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RESEARCH ARTICLE

A 3D molecular map of the cavefish neural plate illuminates eye-field organization and its borders in vertebrates

François Agnès*,†, Jorge Torres-Paz*, Pauline Michel and Sylvie Rétaux‡

ABSTRACT

The vertebrate retinas originate from a specific anlage in the anterior neural plate called the eye field. Its identity is conferred by a set of ‘eye transcription factors’, whose combinatorial expression has been overlooked. Here, we use the dimorphic teleost Astyanax mexicanus, which develops proper eyes in the wild type and smaller colobomatosus eyes in the blind cavefish embryos, to unravel the molecular anatomy of the eye field and its variations within a species. Using a series of markers (rx3, pax6a, cxxcr4b, zic1, lhx2, emx3 and nkx2.1a), we draw a comparative 3D expression map at the end of gastrulation/onset of neurulation, which highlights hyper-regionalization of the eye field into sub-territories of distinct sizes, shapes, cell identities and combinatorial gene expression levels along the three body axes. All these features show significant variations in the cavefish natural mutant. We also discover sub-domains within the prospective telencephalon and characterize cell identities at the frontiers of the eye field. We propose putative fates for some of the characterized eye-field subdivisions, and suggest the existence of a trade-off between some subdivisions in the two Astyanax morphs on a micro-evolutionary scale.

KEY WORDS: Eye-field transcription factors, Telencephalon, Hypothalamus, Cell identity, Astyanax mexicanus, Natural variation

INTRODUCTION

In vertebrates, the bilateral retinas emerge at the end of gastrulation from a single territory in the neural plate called the eye field and located between the prospective telencephalon and diencephalon (Varga et al., 1999; Woo and Fraser, 1995). At such early stage, the eye field already displays significant variations in size and shape across species, which likely prefigure their future morpho-anatomical differences and probably result from species-specific fine-tuning of earlier inductive and signalling events (Bielen et al., 2017; Rétaux et al., 2013).

During gastrulation, anterior neural fate acquisition and distinction between telencephalon or eye-field identities require antagonizing posterior Wnt signals from the anterior neural border (Heisenberg et al., 2001; Houart et al., 2002, 1998) and restricting rx3 expression to a smaller eye field territory; the latter protects the future telencephalon from acquiring eye identity by repressing the expression of the key eye-field transcription factor rx3 (Bielen and Houart, 2012; Fish et al., 2004; Stigloher et al., 2006; reviewed in Giger and Houart, 2018).

Prior to neurulation, a set of transcription factors, rx, pax6, six3, six6 and lhx2, which are expressed in dynamic and overlapping patterns, define eye-field identity and constitute a self-regulating feedback genetic network, in which otx2 initially primes the anterior neural plate (ANP) for eye-field formation (Zuber et al., 2003).

During neurulation, ANP cells adopt specific and drastically different migratory behaviours and trajectories according to their identity: dorso-medial convergence for telencephalic progenitors; evagination for eye-field cells; and anterior ward movement for hypothalamic precursors (reviewed by Bazin-Lopez et al., 2015; Sinn and Wittbrodt, 2013; Wilson and Houart, 2004). Eye progenitors undergo complex movements, starting even before the onset of evagination of the bilateral optic vesicles (Englund et al., 2006). Subdomains of the optic vesicles do maintain their relative positions during morphogenesis (Kwan et al., 2012), suggesting that related, adjacent retina territories might already be determined at late gastrula before evagination.

Anteriorly, the eye field juxtaposes the prospective telencephalon, as revealed by specific markers in zebrafish (Stigloher et al., 2006). By contrast, eye-field posterior limits are less clear, as suggested by pax6 extending posteriorly to rx3 (Loosli et al., 2001; Zuber et al., 2003) and overlapping with diencephalic markers (Macdonald et al., 1997; Staudt and Houart, 2007). Thus, pax6 and rx3 do not appear to form a uniform optic domain and the other eye-field transcription factors likely add additional combinatorial patterning complexity to the eye field that has yet to be investigated.

Here, we sought to define a ‘molecular portrait’ of the eye field and determine its exact frontiers with surrounding territories in 3D. For this purpose, we used the embryos of the dimorphic fish Astyanax mexicanus, which comes in wild-type (surface fish) and blind troglodytic (cavefish) forms; the comparison of these forms offers an exquisite model system to unravel subtle brain anatomical variations (Rétaux et al., 2016). Our results shed new light on what the eye field is, in terms of size, shape, molecular identity and spatial limits. We discovered an unanticipated degree of eye-field regionalization and atypical zones at the frontiers between major forebrain divisions, and significant variations in the natural cavefish mutant.

RESULTS

Viewing eye-field shape and size in 3D with rx3

To date, most reports of eye-field size and shape have used 2D imaging (Fig. 1A-D). In Astyanax, the eye field-specific marker rx3 (Stigloher et al., 2006) delineated a smaller bean-shaped domain, convex anteriorly and concave posteriorly, in cavefish compared with the wild-type surface fish embryos (Fig. 1A-D,K,L). 3D rendering revealed a similar shape ventrally with a midline indentation partially separating the eye field in two lobes; this was less pronounced in cavefish (Movie 1) and undetectable by 2D analyses (Fig. 1E-H). The rx3 domain showed a 25% reduction of volume in cavefish (Fig. 1M), likely corresponding to a lack of posterior rx3 expression or a more isotropic size reduction (Fig. 1J-J’’).

Footnotes:

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Eye-field shape and size changes are highly dynamic

We noticed a significant dispersion of eye-field shapes and volumes between samples, all fixed upon their developmental stage and global tailbud morphology at ‘theoretical’ 10 hpf (Fig. 1I-I‴). To follow eye-field size and form according to time, we decided to stage ANP a posteriori by estimating the advancement of neurulation for each sample. As the anterior ANP curvature resembles a parabola, we designed a simple method to assign...
each 10 hpf sample with a coefficient (d) that was the inverse of the vertical scaling factor for a parabola. This allowed the classification of samples into subgroups according to their intrinsic morphogenesis progress (Fig. 1I′-F′). Using this method, we found a significant shift towards higher curvatures (lower d factor) for cavefish ANP (Fig. 1N,O). This suggested a heterochrony between the two morphs, with neurlulation progression or onset being slightly more advanced in cavefish. To compensate for this bias in sample distribution at 10 hpf, we included older 10.5 hpf surface fish samples in the dataset. This allowed the comparison of eye-field size within the same ranges of d factor for the two morphs (d=2-3 to d=8-9). Whatever the range of d factor, the eye-field volume was always smaller in cavefish, confirming the trend observed at 10 hpf with pooled embryos (Fig. 1P). Furthermore, when comparing size according to d factor, we found a progressive 30% reduction of rx3+ eye-field volume for surface fish (Fig. 1P), whereas the rx3+ volume was constant within the same time interval for cavefish (R²=0.02). This suggests that the cavefish rx3 domain is smaller and does not condense in the same way as it does in surface fish at the onset of neurlulation.

Expression of rx3 within the eye field

Previous analyses showed reduced levels of rx3 transcript levels at 10 hpf in cavefish (McGaugh et al., 2014), which we confirmed by RNaseq and fluorescent in situ hybridization (not shown; Fig. 1Q). To further investigate intrinsic rx3 expression pattern and achieve comparisons of spatial distributions of pixel intensities, we used image stack acquisitions to reveal optimized pixels intensities and normalized intensities (see Materials and Methods). Strikingly, rx3 signals distributed heterogeneously in cavefish embryos when compared with the homogeneous distribution in surface fish (Fig. 1Q-V). Cell-level quantification (Fig. S1, see Materials and Methods) of relative rx3 signals at 80% epiboly and 10.5 hpf ruled out the hypothesis that cavefish rx3 expression was blocked in its early expression stage, by distinguishing patchiness at the onset of expression (identical in the two morphs; pale colours, Fig. 1W-Y) from true expression heterogeneity seen only in cavefish. Accordingly, rx3 fluorescent signals at tail bud stage showed higher dispersions of pixel intensities (Fig. 1V,W) and variance (Fig. 1V,X) in cavefish, with distinct patches of cells, even adjacent, expressing highly variable levels of rx3 (low to high). This fully penetrant, expression heterogeneity phenotype (n=150) was prominent throughout the whole structure (Fig. 1C,D,S′,T,X).

At the tissue scale, rx3 signals also defined concentric patterns in the A/P and D/V axes (Fig. 1S-T). This pattern was observed at every confocal plane, ruling out maximum intensity projection artefacts (data not shown), and was detected in cavefish, to a lesser extent. In summary, the rx3 expression domain appears more patterned and complex than previously thought, and shows variation in size, shape, expression intensity and homogeneity between the two Astyanax morphs.

cxcr4b identifies a ‘core’ anterior subdomain within the rx3-positive eye field

In zebrafish, cxcr4a functions in the segregation of optic vesicles and telencephalon, and its expression depends on rx3 (Bielen and Houart, 2012). As rx3 expression was strongly affected in cavefish, we reasoned that this chemokine receptor-encoding gene might be abnormally expressed in cavefish, potentially explaining optic morphogenetic defects. We retrieved cxcr4a and cxcr4b Astyanax cDNA orthologues (Fig. S2) from our EST library and analysed cxcr4b (cxcr4a was not detected at tailbud stage). In Astyanax, the cxcr4b bean-shaped expression domain was included within the rx3+ eye field in both morphotypes (Fig. 2A-D, Movies 2 and 3) and was 25% smaller in cavefish (Fig. 2H). The cxcr4b domain mapped to the anterior dorsal rx3+ region (Fig. 2A-F), identifying a core domain of co-expression, the volume of which was about half that of rx3 in both morphs. This core region was surrounded in 3D by cells expressing only rx3 (Fig. 2A-F, Movies 2 and 3), thus highlighting further concentric eye-field patterning (Fig. 1S-T).

Low level rx3-expressing cells generally express higher levels of cxcr4b in cavefish

As rx3 expression lacked homogeneity in cavefish (Fig. 1), it became evident that cxcr4b and rx3 expression patterns/levels were complementary within the co-expression domain in cavefish (Fig. 2B-B′,F-F′, Movie 3). Line histogram quantifications confirmed that rx3-low zones were cxcr4b-high, and vice versa (Fig. 2G-G′). We next quantified pixel intensities in individual cells and plotted relative levels of rx3 and cxcr4b (Fig. 2I-L and Fig. S3). cxcr4 levels in cxcr4-expressing cells were similarly distributed in surface fish and cavefish (Fig. S3B). Nevertheless, the distributions in surface and cavefish were different when plotting cxcr4b levels according to rx3 levels, because of the globally lower rx3 expression levels in cavefish (Fig. 2J,K and Fig. S3CD). However, in surface fish, the two genes varied together, i.e. rx3 expression was higher in cxcr4b-high cells and cxcr4b expression was higher in rx3-high cells (Fig. 2L and Fig. S3A). This was not the case in cavefish, where the levels of the two genes varied in an opposite manner, i.e. rx3 expression was lower in cxcr4-high cells and cxcr4 expression was lower in rx3-high cells. These results highlight potential opposing regulatory interactions between rx3 and cxcr4b at the cell population scale in the two morphs.

Shape and size of the pax6a domain

We next used the eye-gene marker pax6, which, together with rx3 and six3, forms the eye field-specific transcription factor network (Sinn and Wittbrodt, 2013). Specifically, we used pax6a (Fig. S4), which is expressed in the eye field (Staudt and Houart, 2007) and in the future posterior retina and diencephalon in zebrafish at 8 somites (Macdonald et al., 1994). At the tailbud stage, in agreement with Strickler et al. (2001), pax6a delineated ventral bilateral triangular-shaped domains that were anteriorly and dorsally connected at the midline and showed no expression at the posterior midline, and hence were markedly different from rx3 (Fig. 3A-B′). In addition, dorsal and ventral projections highlighted pax6a domain shape in 3D (Movie 4). The volume of the pax6a domain was similar in the two morphs (Fig. 3I). pax6a partially overlapped with rx3 anteriorly and was distributed posteriorly to rx3 (Fig. 3C,D). The size of the pax6a/ rx3+ overlapping region was the same in the two morphotypes (Fig. 3I); however, the pax6a/ rx3+ posterior region was slightly bigger in cavefish (Fig. 3L), suggesting potential trade-off between the anterior and posterior domains. The medio-lateral ‘shrinkage’ of the pax6a from high to low d factors occurred at the same pace in both morphs, ruling out the possibility that neurluration itself was slower in cavefish, and confirming our interpretation that condensation over time is compromised in CF (Fig. S5).

In the medial ANP, the region devoid of both pax6a and rx3 expression (Fig. 3E) was 25% longer in cavefish (Fig. 3J; d=3.5-4.5) but its width was similar on the same samples (Fig. 3J). The resultant 1.6-fold increased aspect ratio (height/width) in
cavefish (Fig. 3K), together with the difference in ventral midline shape (Fig. 3), strongly suggested that this ventral medial ANP domain was larger in cavefish. Altogether, these results strengthen the hypothesis that the medial posterior region of the cavefish rx3 domain is smaller.

*pax6a shows anteroposterior and dorso-ventral graded expression patterns*

*pax6a* transcripts distributed in a gradual manner along the anteroposterior axis, with posterior cells showing higher levels than anterior cells (Fig. 3A,B,F). *pax6a* fluorescence intensity in posterior rx3 cells (A-P position 0.7-0.9 in the x-axis) was six times higher than in anterior rx3 cells (A-P position 0.1-0.3), which was similar in both morphotypes (Fig. 3F). In the intermediate region (0.4-0.6 in the x-axis), where the graded expression of *rx3* and *pax6a* shows opposite trends, *pax6a* levels were slightly higher in cavefish, suggesting subtle variations in the control of its expression between the two morphotypes (Fig. 3F, right).

*pax6a* expression also showed polarization along the D/V axis (Fig. 3G,H). Resliced lateral projections of a region of interest (ROI) (rectangle in Fig. 3E) allowed *pax6a* pixel intensity quantification at four different positions along the A/P axis within the *rx3*-expressing domain [anterior (A), middle (M) and posterior (P)] and outside the *pax6a* domain for background noise (BG) calculation (Fig. 3G) (see Materials and Methods). Line histograms depicted similar D/V graded expression in the anterior, middle and posterior regions of the *pax6a*/*rx3* domain (Fig. 3H). In all three regions, dorsal eye-field cells expressed higher *pax6a* levels than ventral cells. In middle sections, higher *pax6a* levels were observed dorsally in cavefish embryos (Fig. 3H, middle), similar to differences observed in anteroposterior measurements (Fig. 3G, right).

Altogether, these data uncover several additional levels of eye-field regionalization. The graded expression pattern of *pax6a* along the A/P and D/V axes, together with the concentric pattern of *rx3*, suggest unsuspected levels of combinatorial specification within the ANP, which are subtly affected in cavefish.

**Defining the posterior limit of the eye field with *barhl2***

As the eye-field markers *rx3* and *pax6a* overlapped only partially, we asked whether the limit of the eye field extended posteriorly to *rx3*. Assuming that *barhl2* is a bona fide marker of the anterior limit of the prospective diencephalon (Staudt and Houart, 2007; Young et al., 2019), we thus used *barhl2* together with *pax6a* and *rx3* to define the posterior eye-field border. As in zebrafish, *barhl2* did not overlap with *rx3* (Fig. S6) and co-labelled several rows (up to eight) of *pax6a* cells at the posterior border of the *pax6a* domain at different stages of neurulation, similarly in both morphotypes.
(Fig. 3M-P). Thus, we deduced that at least two large domains may subdivide the eye field along the anteroposterior axis: an anterior domain containing cells expressing \( \text{rx3} \) with graded levels of \( \text{pax6a} \); and a posterior domain populated by cells expressing only \( \text{pax6a} \) at high levels. These results suggest that the eye field is not a single territory but a composite tissue.

**The ventral limit of the eye field: relative size and position of the hypothalamus**

At tailbud stage, the prospective hypothalamus lies just above the prechordal plate and beneath the eye field (England et al., 2006; Pottin et al., 2011; Varga et al., 1999; Wilson and Houart, 2004; Yamamoto et al., 2004). To better characterize the ventral medial frontier of the eye field, we used the hypothalamus marker \( \text{nkn2.1a} \) (Menuet et al., 2007; Yamamoto et al., 2004). The \( \text{nkn2.1a}^- \) prospective hypothalamus showed expansion in colorimetric in situ hybridization (data not shown; Menuet et al., 2007; Yamamoto et al., 2004) and significant increase in volume in cavefish (Fig. 4E).

Assessing the relative spatial arrangement of \( \text{rx3} \) and \( \text{nkn2.1a} \) (Fig. 4A,B) showed no correlation between eye-field curvature (neurulation advancement) and position of the prospective hypothalamus along the A/P axis during the analysed stages (Fig. S7). This suggested that the more anterior position of the \( \text{nkn2.1a} \) domain in cavefish was not due to faster convergence/extension during gastrulation (Torres-Paz et al., 2019), but more likely to enhanced Shh signalling (Yamamoto et al., 2004). Interestingly, the prospective hypothalamus at 10 hpf had a more-anterior position in cavefish (Fig. 4C,F), showing yet another heterochrony between the two morphs and prompting us to analyse the \( \text{rx3}/\text{nkn2.1a} \) frontier zone.

**\( \text{nkn2.1a} \) and \( \text{rx3} \) reveal mixed cell identities at the cavefish hypothalamus/eye-field boundary**

\( \text{Rax} \) and \( \text{rx3} \) expression have been described in the nascent hypothalamus at 14 hpf and E7.5 in zebrafish and mouse, respectively (Loosli et al., 2003; Orquera and de Souza, 2016). In zebrafish tailbud, one study using double \( \text{rx3}/\text{nkn2.1a} \) allele indicated that some cells might already express the two markers (Tessmar-Raible et al., 2007). The \( \text{nkn2.1a}^- \)/\( \text{rx3}^- \) overlapping domain was found in Astyanax samples where at least one-third of the cells were mainly located at the \( \text{nkn2.1a}^-/\text{rx3}^- \) frontier zone.
existence of a very small subset of specifications (Fig. 4E,I,J). Altogether, these results reveal the characterize the eye field molecularly. In zebrafish forebrain (Maurus and Harris, 2009), would help further Varga et al., 1999) and also responds to midline signalling in cavefish at this stage (Pottin et al., 2011; Torres-Paz cavefish, which could be a direct consequence of increased Shh signalling in cavefish at this stage (Pottin et al., 2011; Torres-Paz et al., 2019; Yamamoto et al., 2004).

Finally, on the lateral sides of the nascent basal hypothalamus, a clear gap separated the nkx2.1a+ cells from the rx3+ cells anteriorly (Fig. 4D,D′, arrows; n=15/15 surface fish) or from the pax6a+ cells more posteriorly in all embryos (Fig. S8). This suggests an additional unknown molecular identity in this thin tissue layer that could be the prospective alar hypothalamus based on absence of nkx2.1a expression.

Shape and size of the zic1 domain
We reasoned that zic1, which is expressed in retinal precursors at tailbud stage (Devos et al., 2021; Grinblat et al., 1998; Varga et al., 1999) and also responds to midline signalling in zebrafish forebrain (Maurus and Harris, 2009), would help further characterize the eye field molecularly. In Astyanax, zic1 expression delineated an anteriorly convex bean-shaped domain at tailbud stage, similar in shape but more indented ventro-medially than the rx3 domain (Fig. 5A,B; Movie 6). The zic1 domain was larger than rx3 in both morphotypes (Fig. 5A-B′; compare Figs 1M and 5F) regardless of the d factor range considered (not shown). The zic1 domain showed a 25% reduction in volume in cavefish (Fig. 5F), which likely corresponded to the lack of a posterior medial region (Fig. 5A,B, arrows, Movie 6). We also found a 35% progressive reduction of zic1+ domain volume for surface fish as neurulation progressed, whereas it was constant within the same interval for cavefish (surface fish R2=0.4039, P<0.001; cavefish R2=0.0456, non significant). As for rx3, the cavefish zic1+expressing domain did not reduce in size in relation to the d factor as it did in surface fish embryos at the onset of neurulation.

zic1 identifies two major domains in the eye field
The zic1 marker, together with rx3, identified three subdivisions within the ANP (Fig. 5A,B). Anteriorly, zic1 extended beyond rx3 in both morphs (Fig. 5D,E, blue, Movies 6 and 7), in a zone corresponding to enx3+ telencephalic precursors (see Fig. 7). The size of this zic1+/rx3+ telencephalic region was 30% smaller in cavefish regardless of the d factor considered (Fig. 5H). A second zone, composed of zic1−/rx3+ cells, corresponded to a sub-territory of the rx3 domain and was also smaller in cavefish (Fig. 5D,E, red; Fig. 5H, Movie 7). The third medial region (Fig. 5A-C) was populated with rx3+/zic1− cells (Fig. 5D,E, white and arrowheads, Movie 7) and was twice as large both in surface area and volume in cavefish embryos (Fig. 5G,J). Proportionally, this domain was fourfold larger in size relative to either zic1+/rx3+ or zic1−/rx3+ domains in cavefish.

Thus, two major subdivisions arise in the eye field based on the combination of zic1 and rx3 expression (Fig. 5D,E, red/white) and the relative proportions of these two subdivisions vary between the two morphs, suggesting a potential trade-off mechanism within the optic primordium.

Refining eye field lateral posterior subdivisions with zic1
Closer inspection of the posterior lateral eye field revealed that zic1 expression extended slightly posteriorly to rx3 in the lateral region of surface fish embryos (Fig. 5, arrows; Fig. S9).
A `low-zic1 crescent' in the anterior ANP is affected in cavefish

We then focused on the eye field/telencephalic boundary. *zic1* showed a thin zone of lowered expression at the putative telencephalic/eye-field border in some samples (Fig. 6L-L‴). This phenotype was rare in surface fish (*n* = 3/50, 6%) and more penetrant in cavefish (*n* = 15/58, 25%). This was reminiscent of a potential neurulation heterochrony effect in sample distribution (see Fig. 1O). Accordingly, the presence of the `low-zic1 crescent' was related to the d factor and its penetrance increased at later neurulation stages in the two morphs (Fig. 6K). Using a qualitative classification of absent, weak, mild or strong (Fig. 6L-L‴), we found a significant increase and full expressivity of the ‘low-zic1 crescent’ in cavefish for samples with a low d factor (Fig. 6M). Thus, *zic1* downregulation in this particular domain is temporally similar in the two morphs but spatially different.

To characterize molecularly the thin ‘low-zic1 crescent’, we performed 3D renditions, which suggested that it matched the...
eye field/prospective telencephalon border, forming a gap between the two territories (Fig. 6N,O; Movie 6). Line histograms drawn across the zone of interest in 2 µm projections of ANPs stained for zic1/emx3 or zic1/rx3 (Fig. 6P) showed similar profiles for zic1 in the two morphs (Fig. 6Q, left), further confirming the shared characteristics of the zic1-depleted region for samples of similar morphogenetic stages. Intensity profiles of emx3 and rx3 showed that the ‘low-zic1 crescent’ potentially had a prospective telencephalic identity in surface fish, as deduced from intermediate emx3 levels and very low or no rx3 expression (Fig. 6R, first and third plots, respectively).

Strikingly, both the relative levels of emx3 and rx3 were elevated in the ‘low-zic1 crescent’ in cavefish (Fig. 6Q,R, second and fourth plots, respectively), suggesting improper cell specification in this eye field/telencephalon intermediate region. These profiles may also be interpreted as the result of posterior and anterior shift of the emx3 and rx3 limits, respectively, in cavefish. We thus used ‘single cell’ analysis to quantify relative pixel intensities in the zic1-depleted zone (Fig. 6S). In this domain (indicated as ‘Low’ in Fig. 6T,V), a fraction of cells expressed significant levels of rx3 (∼20% in surface and 40% in cavefish; Fig. 6T,U). We also identified some emx3-expressing cells within this domain, which corresponded to ~40% in surface fish.
embryos and 80% in cavefish (Fig. 6V,W). Such a high proportion of cells expressing \textit{emx3} in the ‘low-	extit{zic1} crescent’ strongly suggested that the \textit{emx3} posterior boundary was slightly shifted posteriorly in cavefish. Altogether, these data characterize a previously unreported zone in the ANP, showing subtle differences in cavefish, with temporal and molecular shifts of the ‘low-	extit{zic1} crescent’.

**Mixed cell identities at the eye field/telencephalon boundary in cavefish**

Finally, we analysed the telencephalic/eye-field boundary proper. In surface fish, we observed a fully penetrant gap between the \textit{rx3} and \textit{emx3} domains (Fig. 7A, Movie 9), which was independent of the neurulation stage/d factor (not shown). Line histogram quantifications confirmed low (<0.2) relative pixel intensities at the intersection of the two curves (Fig. 7D,E, first plot). Strikingly, this \textit{emx3}/\textit{rx3} gap zone was never detected in cavefish (Fig. 7B, Movie 9), and line histograms revealed significantly higher relative pixel intensity levels for both markers at the intersection zone (Fig. 7E, second plot).

Comparison of \textit{emx3} and \textit{rx3} fluorescence intensity curves revealed a slight posterior and anterior shift, respectively, in cavefish (Fig. 7E, right plots). Accordingly, volume analyses detected a significant \textit{emx3}/\textit{rx3} overlapping domain in cavefish (Fig. 7C). Furthermore, ‘single cell’ analysis at the interface zone (Fig. 7F) showed a threefold increase in the proportion of cells expressing medium to high relative levels of both \textit{emx3} and \textit{rx3} in cavefish (Fig. 7G,H, grey), indicating a high degree of co-expression. We also found in cavefish a fivefold decrease in the percentage of cells expressing low levels for both markers (Fig. 7G,H, white), consistent with the absence of gap observed at the telencephalic/eye-field interface in this morph. In sum, this peculiar ‘gap zone’ expressing the lowest levels of \textit{rx3} and \textit{emx3} in surface fish might correspond to another yet undescribed ANP subdivision that, again, seems phenotypically affected in cavefish.

**DISCUSSION**

**What is the eye field?**

Studies in different models have suggested that the vertebrate eye field is specified by a combination of several transcription factors (Varga et al., 1999; Zuber et al., 2003). Accordingly, \textit{pax6}, \textit{lhx2} or \textit{rx3} are considered as interchangeable markers and universal ‘master’ eye genes, a notion that fits well with the absence of eye development and anophthalmic phenotype of knockout/mutant animals (Gehring, 1996; Loosli et al., 2001; Porter et al., 1997). Our 3D expression map reveals unsuspected and extensive eye-field regionalization at late gastrula stage. Using combinations of five markers, we identified six subdomains in which \textit{pax6a} graded expression patterns (A/P, D/V) superimposed an additional degree of patterning complexity (Fig. 8).

The \textit{rx3}/\textit{pax6a} coupling identified three major eye-field subdivisions in the A/P axis: an anterior \textit{rx3}-only zone; an intermediate \textit{rx3}/\textit{pax6a} zone and a posterior \textit{pax6a}-only zone. The last was distinct from the anterior prospective diencephalon...
barhl2− border at tail bud stage. The rx3/zic1 coupling defined three subdivisions in the medio-lateral axis: a medial rx3−only zone, an intermediate rx3+/zic1−/pax6a+ zone and a small lateral posterior zic1-only zone. Additional positional information is given by the pax6a− graded expression patterns (D/V and A/P), the rx3/zic1 combination in the medial region and the anterior cxcr4-expressing core. These molecularly defined eye-field subdomains integrate in a theoretical framework of forebrain development, which results from potential causal signalling effects (Rubenstein et al., 1998). The eye field should thus be modelled as a highly regionalized structure, well before its bilateral evagination into two optic vesicles. It is likely that many more distinct eye-field cell identities, defined by combinatorial expression of additional transcription factors, are already specified at late gastrula.

The optic recess region (ORR) has been proposed as a morphogenetic entity organized around the optic recess, between the hypothalamus and telencephalon, that could be derived from the eye field (Affatatici et al., 2015). Under this framework, we suggest fates for some of the eye-field subdivisions, in view of the comparative and quantitative observations made in our model. Importantly, these hypotheses are based solely on expression patterns and will need further tracking and lineage analyses for confirmation. The rx3+/zic1−/pax6a− cells would represent the anterior eye fields potentially fated to become retinas, which are smaller in cavefish (Alunni et al., 2007; Devos et al., 2021; Yamamoto and Jeffery, 2000). The posterior rx3+/pax6a+/barhl2−/emx3− eye-field cells, subdivided in at least two populations (zic1+ and zic1−), would be potentially fated to become posterior retinas and RPE. By contrast, the medial rx3+/zic1− cells, which occupy a larger territory in cavefish, could prefigure the medial ORR/alar hypothalamus and the optic stalk region, both larger in cavefish at 24 hpf (Torres-Paz et al., 2019). The relative sizes of these eye-field derivatives would vary between the two morphs, as a trade-off within the optic primordium, likely because of variations in signalling (Torres-Paz et al., 2019). Given the homology and similar molecular organization of the ANP in vertebrates, our data also let us predict a similar extent of ANP regionalization and variation in different species (Fig. 8C).

Our study also highlights subdomains of the prospective telencephalon, which, like the prospective diencephalon (Staudt and Houart, 2007) and eye field (this study), is composed of several concentric territories of specific cell identities, suggesting distinct cell fates. Our proposed ANP molecular map, which integrates concentric gene expression, as previously suggested (Toro and Varga, 2007; Zuber et al., 2003) (Fig. 8), will be relevant to spatially interpret future single cell transcriptomics analyses of developmental trajectories.

**Variations at the borders**

**A prospective telencephalon/eye-field boundary**

Surprisingly, our 3D confocal analysis reveals a small previously unreported, two or three cells wide circumferential territory separating prospective telencephalon (emx3+) and eye field (rx3−), and expressing both markers at low levels in surface fish tailbud (Fig. 7, Movie 9). In cavefish, this region showed cells with mixed identity, i.e. higher relative levels for both markers compared with surface fish. As higher emx3 relative levels might repress rx3, which is itself expressed at low levels in cavefish, this suggests that progenitors at the border are likely to adopt a telencephalic fate later on. Similar prospective telencephalon size in both morphotypes invalidates a slight posterior telencephalic expansion hypothesis, but not a potential progressive telencephalic fate adoption in cavefish. Interestingly, this tiny region also corresponds to the ‘low-zic1 crescent’, which is larger and has different expression dynamics in cavefish. Together, these findings suggest two non-mutually exclusive models: (1) progenitors at the border region maintain dual cell fate potentiality at tailbud stage that is resolved later on; or (2) this region would constitute an uncovered anterior subdivision of the neural plate with specific (unknown) cell fate. Fgf and Hedgehog signalling (Cavodeassi and Houart, 2012), which are both subtly modified in cavefish (Pottin et al., 2011; Yamamoto et al., 2004), are good candidates for explaining variations observed at the telencephalon/eye field boundary between the two morphs.

**Mixed identities at the hypothalamus/eye-field boundary**

In surface fish, only a few cells at the eye field/hypothalamus contact zone express rx3 and nkx2.1a at tailbud stage. Therefore, they may prefigure anterior/tuberal hypothalamic neuropeptidergic fates (Muthu et al., 2016). In cavefish, this intersection is doubled in size, which agrees with reported variations in neuropeptidergic
neuroanatomy in the basal forebrain of the two Astyanax morphs. Shh, the expression of which is expanded in cavefish prechordal plate at this stage (Pottin et al., 2011; Ren et al., 2018; Torres-Paz et al., 2019; Yamamoto et al., 2004) and the manipulation of which affects nkx2.1a expression in vertebrates (including cavefish; Pabst et al., 2000; Rétaux et al., 2008), constitutes a likely candidate for this previously unreported phenotype. As Shh signalling during hypothalamus development controls the future numbers of specific peptidergic neuronal populations (Alié et al., 2018), it is tempting to hypothesize that these neuronal subsets might already be primed at tailbud stage. Lineage analyses should confirm these propositions.

**rx3: an exemplary read-out of eye-field patterning and cell identity variations in cavefish**

More striking than a systematic reduction of mRNA levels in cavefish, as described via transcriptomics (McGaugh et al., 2014; Julien Leclercq and S.R., unpublished), rx3 expression is affected in many additional ways. Its domain of expression is smaller and is especially affected in the posterior part. This potentially explains the small size of early evaginating cavefish optic vesicles (Devos et al., 2021). Its fine regulation during co-expression with other transcription factors is modified at the eye-field boundaries (see above), with possible consequences for cell identities. Even more striking, rx3 expression is always heterogeneous at the cell population level, a phenotype that is never observed for the other transcription factors studied: pax6a, zic1 and lhx2. In cavefish prospective retinas, rx3 levels are not only reduced compared with surface fish, but are also variable and unpredictable, and most likely affect cell programs and behaviours differently in neighbouring cells in a given sample and between samples. Finally, this low and heterogeneous cell level rx3 expression could potentially affect expression of downstream eye-field effector molecules (Stigloher et al., 2006; Yin et al., 2014), as suggested by increased proportions of cells with medium rx3 levels and high cxcr4b levels in cavefish. These quantifications in the natural cavefish mutant suggest a more-complex epistasis between rx3 and cxcr4 genes than previously described and/or additional regulatory modes. In zebrafish, rx3-dependent cxcr4a expression demarcates the telencephalic border of the eye field and confers specific segregative properties to the eye field at the onset of neurogenesis (Bielen and Houart, 2012). In cavefish, rx3-dependent cxcr4b regulation might also affect adhesive or migratory properties of optic cells during early eye evagination, as observed through live imaging (Devos et al., 2021).

We propose that the different aspects of rx3 dysregulation have distinct developmental and genetic origins. The size of the rx3 expression domain would be a ‘simple’ consequence of the many signalling modifications that have been described in cavefish during and at the end of gastrulation (Hh, Fgf, Bmp and Wnt: Hinaux et al., 2015). The rx3 expression levels, on the other hand, and mostly its heterogeneity aspect, would probably result from intrinsic cis-regulatory changes at the level of the rx3 locus.

In zebrafish, medaka and Astyanax surface fish, the paired-type homeodomain transcription factor rx3 is crucial for eye development, as all rx3 mutant fish are eyeless (Kennedy et al., 2004; Loosli et al., 2003, 2001; Warren et al., 2021). In these species, rx3 controls survival of eye progenitors (Kennedy et al., 2004) as well as optic vesicle evagination and neuronal differentiation (Loosli et al., 2003), as revealed in the zebrafish chokh/rx3 loss-of-function background. In cavefish, the optic vesicles and eyes formed are always smaller than in surface fish and very little, if any, compensatory growth is observed (Devos et al., 2021), which contrasts with the tfc71a mutant embryos (Young et al., 2019). In the cavefish morph, no major apoptosis is recorded in optic vesicle cells during morphogenesis (Devos et al., 2021), and retinal apoptosis starts later, around 36-48 hpf (Alunni et al., 2007), in a lens-dependent manner (Yamamoto and Jeffery, 2000). It is possible that low and heterogeneous levels of rx3 could allow cavefish optic cells to survive and might prime them for future apoptotic programs. At the tissue level, rx3 heterogeneity may thus participate in a potential mechanism that allows progressive and controlled degeneration.

**Heterochronic cavefish**

Our study reveals substantial and more-subtle intra-species ANP differences, both in terms of eye-field regionalization/subdomains and at the single cell level. It also reveals striking differences in terms of apparent tissue dynamics: although some extent of eye-field condensation seems to occur at the onset of neurogenesis in surface fish, this global process of size reduction does not seem to take place in cavefish.

We developed a method of anterior ANP curvature normalization to allow the comparison of eye-field domain shape and size at similar stages of neural development. This uncovered two heterochronic processes in cavefish: a more advanced ANP curvature and a more anterior position of the prospective hypothalamus, which are likely to be uncorrelated. Importantly, the observed patterning differences were not subject to timing (i.e. they were observed for all d factors and were not delayed) and thus did not depend on neurogenesis advancement and keel formation.

In zebrasfish, during the initial step of optic vesicle evagination, some eye-fated cells behave like the nearby telencephalic cells and converge towards the midline to form the neural keel, while others lag behind and keep the eye field wide (Ivanovitch et al., 2013; Rembold et al., 2006; reviewed by Bazin-Lopez et al., 2015; Sinn and Wittbrodt, 2013; Wilson and Houart, 2004). This midline convergence may underlie the decrease in volume specifically observed in surface fish for several markers, as a function of the d factor. Such predicted neural plate condensation is likely to take place over a short period in surface fish (~30 min) and may rely on intrinsic tissue properties that are impaired in cavefish, such as heterogeneous rx3 expression and/or dysregulation of cxcr4, which normally influence the cohesion of eye-field cells (Bazin-Lopez et al., 2015).

**Conclusions**

All new ANP subdivisions found in Astyanax are likely present in other vertebrate species (Fig. 8). Detailed fate maps, lineage analyses and pseudo-time single-cell transcriptomics analyses will decipher their respective identities and outcomes. Intra-species differences observed here help to formulate hypotheses regarding the genetic specification of optic tissues, suggesting a substantial degree of variations between species. ANP phenotypes will guide the identification of the genetic basis of cavefish eye defects.

**MATERIALS AND METHODS**

**A. mexicanus embryos**

Our surface fish colony originates from rivers in Texas (USA) and our cavefish colony is derived from the Pachón cave in the state of Tamaulipas (Mexico). Embryos were obtained by *in vitro* fertilization, after induction of the breeding colony for gamete maturation and reproduction by changing the water temperature (Elipot et al., 2014). The embryonic development of
A. mexicanus at 24°C is similar and synchronous for both morphotypes (Hinaux et al., 2011).

Animals were treated according to French and European regulations of animals in research. S.R.’s authorization for the use of animals in research is 91-116. The protocol did not require authorization from the Paris Centre-Sud Ethic committee, as all experiments were performed on early [10 hpf (hours post-fertilization)] embryos, which are non-autonomous and have no nervous system.

Embryo staging and fixation
Morphological criteria were taken to stage 10 hpf and 10.5 hpf embryos, in addition to their known developmental time. These included extent of blastopore closure (100% epiboly), the prominent tail bud and the lateral flat triangular shape of the ANP (Hinaux et al., 2011). Embryos were fixed in 4% paraformaldehyde in PBS, dehydrated in graded ethanol/PBS steps and stored in methanol at −20°C.

Whole-mount in situ hybridization
Whole-mount in situ hybridization was carried out as previously described (Alê et al., 2018). Digoxigenin- and fluorescein-labelled riboprobes were prepared using PCR products as templates. cDNAs of interest were searched in our EST (expressed sequence tag) library (Hinaux et al., 2013). Clones in the library (pCMV SPORT6 vector) were: rx3 (FO289986), cxcr4b (ARA0AAA96YA18), pax6a (ARA0AAA41HY22), barhl2 (ARA0AEE6YL01), nkkx2.1a (AY661435; Menuet et al., 2007), zic1 (FO290256) (Devos et al., 2021), lhx2 (EF175373) (Pottin et al., 2011), enms (FO263072) and dlx3b (Hinaux et al., 2016). For fluorescent in situ visualization, FITC- and Cy3-tyramides (excitation peaks at 491 and 569 nm and emission peaks at 516 and 569 nm, respectively) were prepared as described previously (Zhou and Vize, 2004). Embryos were incubated in PBS containing 10 µM DAPI and 1% DMSO 24 h before dissection to stain nuclei, and washed in PBS.

Colorimetric in situ hybridization and image acquisition
Whole-mount embryos stained using colorimetric in situ hybridization were imaged on a Nikon AZ100 multizoom macroscope coupled to a Nikon digital sight DS-Ri1 camera, using the NIS software.

Fluorescent in situ hybridization and neural plate dissections
Fluorescently labelled embryos were transferred to a Sylgard-containing Petri dish bathed in PBT (PBS+0.3% Tween). Embryos were cut using forceps (Fine Science Tools, 5 Ultra) into two pieces at the yolk equator. After yolk removal, green and/or red staining of the ANP served to dissect out and isolate a piece of tissue containing the labelled ANP, under a fluorescent dissecting microscope. Dissected ANP were mounted in dorsal views in Vectashield (Vector) between glass slides and coverslips inside wells built with one reinforcement ring to limit neuro-ectodermal tissue flattening.

Fluorescent image stack acquisition
Confocal image stacks were captured on a confocal Leica SP8 microscope using the Leica Application Suite software. Water immersion objective (HC FLUOTAR L 25×/0.95 W VISIR 2.50 Water) was used for all experiments except for ‘single cell’ analyses, which required a 40× objective (HC PL APO 40×/1.0 W CORR CS2 0.65 Water 0.14–0.18) to improve resolution and hence better separate nuclei pixel intensities. As we did not compare mean intensities between samples but relative fluorescent levels within each normalized sample, we chose the optimal laser power and photomultiplier gain (PMT, ranging from 450 V to 630 V) for each sample acquisition. PMT gain compensation in the z-axis was also applied to capture signals in ventral deeper regions when stacking the entire eye field. Acquisitions were performed using sequential modes with optimization of emission wavelength range to avoid bleed-through of signals. Image stacks depth of entire neural plate typically ranged between 70 and 90 z steps depending on samples. A z step of 1 µm was used for each confocal acquisition. Image size and format were of 512×512 pixels and 8 bit, respectively.

Neural plate staging: d factor
The anterior curvature of the ANP looks like a parabola (z = ax^2), where (a) corresponds to the vertical scaling factor. For convenience of our experimental design we used the formula (z = ax^2/d), where factor d is the inverse of a. Therefore, the higher d is, the flatter the curve of the ANP is and the less advanced convergence is. Conversely, a lower d factor indicates a strong curvature of the ANP and thus advanced tissue convergence. We generated a single image (512×512 pixels) containing a merge of nine parabolas ranging from d factors 1 to 9, in an orthonormal system (Fig. 11−1’). This allowed us to generate composite 2-channel images containing a maximum intensity projection of single fluorescent in situ hybridization image stacks superimposed on the parabolas grid, in which the ANR medial anterior point was positioned at (x=0, y=0). We then assessed to which parabola/d factor each sample was best fitted (by eye). As ANP anterior curvatures were sometimes slightly asymmetric, we calculated the d factor for each sample as the mean of the two values estimated for the left and right side. We validated the accuracy of this method by merging images of similar d factors and controlling their shape correspondence (by eye). Overall, this method allowed the assessment of intrinsic neural temporality and staging for each sample, and compared them according to this criterion.

Morphometric analyses
All images were obtained from flat-mount dissected ANP (except for Fig. 1A,B).

2D and 3D size analyses
Size areas on colorimetric and fluorescence images were measured by tracing contours manually using FIJI (Schindelin et al., 2012). Volumes on image stacks were calculated as follows: for each marker, objects were generated after segmentation with Imaris software (Bitplane) using a common method (the same threshold method and morphological filters). For each channel, we created a corresponding cell using the default mode (settings: volume display). Cell body detection used the smooth option (filter width 2 µm). Cell threshold (absolute intensity) did not split touching cells and was adapted for each sample. Object properties were thereafter extracted (i.e. volumes). To compute the volume of the overlapping region between two markers, we used the ‘Surface-surface colocalization XTension’ (Imaris).

For Fig. 5, the volume of the domain expressing only rx3 (zic1−) was calculated by adding the areas of binarized objects at each plan. For Fig. 4E, we used the measure stack plug-in (FIJI), which allows the semi-automatic drawing of nkkx2.1a domain contours in each z step, the sum of which allowed the calculation of the volume of prospective hypothalamus.

For Fig. 5D,E and Movie 7, we applied the Make Binary plug-in (FIJI) on image stacks, setting identical minimum and maximum pixel intensities for all samples compared. Symmetrical subtraction of binarized zic1 and rx3 stack channels using the Image Calculator plug-in allowed the generation of zic1+/rx3− and rx3+/zic1− images. Segmented areas were calculated from maximum pixel intensity ventral half-stack projections using the Wand Tool. Volume of the midline ventral rx3/zic1− domain was calculated by summing all z segmented areas from rx3/zic1− images.
ectoderm). All images of the ANP show anterior towards the top except for Fig. 11-I” (towards the bottom).

Plot profiles
We generated plot profiles from maximum intensity projections of 10 µm sections at a position close to mid-z-depth of the expression domains considered in the majority of the experiments. For Figs 4, 6 and Figs S9 and S10, maximum intensity projections corresponded to entire stacks. In Fig. 4, we considered this variant for measurement as it clearly showed the relative position of the two tissues: eye field and prospective hypothalamus. The ROIs selected for measurements are indicated in each figure. Owing to inter-individual size differences, the length of each plot for rx3 (Fig. 1), pax6a/rx3 (Fig. 3) and nkt2.4/a/rx3 (Fig. 4) was normalized by the total distance, thus allowing inter-sample relative pixel intensity comparisons. For specific analyses at domain boundaries, the ROIs for pax6a/rx3 (Fig. 2), emx3/zic1 and zic1/rx3 (Fig. 7) were 20 µm wide and 50 µm long.

For analyses in the D/V axis (Fig. 3), we resliced image stacks entirely according to the indicated scheme to generate 3 µm sagittal projections. We next quantified relative pixel intensities in D/V line histograms equally spaced in the A/P axis inside the rx3 domain.

Plot profiles averaging
In order to compare normalized pixel intensities within the same relative zone between samples of different sizes, we averaged intensity values within intervals of 0.02 or 0.05 in the x axis.

Single cell analyses
For individual cell fluorescence quantifications, we used 2 µm maximum intensity projection images. Nuclear staining (DAPI) served to segment ‘individual particles’ in the ROI, allowing measurement of pixel intensity mean levels for all channels (single cells). As shown in Fig. S1, fluorescence levels measured in individual nuclei areas reflected the fluorescence levels in the corresponding surrounding concentric zones, thus showing that measuring pixel intensity levels in the nuclear zones was a good proxy of individual cell fluorescence levels. Again, as all fluorescence measurements were normalized, this allowed the comparison of relative fluorescent intensity levels – taken as a proxy of gene expression level – between cells within a given sample.

Statistical analyses
Mann-Whitney U-tests were used in Fig. 1K–N,R,V, Fig. 2H,K,L, Fig. 3L–I, Fig. 4E,G, Fig. 5F–I, Fig. 6E,F,H and Fig. 7C. P values are indicated in respective figures. A two-sided t-test with unequal variance was used for Fig. 5A’. Spearman correlation was used and R coefficient calculated in Figs 1P and 6J. Two-way ANOVA tests were used for Fig. 1Q,U, Fig. 3F,H,P, Fig. 4F, Fig. 5C, Fig. 6Q and Fig. 7E. Black bars and asterisks indicate significant differences. Chi-squared tests were performed in Fig. 2M, Fig. 4L and Fig. 7H. P value are indicated in each figure. Fisher’s exact test was performed in Fig. 6U,W, P values are indicated at the bottom. No statistical method was used to predetermine sample size. All experiments shown were replicated at least twice. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

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The authors declare no competing or financial interests.

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