13q32.1 as a candidate region for physiological anisocoria

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ABSTRACT

Background Physiological anisocoria is an asymmetry of pupil size in the absence of pathology.

Methods Images of the pupils under standard illumination were collected in the course of a whole-genome association study of a range of visual functions in 1060 healthy adults. DNA for each participant was extracted from saliva samples.

Results We found no relationship between anisocoria and the difference in refraction between the eyes, nor between anisocoria and difference in acuity. There was a small but significant relationship with lightness of the iris, in that the eye with the smaller pupil was associated with the lighter iris. There was a strong association between anisocoria and a local region of chromosome 13 (13q32.1), a region lying between the genes *GPR180* and *SOX21*. The strongest association was with the single-nucleotide polymorphism rs9524583.

Conclusion The very specific region associated with anisocoria is one where microdeletions (or microduplications) are known to lead to abnormal development of pupil dilator muscle and hence to the autosomal dominant condition of microcoria. It is possible that alterations at 13q32.1 act by altering the expression of *SOX21*, which encodes a nuclear transcription factor.

Key messages

What is already known on this topic

⇒ An asymmetry of pupil size is often observed in the absence of pathology and has been termed 'physiological anisocoria'.

What this study adds

⇒ This study found a relationship between anisocoria and chromosome 13q32.1, a region known to be associated with development of the pupil dilator muscle.

How might this study affect research

⇒ Alterations at 13q32.1 may act by altering expression of the nuclear transcription factor gene SOX21.

INTRODUCTION

'Physiological anisocoria' (or 'simple central anisocoria') is an asymmetry of pupil diameter in the absence of any pathology.[1 2] Its incidence has been reported for a number of populations[1–6]: the reported frequency will clearly depend on the criterion difference in pupil diameter that is adopted, but values of the order of 20% have been given for a threshold difference of 0.4–0.6 mm.[4] Physiological anisocoria is known to depend substantially on the illumination conditions used for the measurements[2 4 7 8]; and the size of the asymmetry for a given person may vary from one test occasion to another.[9] An increase with age has been reported.[8]

In a whole-genome study of approximately 1000 healthy young adults, we have found that the degree of physiological anisocoria is associated with a restricted region of chromosome 13q32.1. This finding draws interest from the existing evidence that that deletions or duplications in the same small region are associated with abnormal development of the pupil dilator muscle. It was in the *British Journal of Ophthalmology* a hundred years ago that Holth and Berner identified abnormal development of the dilator muscle as a cause of congenital miosis, or microcoria[10] (see also references[11–13]). In an extended French family with 31 affected members, Rouillac *et al*[14] found microcoria to be inherited as an autosomal dominant condition, and were able to establish linkage to an 8 cM region at 13q31-q32. A subsequent study confirmed the linkage to 13q31-q32,[15] although another showed that the condition is genetically heterogenous.[16] In 2015 Fares-Taie *et al*[17] sequenced the 13q31-q32 region in the French family studied by Rouillac *et al* and in five other families. They found sub-microscopic deletions at 13q32.1 that invariably encompassed or interrupted only two tail-to-tail genes: *GPR180*, which encodes a heptahelical G-protein-coupled receptor 420 amino acids in length[18] and *TGDS*, which encodes the enzyme thymidine diphosphate (TDP) glucose-4,6-dehydratase. Three subsequent studies have independently reported a similar heterozygous loss of these two genes in British, Swiss and Saudi families exhibiting microcoria, in some cases associated with glaucoma.[19–21] In addition, non-syndromic microcoria has recently been found in a child exhibiting a heterozygous 289 kb duplication at 13q32.1 encompassing *GPR180*, *TGDS* and five other genes.[22]

Given the evidence associating 13q32.1 with development of the pupil dilator muscle, we explore in this paper the possibility that variation in the same muscle is a source of anisocoria in the normal population. It is, of course, prima facie unlikely that any single genetic locus will account for more than a small part of the variance in physiological anisocoria. Many factors—genetic or environmental—could potentially give rise to anisocoria, including

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an asymmetry of refractive state, a morphological asymmetry of the anterior segment, or an asymmetry of autonomic innervation. Some evidence for the last of these factors is given by Rosenberg,[23] who performed the 'friction sweat test' on 42 patients with anisocoria and found that 36 of them had less sweating on the side with the smaller pupil.Tramonti Fantozzi *et al*[24] report that anisocoria can be associated with unbalanced dental occlusion and consequent asymmetry of activity of masseter muscles during clenching. And Bremner and Nordström,[25] describing a case where alternating aniscoria is synchronised with alternation of which nostril is the more congested, hypothesise that a central oscillator may concurrently modulate the sympathetic stimulation of dilator muscles and nasal vessels.

METHODS

Our measurement of anisocoria was made as part of the PERGENIC genome-wide association study of perceptual traits.[26–28] A test battery of 80 perceptual measures was conducted, taking approximately 2.5 hours. The photographs used to measure anisocoria were taken during the first 40 min of the battery,[29] as part of a series of optometric assessments, which also included measurements of acuity, stereo acuity, heterophoria and macular pigment density.

Participants

One thousand and sixty participants (647 female), aged between 16 and 40 (mean=22.14; SD=4.09) took part in the PERGENIC study. All participants were of European ancestry and a large proportion were students at the University of Cambridge. Participants were refracted to their best corrected visual acuity (all less than 0.00 logMAR), and were provided with lenses to wear during the test battery if the correction led to an improvement in acuity of at least 0.1 logMAR. If participants usually wore glasses or contact lenses any lenses provided were in addition to their usual correction. Their usual correction was recorded, either using the prescription provided with contact lenses, or for glasses, on the basis of measurements with a focimeter. A randomly selected subset of 105 participants (66 female) completed the test battery for a second time, at least 1 week after their first session, in order to estimate test–retest reliability.

Equipment and procedure

Images were acquired using an EOS 1000D DSLR camera (Canon, Tokyo, Japan), fitted with an A14 telephoto zoom lens (Tamron, Saitama, Japan) and an EF-530 DG ST flash (Sigma, Kanagawa, Japan).[29] The exposure was manual, allowing a fixed F5 aperture, a 5 ms exposure time and an ISO of 100. Images were saved in Raw format.

The camera and flash were mounted above a 24" Trinitron CRT monitor (Sony, Tokyo, Japan) that was used to provide a source of illumination. The full screen of the monitor was illuminated with a uniform field that was metameric to equal energy white and had a luminance of 35 cd/m^2. The viewing distance of the participant was fixed at 1 m from the display by means of a chin-rest and a forehead-rest. He or she viewed the monitor through a rectangular slit in a large screen, which allowed the eyes to be imaged, but obscured the participant's face. Mounted on the face of the screen was a Kodak colour chart, which was included in the image to allow for calibration of size, chromaticity and lightness. Participants were asked to fixate a small black fixation cross at the centre of the display.[29]

The camera was positioned 1.05 m from the participant's eyes (a relatively long distance was chosen to minimise errors in image size, which are proportional to the tangent of the error in distance divided by the distance of the camera). The focal length of the zoom lens was set to be 171 mm, to allow a relatively large distance but good image resolution. The experimenter manually adjusted the final focus to ensure a sharp image of the corneal reflection of the monitor that the participant was viewing. The participant was adapted to the light from the display while the photographer adjusted the focus of the image, allowing adequate time for the pupil size to adjust to the illumination level. The image was illuminated by a low-power flash using the EF-530 DG ST's in-built diffuser, with a manual setting to produce consistent illumination power at each exposure. (The flash would not have affected pupil size, since it was much briefer than the reaction time of the pupil[30]). All images were taken without glasses to avoid reflections of the flash obscuring the pupil in the image. If participants habitually wore contact lenses, these were worn when the images were taken.

Pupil size for each of the two eyes was extracted by fitting a circle to the image of the pupil and taking the radius. To convert the radii from pixels to mm, the measurement was compared with a fixed length derived from the image of the calibration chart, accounting for any changes in the precise focal length of the camera. Average pupil size was quantified as the mean diameter in mm of the two pupils, and absolute anisocoria as the absolute difference between the sizes of the two pupils in mm.

Near and far phorias were measured with plates 5218 and 5219 of a Keystone telebinocular (Mast Concepts, Reno, Nevada, USA).[31]

Genome wide association study (GWAS) methods

About half-way through the 2.5-hour session, all participants provided a 2 mL saliva sample, collected using Oragene OG-500 SNA kits (DNA Genotek, Ottawa, Canada). Details of DNA extraction and quality checks have been published previously.[26] A total of 1008 samples were genotyped using Human OmniExpress arrays (Illumina, San Diego, California, USA). This beadchip array allowed 733 202 single-nucleotide polymorphisms (SNPs) to be characterised. Genotypes were called by custom clustering using GenomeStudio (Illumina).

Twenty individuals were excluded from the genetic dataset. One had a low call rate, 3 had sex anomalies, 15 were related individuals or duplicate samples, and one was a population outlier. A total of 988 individuals remained in the sample, and of these 984 had pupil size available (images were either missing or were not of sufficient quality for pupil size to be extracted for 4 individuals). 12.3% of the genotyped SNPs were excluded because they had either greater than 2% missing genotypes (N=12 706), or had a minor allele frequency below 1% (N=77 738). A total of 642 758 SNPs remained in the analysis.

For each SNP, a quantitative trait analysis was conducted using PLINK[32] for average pupil size and for absolute anisocoria. To control for any residual stratification in our population we used EIGENSOFT[33] to extract the first three principal components (PCs) of the genetic variation in the sample. The 3 PCs, along with sex, were entered as covariates in the regression model for each phenotype.

SNPs that achieved a $p<1\times10^{-5}$ were defined as 'suggestive', and variants were imputed in a region 2.5 Mb centred on each suggestive SNP using the software IMPUTE2[34 35] with the 1000 genomes phase 3 haplotypes. Association analyses of the imputed regions were carried out on the genotype probabilities using the dosage association function of PLINK, adding the three

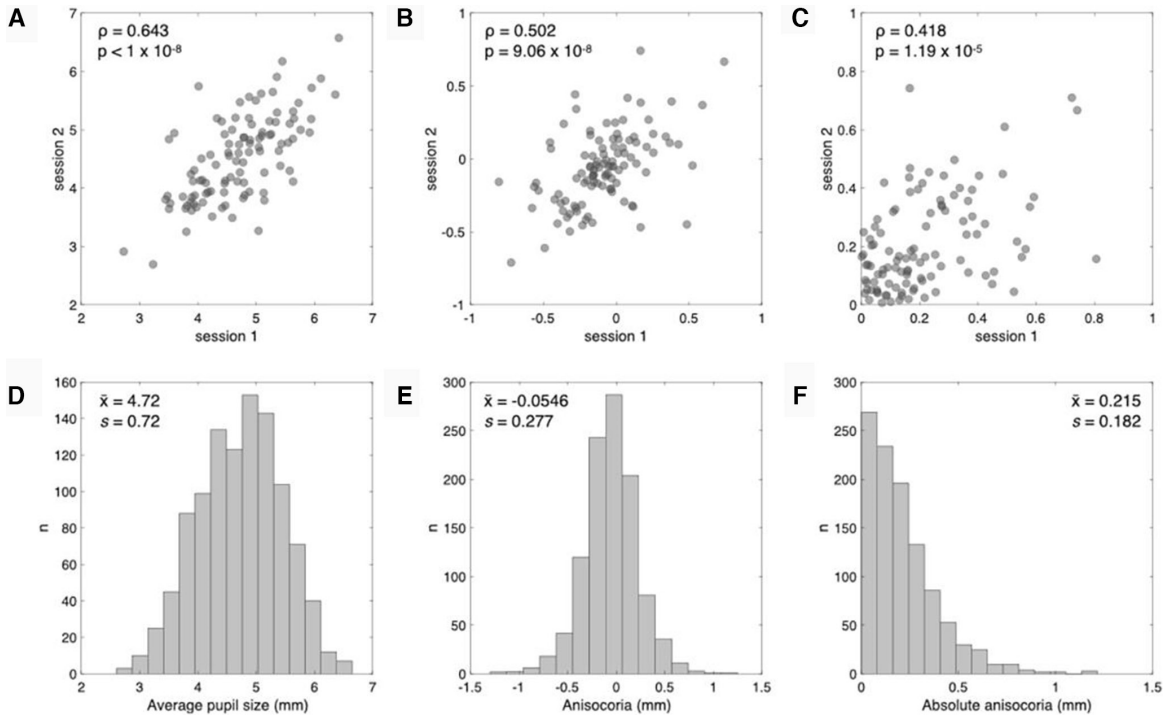

Figure 1 Test–retest reliabilities and distributions for average pupil size (A, D), signed anisocoria (B, E) and absolute anisocoria (C, F).

genomic PCs and sex as covariates as in the initial quantitative trait analysis.

Lastly, we performed a clumping analysis on each region of interest using PLINK's clumping function, with a significance threshold for index SNPs of 1×10^{-5}, a significance threshold for clumped SNPs of 0.01, a linkage disequilibrium (LD) threshold for clumping of 0.1, and a physical distance threshold of 1250 kB. Clumping defines a region in LD with the associated SNP that contains other SNPs also associated with the phenotype with a specified p value, and which is therefore likely to contain the critical variant.

RESULTS

Distributions and test–retest reliability

Phenotypic measurements of pupil size were available for 1057 of PERGENIC's 1060 participants. For the present paper, our primary variables of interest were average pupil size (average diameter in mm of the two pupils), signed anisocoria (the signed difference between pupil diameters) and absolute anisocoria (the absolute difference in the diameters of the left and right pupils).

Mean average pupil size was 4.72 mm (SD=0.72) and was approximately normally distributed in our sample (figure 1D). The test–retest (between-session) reliability was moderate: $\rho=0.64$ ($p<1\times10^{-8}$, n=104; figure 1A), although the correlation between left and right eyes within one session was $\rho=0.92$.

Signed anisocoria, quantified as the difference between the left and right pupils in mm, was also approximately normally distributed (figure 1E). Mean absolute anisocoria was 0.215 mm in our sample (SD=0.182; figure 1F). The anisocoria measures showed moderate test–retest reliability: $\rho=0.50$ ($p=9.1\times10^{-8}$, n=104; figure 1B) for signed anisocoria and $\rho=0.42$ ($p=1.2\times10^{-5}$, n=104; figure 1C) for absolute anisocoria.

Visual acuity

A total of 332 participants habitually wore glasses and 155 habitually wore contact lenses. The habitual correction was unavailable for 22 participants owing to missing data. A total of 343 participants were provided with extra lenses when the lenses improved visual acuity in one or both eyes by at least 0.1 logMAR. Mean total refraction (the sum of the existing correction in dioptres and the additional lenses provided in dioptres) was −1.45 for the left eye and −1.46 for the right eye (SD 2.23 for left eye and 2.22 for right eye; range −11.5 to +6.25 for left eye, −11.75 to +7.75 for right eye).

There was no significant correlation between mean pupil size and mean total refraction ($\rho=-0.033$ $p=0.28$, n=1035), nor was there any significant correlation between the two variables when the refraction due to contact lenses (worn by 155 participants when the images were acquired) was left out ($\rho=-0.038$ $p=0.22$, n=1040).

Relationship of anisocoria to other variables

Duke Elder[36] cites anisometropia as a possible cause of anisocoria and suggests that the more myopic eye will have the larger pupil. In our sample, there was a significant but small relationship between absolute anisocoria and the difference in refraction between the eyes ($\rho=-0.0737$. p=0.02, n=1035). When the refraction attributable to contact lenses was left out, the correlation between absolute anisocoria and the difference in refraction between the eyes was slightly reduced ($\rho=-0.0609$. p=0.05, n=1040).

It is known that sympathetic stimulation is important for development and maintenance of the pigmentation of the iris.[37–39] In our present population of healthy adults, we had measures from the same images of the lightness of the iris[29] and we were therefore prompted to ask whether there was a relationship between signed anisocoria and lightness of the iris. We

record here that there was a small but significant correlation (ρ=−0.088, p=0.004), in that the smaller pupil was associated with the lighter iris. There was also a correlation of ρ=0.097 (p=0.0016) with near vertical phoria.

Poynter[40] has reported that the average physiological anisocoria varies with sex: in his sample of 310 participants aged 18–40, the mean signed anisocoria differed from zero for men, in that the left pupil tended to be slightly larger, but this was not the case for women. Poynter related this finding to greater hemispheric lateralisation in males. We record here that our own large cohort showed a trend in the same direction for signed anisocoria but a t-test between men and women failed to reach significance (t=1.7, p=0.080).

GWAS

A quantitative trait analysis of the genotyped SNPs and average pupil size revealed no strong associations. Since intrinsically photosensitive retinal ganglion cells project to the olivary nucleus and influence pupil diameter, the gene for melanopsin, *OPN4*, has been a candidate gene for pupil size[41 42] : so it is worth recording that we found no association with measured or imputed SNPs in this region, although the candidate SNP (rs1079610) identified in ref[41] is included in the beadchip array that we used.

In the case of absolute anisocoria, we found a strong association with the marker rs9524583 at Chr13q32 ($p=4.8\times10^{-7}$): the minor allele (T) is associated with greater anisocoria. Each additional copy of the minor allele was associated with a 0.05 mm increase in absolute anisocoria (SD 0.26). This association would be considered suggestive or marginally significant by the accepted standards for a GWAS using a beadchip array of the size used here,[43 44] but the fact that 13q32 is a candidate region on the basis of its association with development of the dilator muscle makes this association a priori very plausible.

The minor allele frequency of rs9524583 in our sample was 0.378. A further genotyped SNP (rs9516452) in the same region was suggestively associated with absolute anisocoria ($p=1.1\times10^{-6}$), as were five further imputed SNPs (rs9516453, rs9516455, rs7317537, rs12372828, rs72632663; $9.1\times10^{-6} < p<5.4\times10^{-7}$). The genomic region around rs9524583 is shown in figure 2. The clumped region most likely to contain the causal variant includes three protein-coding genes, *TGDS*, *GPR180* and *SOX21*, as well as the antisense RNA gene SOX21-AS1 and the 'long intergenic non-coding' RNA gene LINC00391.

Twelve further SNPs met our criteria for suggestive associations, three associated with average pupil size and nine with absolute anisocoria. Details of the suggestively associated SNPs, any further associated imputed SNPs nearby and the genes inside the clumped regions around suggestively associated SNPs are listed in table 1.

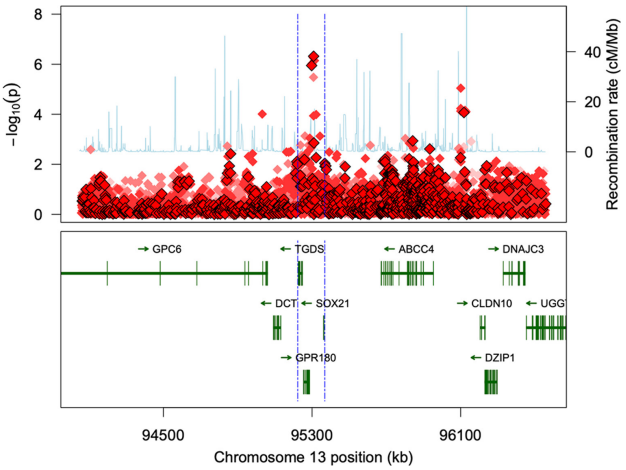

Figure 2 Manhattan diagram for the region surrounding rs9524583 (GRCh37). (Top) association results for genotyped SNPs (red diamonds with black borders) and imputed SNPs (red diamonds, with saturation denoting imputation quality). Recombination rate is plotted with a solid blue line. (Bottom) genes located in the region. Vertical rectangles indicate exons. (Both) vertical blue dashed lines indicate the region identified by clustering, in which the critical variant is likely to lie.

DISCUSSION

Test–retest reliabilities

We found a high correlation between our estimates of left and right pupil diameter when the eyes were imaged concurrently, a result that suggests that our measurement error is low. It is not surprising, however, that test–retest reliabilities (estimated from the 10% of our sample who were tested twice at an interval of >1 week) were only moderate both for average pupil size and for anisocoria (0.64 and 0.5, respectively). We carefully controlled the illumination conditions, but natural pupil size depends not only on light history but also on sympathetic activity; and thus, there are many known time-varying factors that may affect pupil diameter, such as time of day, prescription drugs, cognitive activity and the novelty of the stimulus.[45]

The association of anisocoria with chromosome 13q32.1

By current convention, the p value that we report for the association of anisocoria with 13q32.1 ($p=4.8\times10^{-7}$) falls just above the value for genome-wide significance. However, the independent finding that microdeletions in this local region have been associated with abnormal development of the dilator muscle[17] gives confidence that the association with physiological anisocoria is a real one. The critical region that is common to our association and to the association with abnormal development of the dilator amounts to ~0.000025 of the human genome. Our strongest markers also lie within the microcoria-associated microduplication observed in the case reported in 2020 by Pozza *et al*.[22]

Possible causal mechanisms

Of the two genes common to all the deletions in the families studied by Fares-Taie *et al*[17], *TGDS* looks an unlikely candidate to influence pupil size, since it has no known function in muscle cells and has no preferential expression in the iris. In addition, abnormalities of the pupil have not been reported in cases with pathogenic variants of *TGDS* (Catel-Manzke syndrome).[46 47]

On the other hand, *GPR180* offers itself as a prima facie candidate. Although the function of the corresponding protein is little understood and although no ligand is known, the protein is produced particularly in vascular smooth muscle cells, where it is upregulated in response to experimental injury. Second, *Gpr180*-/- mice do not exhibit the thickening of the intima after injury that is seen in the wild type—a result suggesting a specific role in the growth of vascular smooth muscle.[48] Third, although *GPR180* is not highly expressed in other ocular tissues, it is among the top 20 genes expressed in the iris.[49]

However, the issue proves to be more complicated. Fares-Taie *et al*[17] examined eyes from *Gpr180*-/- and *Gpr180*+/- mice and found that they were indistinguishable from the wild type.

Table 1 Suggestively associated loci with average pupil size and absolute anisocoria

	Lead SNP (number of additional SNPs in brackets)	Chr	Position (GRCh37)	MAF	P value	Clumped region	Genes inside clumped region
Average pupil Size							
1	rs10267716:G (36I) rs10232268:A (1G)	7	52 111 922 52 130 600	0.43 0.41	3.35e-06 5.14e-06	52094115–52 189 529	N/A
2	rs11242183:G (1G)	5	133 127 733	0.45	5.24e-06	133111867–133 227 047	N/A
3	rs1054724:A (G)	5	111 499 732	0.34	9.70e-06	111137944–111 661 139	NREP
Absolute anisocoria							
1	rs12822264:G (4I)	12	91 999 356	0.15	1.76e-06	91179070–92 050 907	EPYC, DCN, KERA, CCER1, LUM
2	rs6135591:A (1G; 2I)	20	15 874 488	0.08	2.90e-06	15818142–15 944 725	MACROD2
3	rs41516347:G (2I) rs17088782:A (G)	4	58 709 806 58 704 924	0.22 0.17	9.32e-08 3.06e-06	58703793 – 58 876 866	N/A
4	rs10851970:G (14I) rs2339747:G (G)	15	35 270 521 35 306 104	0.13 0.11	1.11e-06 3.56e-06	35134106–35 310 789	AQR, ZNF770
5	rs72803437:C (2I) rs2901066:C (G)	2	60 798 459 60 797 896	0.05 0.16	1.04e06 3.70e-06	60642782–60 928 605	BCL11A
6	rs66596684 (49I) rs2016251:A (3G)	1	155 876 971 155 914 988	0.25 0.24	3.51e-06 4.36e-06	155198347–156 016 356	GBA, FAM189B, SCAMP3, CLK2, HCN3, PKLR, FDPS, RUSC1, ASH1L, MSTO1, YY1AP1, DAP3, GON4L, SYT11, RIT1, KIAA0907, RXFP4, ARHGEF2, SSR2, UBQLN4
7	rs3754821:A (1G; 4I)	2	209 013 401	0.32	6.26e-06	208959000–209 024 890	CRYGD, CRYGC, CRYGB
8	rs17117165:C (I) rs17653878:A (G)	5	154 638 558 154 609 080	0.05 0.04	1.25e-06 6.70e-06	154602019–154 680 962	N/A
9	rs2836796:T (1I) rs2836797:G (1G)	21	40 345 391 40 345 484	0.29 0.29	4.91e-06 6.83e-06	40319856–40 367 164	N/A

N/A, not available; SNPs, single-nucleotide polymorphisms.

Normal mydriasis could be induced with drugs. In addition, they identified five human subjects carrying a heterozygous nonsense mutation in *GPR 180* that was predicted to produce a truncated protein (c.343>T[p.Gln115*]). Pupillary responses were normal (although iridocorneal angle dysgenesis was present).

If then microcoria does not necessarily follow the elimination of either *TGDS* or *GPR180*, what are the remaining possibilities? It seems unlikely that the conjoint removal of the two, very different, proteins is the required precondition for idiopathic microcoria, since the inactivation of *TGDS* ought to lead to the Catel-Manzke syndrome (see above). The remaining class of hypotheses are those that suppose that the 13q32.1 deletion affects the expression of other genes, either by removing an enhancer in the selected region or by a distance effect—a change in the physical relationship between a gene and its enhancer(s). Fares-Taie and collaborators have favoured an explanation of the latter kind, on the basis of experiments on mice carrying the deletion or smaller ones.[50 51] In a recent abstract,[52] they suggest that the critical deletion leads to ectopic expression of *SOX21*, which encodes a nuclear transcription factor; and that one target of SOX21 is a trophic factor that is expressed in the pigment epithelium of the iris and acts by paracrine signalling.

It is in the interval between *GPR180* and *SOX21* that we find two genotyped SNPs strongly associated with anisocoria (figure 3). One of these markers, rs9516452, is encompassed by the largest deletion that causes developmental abnormality of the dilator muscle, and both are within 32 kbp of the smallest deletion.[17] The genotyped marker rs9516452 and the imputed marker rs7317537 lie within DNase I hypersensitivity sites (13.844675 and 13.844718, respectively)—sites where the chromatin structure can be altered to allow access to regulatory elements.[53] Such sites, where promoters, enhancers, and silencers can act, are known to be disproportionately associated with phenotypic variation.[53] It may also be relevant that this local region is rich in H3K27ac marks, which indicate sites of higher activation of transcription (see figure 3). Our present results appear very compatible with the hypothesis of Angee *et al*[52] that variations at 13q32.1 act indirectly via an effect on the expression of *SOX21*, but more generally we confirm that anisocoria is associated with this local genomic region, and in

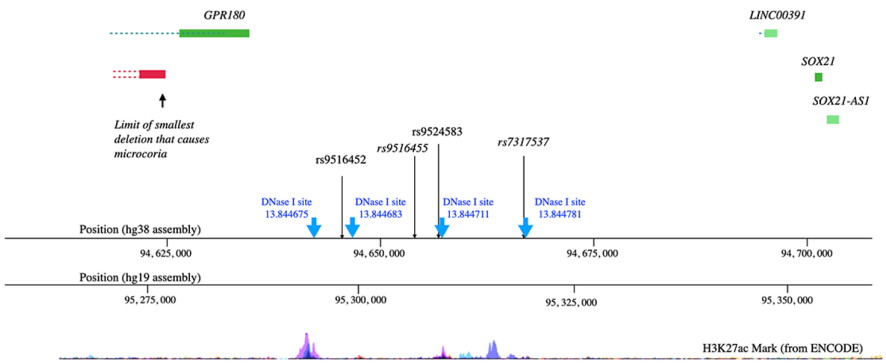

Figure 3 Schematic representation of the region of chromosome 13q32.1 between GPR180 and SOX21, showing the relative positions of markers associated with anisocoria, DNase I hypersensitivity sites and H3K27ac marks. Only selected DNase I hypersensitivity sites are shown. Imputed SNPs are shown in italics. SNPs, single-nucleotide polymorphisms.

particular with the region between positions 94, 625, 000 and 94, 700, 000 (hg38 assembly).

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Contributors JDM secured research funding; JDM led the study; all authors designed the methods; JMB, AJL-O, GB and PTG gathered the data; AJL-O, PTG and JMB analysed the data; JMB and JDM wrote the manuscript; JMB, PTG and JDM edited the manuscript. JDM is the study's guarantor.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval The study was approved by the Cambridge Psychology Research Ethics Committee, and was carried out in accordance with the tenets of the Declaration of Helsinki (2008). All participants provided written informed consent before taking part.

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Data availability statement Data are available on reasonable request. Summary GWAS results and anonymised phenotype data underlying these results are available by request to the corresponding author. Raw genetic data for individual participants are not available under our ethical permissions.

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