disc area and RGC abundance were not significantly different in adults, but heterozygotes had thinner optic nerves and 25–30% fewer RGCs at birth than wild-type littermates ($P < 0.05$). Our results suggest that *Math5* dosage is important for the genesis, but not the ultimate number, of RGCs. Our findings highlight the importance of ganglion cell culling as a compensatory mechanism for retinal homeostasis, and support a quantitative role for *Math5* in RGC specification.

Keywords

apoptosis; *Atoh7*; culling; glaucoma; genome-wide association studies; *Math5*; neurogenesis; optic disc area; optic nerve; retinal ganglion cell

Introduction

Humans and mice show marked intraspecies variations in the total number of retinal ganglion cells (RGCs) and optic disc area, which are directly correlated measures [1–5]. In principle, this variation may arise from differences in RGC genesis or in the extent of developmental culling. In mice, multipotent retinal progenitors exit the cell cycle and differentiate as RGCs between embryonic day E11 and postnatal day P0 (birth), with peak genesis at E14 [6,7]. RGC axons coalesce to form the optic nerve and project to brain visual centers. More than 60% of new RGCs are lost by apoptosis during the period of synaptic refinement, which occurs during the first postnatal week in mice and during late gestation in primates [2,8–11]. RGC survival is believed to reflect the formation of functional synaptic connections in the brain [10].

The *Math5 (Atoh7)* basic helix–loop–helix transcription factor is critical for the specification of ganglion cells [12]. *Math5* mutant mice lack the vast majority (>95%) of RGCs [13,14], and recessive loss-of-function mutations cause congenital retinovascular

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Conflicts of interest

There are no conflicts of interest.

disease in humans [15–17], with optic nerve aplasia being the primary phenotype in both species. Recent genome-wide association studies (GWAS) have identified the *ATOH7* locus as a major determinant of variation in the optic disc area in normal human populations [3–5] and a contributing factor in the susceptibility to glaucoma disease [18,19]. However, a substantial cohort of RGCs are *Math5*-independent, and only a small fraction of *Math5*⁺ cells adopt the RGC fate [20].

Given the importance of *Math5* in the development of RGC, we examined the quantitative effects of *Math5* gene dosage on ganglion cell abundance. We found that mice with a 50% reduction in gene dosage (*Math5*^{+/-}) generate fewer RGCs than *Math5*^{+/+} mice, but postnatal compensatory mechanisms equalize the number of ganglion cells between these genotypes. Our results highlight the robustness of mammalian retinal development, show the buffering capacity of the initial RGC pool, and implicate *Math5* as a limiting determinant of RGC abundance.

Materials and methods

Processing optic nerves for transmission electron microscopy

All mouse procedures were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA). Congenic *Math5*^{+/-} mice (*Atoh7*^{tm1Gla} [13]) were systematically maintained on a C57BL/6J background (N18 generation). Young adult (P30) *Math5* heterozygotes and control littermates were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine and perfused transcardially with 15 ml 1.25% glutaraldehyde (GA) 1% paraformaldehyde (PFA), followed by 10 ml 2.5% GA 2% PFA. For P1 optic nerves, eyelids were incised and whole heads were fixed by immersion in 2.5% GA 2% PFA for 1 h at room temperature. Eyes were then carefully dissected with optic nerves attached and postfixed in 1.25% GA 1% PFA overnight at 41°C.

Fixed eyes were washed three times in 0.1M phosphate buffer (pH 7.4), and optic nerves were transected 2–3mm from the sclera. The cut nerves were washed three times in 50mM sodium cacodylate phosphate buffer, postfixed in 1% osmium tetroxide 50mM sodium cacodylate for 1 h at 41°C, dehydrated through a graded ethanol series, infiltrated with 100% propylene oxide, and embedded in EMBed-812 resin (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA). For light microscopy, semithin (0.5 µm) sections were stained with toluidine blue. For transmission electron microscopy (TEM), ultrathin (75 nm) sections were stained with 7% uranyl acetate and Reynold's lead citrate, and imaged using a Philips CM-100 electron microscope (Andover, Massachusetts, USA).

Quantitative analysis of retinal ganglion cell axons

For each genotype and age, axons were counted in 3–4 optic nerves. Ten fields were systematically sampled from central (four) and peripheral (six) areas at ×10 500 magnification (103 µm²). The optic nerve area was measured from low-power (×380–530) TEM images by manually tracing the perimeter using ImageJ software (NIH, Bethesda, Maryland, USA). The total number of RGCs per optic nerve was extrapolated by multiplying the average RGC density from 10 fields by the optic nerve area. The results were compared between genotypes using Student's *t*-test.

Results

To isolate the role of *Math5* dosage in determining RGC abundance, we systematically measured the optic nerve area and the total number of RGCs in neonatal (P1) and young adult (P30) mice (Fig. 1). These ages were chosen to reflect the size of the RGC population before and after the period of postnatal culling, respectively [9,11]. To identify ganglion

cells definitively, we counted axon fibers in optic nerves of congenic (N18) C57BL/6J *Math5* heterozygous and wild-type mice by TEM [1,2]. In adult mice, the optic nerve area and RGC number did not differ between these genotypes (Fig. 1, Table 1), and were comparable with previously reported values for C57BL/6J strains [1,2]. In contrast, neonatal *Math5*^{+/-} mice showed a marked reduction in the optic nerve area (36%, 0.018 vs. 0.028 mm², $P < 0.03$) and RGC number (20%, 129 000 vs. 178 000 per eye, $P < 0.04$). Surprisingly, the density of RGC axons was higher in P1 *Math5*^{+/-} mice than that in wild-type littermates (7.5 vs. 6.3 million axons/mm², $P < 0.04$), despite a marked decrease in the optic nerve area. This difference did not persist in adults, suggesting a transient deficiency of optic nerve glia.

Given the wide variation in RGC abundance among inbred mouse strains [1], we also compared residual RGC axons between genetically diverse *Math5*^{-/-} mice, which retain only 4% of RGCs [21]. There was no apparent difference in the density of TuJ1+ axons between C57BL/6J congenic (N18) and outcrossed (129S1×C57BL/6J) F2 *Math5*^{-/-} mice (data not shown).

Discussion

The absolute number of ganglion cells per eye, and the photoreceptor-to-RGC ratio, determine the anatomy of the optic nerve and ultimate spatial integration of visual signals in the retina. These parameters appear to be precisely controlled; yet, few genetic factors have been identified for variations in the optic disc area and RGC number. In mice, one quantitative trait locus (*Nnc1*) for RGC abundance was defined between C57BL/6J and DBA/2J inbred strains [22]. Recently, human GWAS identified the *ATOH7* locus as the major single determinant of variation in the optic disc area in Asian and White populations [3–5]. Because no common coding variants were identified in individuals with normal vision or optic nerve disease [17,18], this effect is most likely because of differences in transcriptional regulation. Indeed, the GWAS peak is centered over rs3858145, an SNP located 20 kb upstream from the *ATOH7* promoter, within a conserved transcriptional enhancer [15]. *Math5* mRNA is transiently expressed in retinal progenitors during or immediately following the terminal cell cycle [20] and is reduced in *Math5* heterozygotes [13]. In principle, a specific threshold level may be required during histogenesis to confer RGC competence to individual progenitor cells or the retinal population. The Math5 bHLH protein, for example, may represent the limiting component in a multimeric transcriptional complex that initiates the ganglion cell development program. In this model, decreasing *Math5* expression would decrease RGC genesis. Conversely, *Math5* overexpression would not be expected to alter RGC abundance, and this prediction holds true in *Crx* > *Math5* transgenic mice, which express *Math5* broadly in a large population of retinal progenitor cells [23]. Sequence variants in human *ATOH7* enhancer regions may cause quantitative or qualitative differences in the spatiotemporal pattern of *Math5* expression.

In adult mice, a 50% reduction in *Math5* activity did not alter the number of RGCs or the optic nerve crosssectional area. However, it remains unclear whether humans carrying one null *ATOH7* allele [15,17] have a reduced number of ganglion cells. This is an intriguing possibility because GWAS data connect *ATOH7* to variations in the optic nerve area [3–5], and one patient with optic nerve hypoplasia was found to be heterozygous for an allele (A47T) with reduced function [3,17]. In open-angle glaucoma, patients experience progressive death of ganglion cells during adulthood. Visual impairment is not clinically significant until 40–50% of RGCs are lost, but field differences are detectable by perimetry with a 25–30% reduction in ganglion cells [24]. The potentially discordant effects of the *ATOH7* genotype on adult RGC number between humans and mice may indicate species differences in genetic heterogeneity, which was absent in our comparison between inbred

littermates, or the degree of developmental culling. The overall extent of culling is comparable in mice and humans, involving two-thirds of new RGCs [8,11], but the genetic background can influence birth and death independently [2]. Our results suggest that *Math5* dosage affects RGC genesis, but other mechanisms compensate to ensure the proper number of functional ganglion cells in adults (Fig. 2). Although 25–30% fewer RGCs are generated in *Math5*^{+/-} mice, fewer are lost during the culling period [9,11]; therefore, the adult optic nerve is similar to the wild type (Fig. 1). These results show an important ‘fate-buffering’ capacity in the retina and provide an instructive example of developmental compensation for a genetic defect [25].

Conclusion

Our results show that *Math5* (*Atoh7*) dosage has a significant impact on the genesis of RGCs, but not on the ultimate number of ganglion cells. These findings highlight genetic differences in RGC development and culling, and the role of *Math5* in these processes.

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Fig. 1. Heterozygous *Math5* effect on retinal ganglion cell (RGC) number and optic nerve size. (a) Toluidine blue-stained semithin sections of P30 (left) or P1 (right) optic nerves. (b) High-power (×10 500) transmission electron microscopy images of representative ultrathin optic nerve sections. (c, d) Quantitative analysis of (c) the optic nerve area and (d) the overall RGC abundance. The cross-sectional area and RGC number are significantly reduced in *Math5*^{+/-} mice at P1, but not P30. **P*<0.05. Scale bar, 50 μm in (a), 200 nm in (b).

Fig. 2.

Model of retinal ganglion cell (RGC) genesis and culling in *Math5*^{+/+} and *+/−* mice. In *Math5* heterozygotes, fewer ganglion cells are produced than in wild-type mice during genesis stages (E11–P0). However, adult *Math5*^{+/-} and *+/+* mice have a similar number of RGCs. Therefore, fewer ganglion cells undergo apoptosis during the neonatal RGC culling period in *Math5*^{+/-} mice.

Table 1

Optic nerve metrics in *Math5*⁺ / – and *Math5*⁺ / + animals

<i>Math5</i> genotype	Age	<i>n</i>	Axons counted ^a	Optic nerve area (mm ²) [<i>α</i>]	RGC density×10 ⁶ (axons/mm ²) [<i>β</i>]	Total RGCs [<i>α</i> × <i>β</i>]
+ / +	P30	3	1440±80	0.051±0.002	1.39±0.07	71000±6000
+ / –	P30	3	1200±100	0.056±0.006	1.2±0.1	65000±7000
+ / +	P1	4	6500±400	0.028±0.004	6.3±0.4	178000±16000
+ / –	P1	4	7700±100	0.018±0.001	7.5±0.1	129000±8000

RGC, retinal ganglion cell.

^a Axons were counted in high-quality areas and normalized to the entire image area. Errors are reported as SEM.