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Sharpening of the anterior neural border in the chick by rostral endoderm signalling

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SUMMARY

The anterior border of the neural plate, presumed to contain the prospective peripheral portion (roof) of the prospective telencephalon, emerges within a vaguely defined proneural ectodermal region. Fate maps carried out at HH4 in the chick reveal that this region still produces indistinctly neural, placodal and non-neural derivatives; it does not express neural markers. We examined how the definitive anterior border domain of the rostral forebrain becomes established and comes to display a neural molecular profile, whereas local non-neural derivatives become separated. The process, interpreted as a border sharpening mechanism via intercalatory cell movements, was studied using fate mapping, time-lapse microscopy and in situ hybridization. Separation of neural and non-neural domains proceeds along stages HH4-HH4+, is well advanced at HH5, and is accompanied by a novel dorsoventral intercalation, oriented orthogonal to the border, that distributes transitional cells into molecularly distinct neural and non-neural fields. Meanwhile, neuroectodermal *Sox2* expression spreads peripherally from the neighbourhood of the node, reaching the nascent anterior border domain at HH5. We also show that concurrent signals from the endodermal layer are necessary to position and sharpen the neural border, and suggest that FGF8 might be a component of this signalling.

KEY WORDS: Neural induction, Neural plate, Neural border, Anterior neural ridge, Secondary prosencephalon, Forebrain, Telencephalon, Fate map, Neural patterning, Cell intercalation, Vertical induction, Chick

INTRODUCTION

Patterning of the embryo involves its subdivision into domains with different fates. One early process is the subdivision of the ectoderm into neural plate and epidermis (with placodes), which has been the subject of considerable attention since the first definition of neural induction in 1924 (Spemann and Mangold, 1924). These two primordial regions meet at the neural plate border (Fernandez-Garre et al., 2002; Puelles et al., 2005; Rodriguez-Gallardo et al., 2005; Streit and Stern, 1999). Initially, the neural and non-neural territories do not build a sharp boundary (Puelles et al., 2005): there is fate-mapping evidence that cells within a vaguely defined transitional zone produce both neural (forebrain) and non-neural (neural crest, adenohypophysis, skin, olfactory placodes) derivatives (Bronner-Fraser and Fraser, 1988; Streit, 2002). However, there is evidence for the existence of a 'pre-border' state (Linker et al., 2009; Pinho et al., 2011). We will thus refer to the immature border territory as proneural transitional ectoderm, subdivided into anterior, intermediate and posterior proneural transitional zones (APT, IPT and PPT) according to their position relative to prospective telencephalon, diencephalon-midbrain and hindbrain-spinal cord (supplementary material Fig. S2).

We placed the APT at the centre of the present investigation. Gradually, the APT becomes refined as its pattern sharpens into distinct subregions with differential neural versus non-neural fates. Eventually, transitional border areas fated to contribute differentially to either neural or extra-neural tissues become clonally isolated from each other, leading to full physical separation after neurulation. Separation of neural and non-neural subdomains of the APT presumably precedes differentiation of the anterior neural ridge (ANR), an important secondary organizer for the forebrain. We know very little about how this delimiting process occurs, least of all at the rostral end of the neural plate. This study was designed to explore how cells with distinct neural and non-neural fates separate at the APT, assessing also whether other tissues influence this sharpening process.

In the head, neural crest tissue is absent at the rostral to mid-diencephalic level (Basch et al., 2006; Carmona-Fontaine et al., 2007; Ezin et al., 2009). Cells of the early APT fate-mapped at Hamburger and Hamilton stage 4 (HH4) (Hamburger and Hamilton, 1951) contribute to structures found at the roof of the closed rostral neural tube (telencephalon and rostral diencephalon, to the adenohypophysis, olfactory placodes and neighbouring head skin) (Fernandez-Garre et al., 2002; Puelles et al., 2005; Rodriguez-Gallardo et al., 2005; Sanchez-Arrones et al., 2009). As the border of the late neural plate elevates, forming a limiting ridge and an associated neural head fold (stage HH8 in the chick), part of the APT thickens (acquires a cylindric neuroepithelial structure) and protrudes upwards forming the ANR.

Previous fate-mapping studies attempted to locate as precisely as possible the rostral neural border at late primitive streak stage (HH4) in chick embryos. Cells that end up in the ANR or the dorsorostral part of the forebrain can be labelled reliably using a coordinate reference system centred on the node (Fernandez-Garre et al., 2002). However, at this stage, the APT still contributes indistinctly to neural, non-neural and placodal cells, and it expresses mainly non-neural markers (e.g. *Gata3*, *Dlx5*) (Khudyakov and Bronner-Fraser, 2009; Puelles et al., 2005; Sanchez-Arrones et al., 2009). Therefore, cells with neural and non-neural fates should separate and form the expected border at some later point in development. The prospective neural portion of the APT should be induced to express appropriate neural markers.

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Here, we define the time at which such sharpening of the APT occurs, leading to gradual emergence of the ANR, and explore the cell interactions involved.

We started by labelling very small groups of cells at the APT with DiI/DiO, and followed their progeny and related intercalatory cell movements in either the anteroposterior (AP) or dorsoventral (DV) direction by time-lapse imaging. The embryos were then fixed and processed for in situ hybridization, and the position of labelled cells was related to the expression domains of various markers. The results support the notion that, initially, cells that will ultimately express either neural or non-neural markers are first intermingled within an unrefined transitional border domain. They gradually separate by DV intercalation, with a minor AP component. Prospective neural and non-neural cells of the APT seem to be fully separated after HH5. We identify the nascent anterior neural border at stages HH5/6 as emergent ANR (eANR), and by stages HH7/8 the definitive ANR becomes established, as indicated by activated *Six3* and *Fgf8* expression. Furthermore, we show that the hypoblast and/or rostral endoderm plays a crucial role in this anterior border sharpening process: extirpation and ectopic grafting of the endodermal layer reveals that endodermal signalling is required for DV cell intercalation during APT maturation up to stage HH5, as well as for induction and maintenance of the expression of *Six3* at the neural border. We also show that FGF8 can mimic this inductive effect, and suggest that FGF-related signals might underlie the endodermal influence on the APT.

MATERIALS AND METHODS

Fate map

Fertilized chicken eggs were incubated at 38°C to reach the desired stage. Fate-mapping experiments were performed at stage HH3+/4 (Hamburger and Hamilton, 1992), with focal injections of DiI and/or DiO into the anterior ectoderm. Alternatively, homotopic grafts labelled with green fluorescent CFSE (Molecular Probes, Carlsbad, CA, USA) (Fernandez-Garre et al., 2002) were performed in New-cultured chick host embryos (New, 1955; Stern and Bachvarova, 1997). Although grafted cells need two hours to integrate fully in the host epiblast and participate in local cell movement, their intercalation movements were thereafter very comparable to those observed after cell labelling with DiI. Injections and grafts were performed to map overlapping territories across the entire rostral neural/non-neural domain, including the APT zone (Fernandez-Garre et al., 2002). Immediately after labelling, the operated embryos were photographed under fluorescent illumination using an Axiocam camera (Carl Zeiss Vision, München-Hallbergmoos, Germany). After appropriate survival, embryos were fixed overnight in 4% buffered paraformaldehyde (pH 7.4, 4°C) and stored in cold PBS (for a maximum of 4 hours) until further processing. Some specimens were processed for in situ hybridization or immunochemistry as described below. Some embryos were followed by time-lapse microscopy in New culture.

Endoderm manipulations

A small region of the endoderm under the anterior neural plate was removed at stage HH4. The ablation was repeated twice at two-hour intervals to overcome the observed rapid regeneration of the endoderm. For time-lapse video microscopy, the endoderm was ablated twice at two-hour intervals and embryos were immediately recorded for two hours. In some cases, endoderm-ablated embryos were cultured with FGF8- or PBS-impregnated beads and cell behaviour was followed with DiI/DiO labelling and time-lapse microscopy. Anterior endoderm grafts were positioned ectopically under the lateral non-neural ectoderm at stage HH4+/5. The embryos were allowed to develop up to HH8 and then processed for in situ hybridization or cryoprotected in 15% sucrose solution in phosphate buffer overnight and cryostat-sectioned at 15 μm thickness in the transversal plane.

Beads

HH4 (control) and HH4+ (with ablated endoderm) chicken embryos were cultured after applying heparin-coated beads washed in PBS and soaked in FGF8b (50 μg/ml; R&D Systems). FGF signalling was inhibited for 1 hour with AG1X2 beads (Biorad) soaked with 1 mM, 2 mM or 5 mM SU5402 (Calbiochem) dissolved in DMSO. PBS-loaded AG1X2 or heparin beads were used as controls.

Electroporation

The activities of the *Sox2* enhancers N1 and N2 were visualized with the mRFP1 and EGFP expression vectors (Campbell et al., 2002; Uchikawa et al., 2003). The constructs N2-ptk-EGFP and N1-ptk-mRFP1 contained the corresponding *Sox2* enhancer sequence and a minimal promoter (TK) driving the expression of green (EGFP) or red (mRFP1) fluorescent protein. Vectors were co-electroporated in HH3+ chick embryos then transferred to New culture (Voiculescu et al., 2008). Fluorescent signal was usually seen 4 hours after electroporation. Some specimens were followed by time-lapse microscopy and then fixed between HH4+ and HH9 for further processing.

Time-lapse microscopy

Some DiI and/or DiO injected, grafted or electroporated embryos were filmed using a Leica SP2 microscope equipped with a Tsunami XI infrared laser and a thermoregulated hood, or using Zeiss upright or inverted fluorescence microscopes equipped with a Hamamatsu camera and Simple PCI time-lapse software. Embryos with endodermal ablations and DiI/DiO injections were filmed or photographed at regular intervals using a Zeiss Axiovert 200 microscope equipped with fluorescence optics. Embryos were thereafter fixed and processed further.

In situ hybridization (ISH)

Embryos were hybridized following the protocol described by Stern (Stern, 1998). Digoxigenin- or fluorescein-UTP-labelled antisense chicken riboprobes were used to detect the following markers: *Sox2*, *Six3*, *Fgf8*, *Otx2*, *Dlx5*, *Gata3*, *Fgf8*, *Bmp4*, *Pax6* and *Msx1*. Probes were visualized with NBT/BCIP (dark blue) or INT/BCIP (red).

GFP and CFSE signal detection

To visualize GFP and CFSE signal in fixed embryos, the samples were immunostained with rabbit anti-GFP (1:2000) and anti-fluorescein-POD (1:500) following standard protocols (Stern, 1998; Fernandez-Garre et al., 2002). HRP-coupled goat anti-rabbit (1:2000) was used as a secondary antibody.

Imaging

Images were captured with an Axiocam digital camera (Carl Zeiss Vision). Brightness and contrast were adjusted using Adobe Photoshop 7.0.1 (Adobe Systems, San Jose, CA, USA). Representative images were used as imported templates in Canvas 9.0 (Deneba, Miami, FL, USA) in order to draw vectorial schematic diagrams. Embryos subjected to ISH shrink significantly when heated in the presence of formamide and detergent (Streit, 2002). To overcome this problem and to compare ISH signal with the position of DiI-labelled cells, the images of hybridized embryos were magnified by 10% and aligned to landmarks visible in the fluorescence image.

Note on terminology

The conventional terminology for spatial direction or position in the neural plate becomes unwieldy when applied to the rostral-most positions, because of the circumferential shape of the rostral neural plate. The conventional term ‘mediolateral’, used to distinguish neural from non-neural territories, is contradictory at the rostromedian APT area, because there the non-neural territory lies in front of the neural plate, rather than laterally, and actually crosses the midline. This semantic problem can be conveniently resolved by referring to the prospective dorsal topological position of the whole neural plate border, considering its direct derivative, the neural tube roof. We therefore refer to planar cell movements orthogonal to the neural plate border as ‘dorsoventral intercalation’ or ‘dorsoventral convergence/divergence’, distinguishing centripetal/ventral/convergent from centrifugal/dorsal/divergent directions. Dorsoventral

convergence is conceptually equivalent to mediolateral convergence at more caudal parts of the neural plate. For the same reason, elongation, i.e. ‘anteroposterior intercalation’, occurs mediolaterally at the APT.

RESULTS

Cell movements at the anterior proneural transition zone and their relation to gene expression domains

We define the APT as a band of ectoderm at the periphery of the rostral neural plate, derivatives of which, mapped at neural plate stages (by transplantation or DiI-labelling), consistently contribute to both neural and non-neural tissue. This transition zone was estimated to be roughly 30-35 μm wide by Fernandez-Garre et al. (Fernandez-Garre et al., 2002). At stage 4 (late primitive streak), the APT largely expresses non-neural markers such as *Bmp4*, *Dlx5* and *Gata3*, and does not yet express *Sox2*, *Sox1*, *Six3* or other selective neural markers (Fernandez-Garre et al., 2002; Puelles et al., 2005; Sanchez-Arrones et al., 2009). The APT is expected to sharpen its fate and molecular pattern subsequently. To explore the timecourse of how this happens in more detail, we performed fate-mapping experiments and compared the results with the patterns of gene expression for neural and non-neural markers. Small groups of prospective chick APT cells were labelled with DiI/DiO at different stages to analyse the subsequent distribution of labelled cells in successive steps from HH4 to the early neural tube stage (HH8), when anterior neural and non-neural cells clearly are completely separated (Cobos et al., 2001).

Generally, neural plate cells at the proneural transition zone intermix extensively by intercalation. Some cases were followed with time-lapse analysis to determine the movements of labelled APT cells (Fig. 1; Fig. 2G; supplementary material Fig. S1A, Movies 1, 2). The experimental embryos were then processed by ISH for neural and non-neural markers to identify the stage at which the fate and molecular maps first become congruent.

Cells labelled within the lateral part of the APT at HH4 first disperse longitudinally along the emergent border of the neural plate (the prospective axial roof plate of the neural tube; Fig. 1A,D; Fig. 2D). We identify this process contributing to brain elongation as ‘longitudinal or AP intercalation’. A few labelled cells end up outside the neural plate (Fig. 1B,C). However, when cells labelled in this lateral domain at stage 4 are examined at HH5+, they are found to have dispersed orthogonally to the APT; that is, both centrifugally into the immediately adjacent non-neural territory and centripetally within the neural plate, as if moving towards the node (Fig. 1D,E;

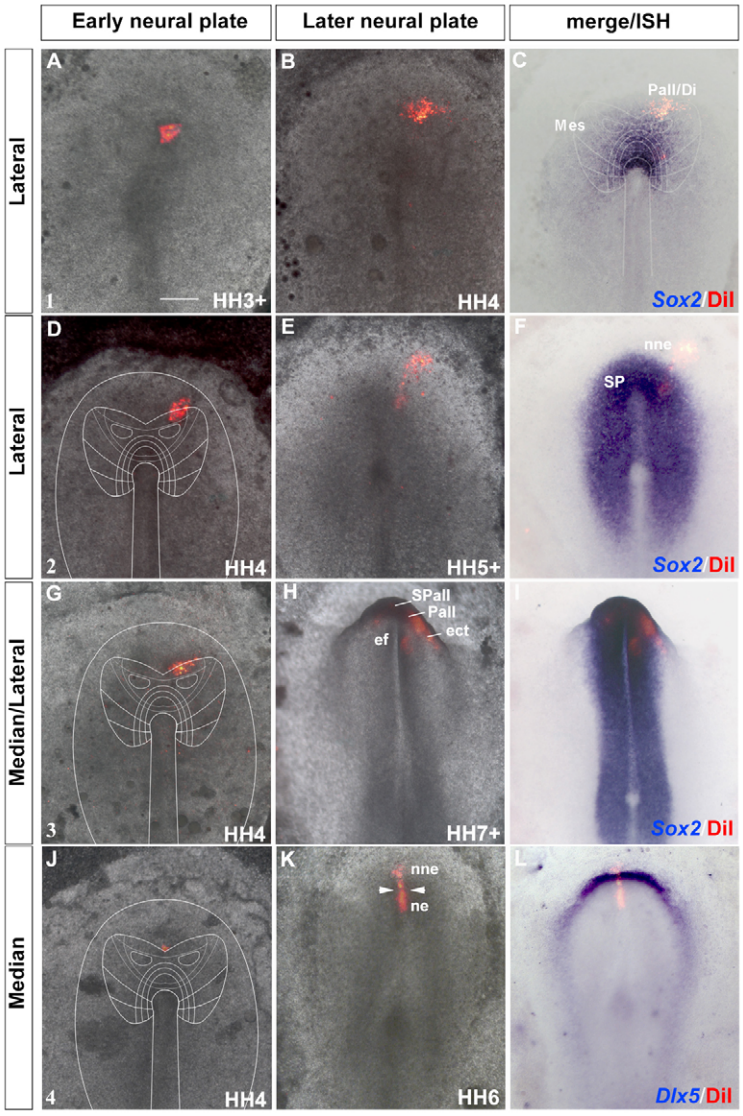

Fig. 1. DiI tracing of cell movements at the APT. (**A-L**) Combined fluorescent and bright-field images of HH3+/4 chick embryos, in which cells at the lateral, intermediate and median parts of the APT were labelled with DiI and immediately recorded (A,D,G,J) and thereafter grown up to the stage indicated in the panels and re-recorded (B,E,H,K). Embryos 1, 2 and 3 (A,D,G) were hybridized for the neural marker *Sox2* (C,F,I), whereas embryo 4 (J) was hybridized for the non-neural marker *Dlx5* (L). Note that cells marked in the lateral APT undergo both longitudinal and dorsoventral intercalation, whereas rostromedial cells intercalate only dorsoventrally (orthogonal to the presumptive neural border). Labelled cells end up in both neural (C,F,I) and non-neural domains (L). Scale bar: 250 μm. Di, diencephalon; ect, ectoderm; ef, eye field; Mes, mesencephalon; ne, neural ectoderm; nne, non-neural ectoderm; Pall, pallium; SP, secondary prosencephalon; Spall, subpallium.

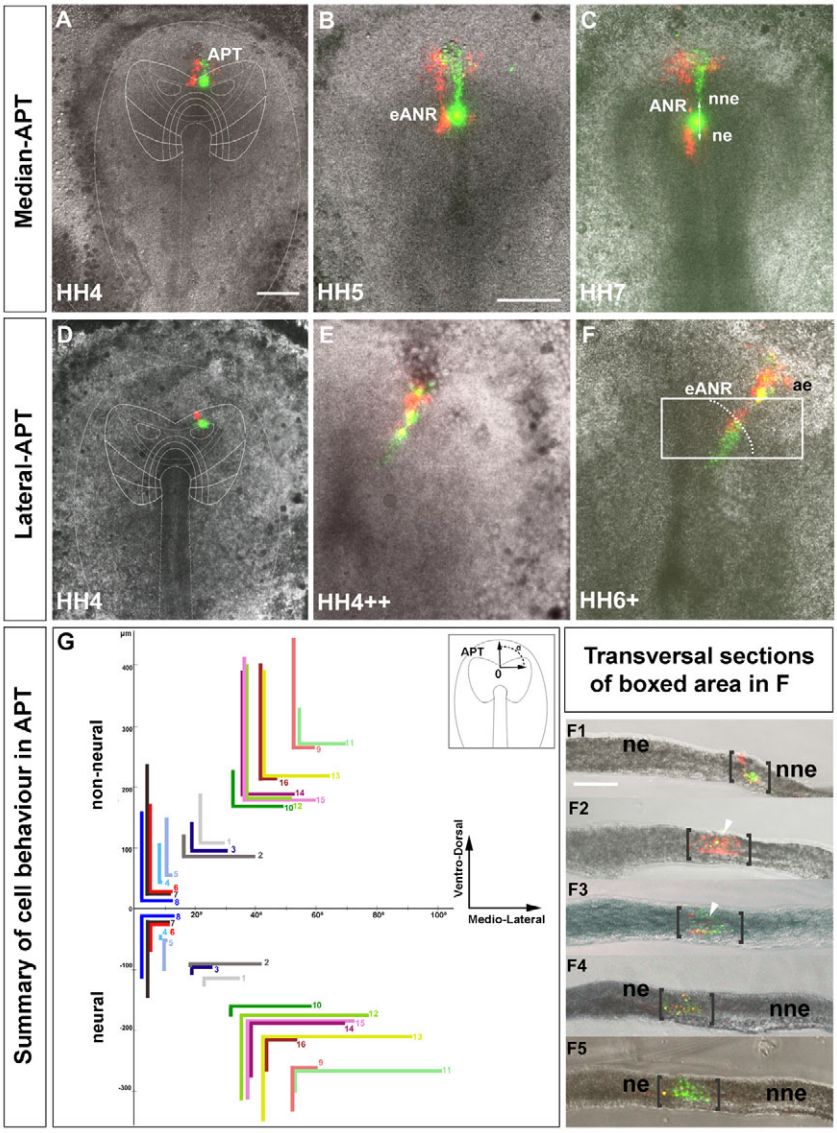

Fig. 2. The neural border becomes established at stage HH5. (**A-C**) Combined fluorescent and bright-field images of HH4 chick embryos labelled with DiI/DiO (red and green, respectively) at the median APT. Note how neural and non-neural subpopulations are largely separated dorsoventrally at HH5 (B); this becomes more evident at later stages (C). (**D-F**) Equivalent views of embryos in which cells in the lateral APT were labelled with DiI and DiO, and recorded by time-lapse video microscopy (E,F). The partial neural border in F (dotted white line) is taken from the limit of *Sox2* expression at this stage. Images in F1-F5 show transversal sections of the embryo in F (HH6+). Brackets in F1-F5 delimit the region occupied by labelled cells (white arrowheads). (**G**) Graphical representation of the initial (HH4) and final (HH5-6) position of neural and non-neural cells labelled with DiI and DiO at different mediolateral and dorsoventral positions. Only embryos labelled at HH4 (t=0) and fixed at HH5-6 are represented in the graph. The topography of each labelled cell group is represented with colour-coded horizontal and vertical bars (with embryo number) where mediolateral initial and final positions are scored in degrees (*x*-axis) to respect to the median neural border (0°; see diagram on the right) whereas dorsoventral elongation is measured in microns (*y*-axis). Longitudinal intercalation is minimal at the midline (embryos 4-8) but increases in more lateral positions (embryos 10-15). Scale bars: 250 μm (A for A,D; B for B,C,E,F); 100 μm (F1-F5). ae, anterior endoderm; APT, anterior proneural transition zone; eANR, emergent ANR; ne, neural ectoderm; nne, non-neural ectoderm.

Fig. 2D). Cell patches labelled slightly outside the APT also stretch in the same way and partially disperse into neural territory (Fig. 4A,B). Thus, lateral APT polyclones labelled at HH4 tend to become separated into two dorsoventrally stretched subgroups at HH5, which lie respectively inside and outside the neural primordium. The separation between the inner and outer subgroups apparently increases at stage HH6, though sectioned material reveals no real separation, but rather a progressive dilution of labelled cells in the intervening area (Fig. 2F, sections F1-F5; supplementary material Movie 2). We call this non-axial dispersion process ‘dorsoventral or DV intercalation’ because after neurulation the neural-labelled domains lie topologically ventral to the non-neural ones (see section on topologic terminology in Materials and methods).

Serial transverse sections of the lateral eANR region at HH6 confirmed a final more limited AP dispersion of DiI/DiO labelled neural and non-neural cells (Fig. 2F, sections F1,F2,F5) and a more marked DV dissociation across the boundary (Fig. 2F, sections F3, F4; supplementary material Movie 2). Note that at this stage the pre-placodal marker *Dlx5* and the anterior neural marker *Six3* appear to be co-expressed at the eANR (Fig. 4C, section C3). Labelled cells are also found in the rostral ectoderm overlying the labelled neural domains (Fig. 2F, section F1).

At HH4, the early neural marker *Sox2* is expressed in the immediate vicinity of Hensen’s node, but barely reaches the APT (Fig. 1C; Fig. 3A′; supplementary material Fig. S2A). In the following few hours (stages 4+-5), its expression domain first expands anteriorly and laterally, before it starts to extend caudalwards as well (Fig. 1F; supplementary material Fig. S2E) (Rex et al., 1997). To determine the precise relationships of the lateral APT-derived cells to this marker, APT-labelled embryos were processed for in situ hybridization after recording the position of the labelled cells. Compared with the *Sox2* expression pattern at HH5, the centrifugally (dorsally) displaced DiI-labelled cells lie within a *Sox2*-negative region, whereas centripetally (ventrally) displaced DiI-labelled cells are found within the *Sox2*-expressing domain inside the neural plate (Fig. 1E,F).

The specificity of the observed DV intercalation process was further supported by DiI/DiO injections in the median portion of the APT. When this peripheral region of the neural plate is labelled very close to the midline at HH4, massive DV intercalation is detected, with marked dispersion of labelled cells orthogonal to the eANR, but there is virtually no longitudinal or AP dispersion (Fig. 1J, Fig. 2A-C; see also embryos 4-8 in Fig. 2G at stages HH4 and HH5-6; supplementary material Fig. S1A,B and Movie 1). At stage

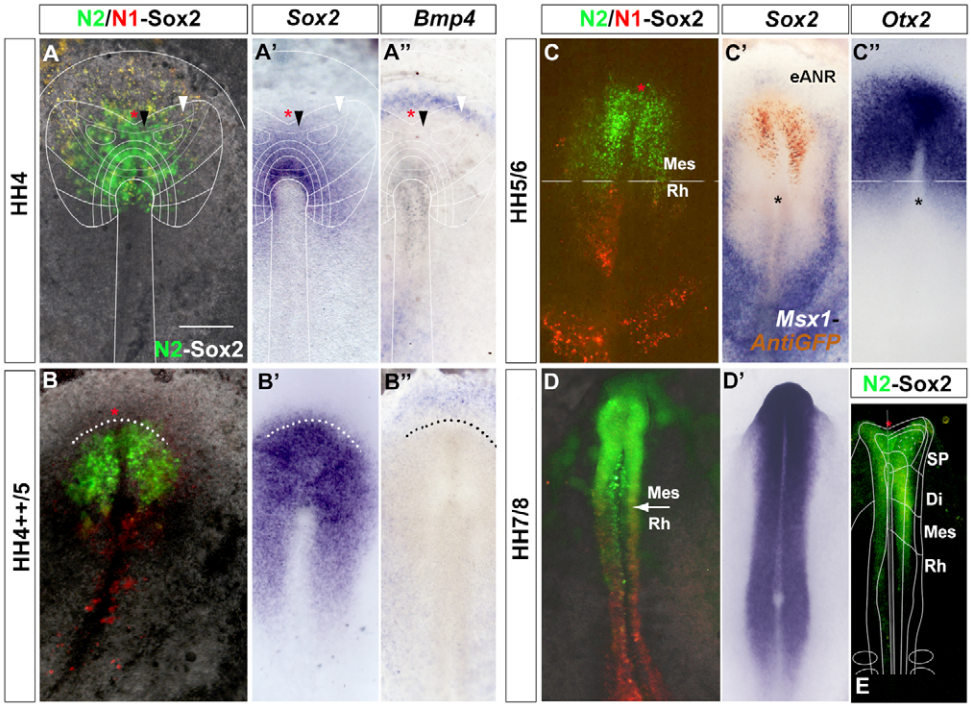

Fig. 3. Correlation of expression patterns with activity of the *Sox2* regulatory sequences. (**A-E**) Combined fluorescent and bright-field images of chick embryos grown in New cultures and electroporated at stage HH3 with a plasmid containing either the N2-*Sox2*-EGFP enhancer reporter (green, A,E) or the N1-*Sox2*-RFP1 enhancer reporter (red, B,C,D). Embryos were analysed from HH4 onwards as indicated in the panels. The embryo in C was hybridized for the non-neural marker *Msx1* and immunostained with anti EGFP (C′). In situ hybridization for neural (*Sox2* and *Otx2*, A′,B′,C″,D′) and non-neural (*Bmp4*, A″,B″; *Msx1*, C′) markers were compared with *Sox2*-reporter expression. Red asterisks represent the median APT locus. Black and white arrowheads point to the prospective subpallial and pallial telencephalic domains, respectively, whereas the white arrow indicates the prospective midbrain-hindbrain boundary (MHB). A sketch of the ANP fate map previously determined at stages HH4 and HH8 was superimposed in A-A″ and G. Scale bar: 250 μm. APT, anterior proneural transition zone; Di, diencephalon; eANR, emergent; Mes, mesencephalon; Rh, rhombencephalon; SP, secondary prosencephalon.

HH6, the DiI-labelled cells still form a compact group stretched across the eANR along the ectodermal midline (Fig. 1K; Fig. 2A-C,G; supplementary material Fig. S1A,B). Some labelled elements move centrifugally, entering the non-neural territory (which expresses *Dlx5*; Fig. 1L) and extending significantly within it, and others move centripetally, into the *Sox2*-positive neural domain (Fig. 1K,L). At HH6/7+, the DiI-labelled median neural cells lie largely within the *Sox2*-expressing prospective preoptic area, whereas non-neural derivatives are found mainly in the median *Sox2*-negative ectoderm corresponding to the future adenohypophysis (Fig. 1H,I; see 10-12 hour time points in supplementary material Movie 1).

When similar DiI-labelling experiments were performed in HH5 embryos, no significant cell dispersion was observed in any direction and the polyclones remained as coherent groups (not shown), suggesting that intercalation movements characteristic of the APT have finished by this stage, coinciding with the stage at which *Sox2* expression expands into the eANR (see below).

The molecular profile at the APT changes from HH4 to HH7

To explore when the prospective neural plate border acquires a molecular identity consistent with a neural fate, we re-examined in more detail the expression of several marker genes from HH4 to headfold stages (HH7/8), and correlated this with the positions of labelled cells from our fate-mapping experiments (supplementary material Fig. S2). At stage HH4, expression of *Sox2* is strongest around the node (prospective ventral CNS); strong expression barely reaches the prospective median chiasmatic area (supplementary material Fig. S2A,A′). More peripherally, *Sox2* decreases in intensity progressively towards the APT (supplementary material Fig. S2A,A′). The broader lateral part of the neural plate, representing prospective caudal telencephalic, diencephalic and midbrain regions, does not express *Sox2* at this stage. Rather, these regions express the non-neural markers *Gata3* and *Dlx5* (supplementary material Fig. S2A-B; compare with *Pax6* in supplementary material Fig. S2D) (Sheng and Stern, 1999). Therefore, the expression of genes considered as neural and non-neural markers is not yet consistent with the partial neural fate of the APT mapped at HH4.

At stages HH5-HH7, *Sox2* expression expands to the whole of the region fate-mapped as prospective neural tube; the characteristic wedge-shaped median indentation of the fate-mapped neural plate anterior border (Fernandez-Garre et al., 2002) disappears as the anterior neural plate rounds up and the adenohypophysial anlage moves away (supplementary material Fig. S2A,B,E,F). Simultaneously, the *Sox2* signal disappears from the ventral epichordal midline of the neural plate, the future floor plate (supplementary material Fig. S2E,J) (Ruiz i Altaba et al., 1995). Expression of *Gata3* and *Dlx5* is gradually excluded from the rostral neural domain and particularly from the prospective dorsal diencephalic and midbrain areas, where these non-neural

markers persist at HH4 (supplementary material Fig. S2 A,B,E,F). The strongest inner part of the *Dlx5* expression domain overlaps with the inner edge of the band of *Gata3* placed at the rostral edge of the *Six3*-positive neural plate (supplementary material Fig. S2E,F,J,K). This non-neural overlap area might correlate with the rostral pre-placodal domain (Fig. 4C). In regions caudal to the prospective midbrain-hindbrain border, *Dlx5* expression is much weaker at stage HH6, but that of *Msx1* appears in the equivalent region and its domain increases caudalward (supplementary material Fig. S2F,G). Subsequently, non-neural *Msx1* signal expands rostralwards parallel to the mesencephalic part of the neural plate border, coinciding with *Bmp4* (supplementary material Fig. S2L,N). Comparison of the fate maps with the locations of these expression domains suggests that the molecular boundary formed between the *Sox2* and *Gata3-Dlx5-Msx1* expression domains at HH5-7 corresponds to the frontier that divides groups of cells destined to adopt neural versus non-neural fates. We therefore conclude that a sharp neural/non-neural molecular boundary first becomes defined at stage HH5 or shortly thereafter.

The expression patterns of *Pax6* and *Bmp4* reveal further subdivision and refinement of the neural plate border after this stage. *Pax6* expression, apparently first overlapping both neural and non-neural sub-regions of the APT and IPT (Sanchez-Arrones et al., 2009), later localizes to a territory lying rostral to the prospective diencephalo-mesencephalic boundary (supplementary material Fig. S2H). At HH6, non-neural expression of *Bmp4* is observed along the whole neural border, including the eANR (supplementary material Fig. S2I); thereafter, *Bmp4* becomes downregulated at the anterior part of the head fold (Fig. 3A-A″,B,B″, arrowheads), next to the prospective secondary prosencephalon (i.e. ANR), and remains restricted to diencephalic (weak signal) and midbrain (stronger signal) territories of the neural border (supplementary material Fig. S2M,N). These domains coincide approximately with areas in which *Msx1*, a downstream target of *Bmp4* (Streit and Stern, 1999), is likewise expressed in non-neural ectoderm (supplementary material Fig. S2L; Fig. 3C,C′). At HH7+, the ANR lacks both *Bmp4* and *Msx1* but expresses *Six3* (supplementary material Fig. S2L,M). Shortly afterwards, at HH8, *Fgf8* expression appears at the ANR.

Correlation of expression patterns with activity of *Sox2* regulatory sequences

Early expression of *Sox2* is controlled by two enhancers: N1 and N2 (Papanayotou et al., 2008; Uchikawa et al., 2003). To determine their activity domains in relation to the fate map and to gene expression patterns, we electroporated reporter constructs containing each of these enhancers. At stage HH4, the N2 enhancer already shows activity in the anterior neural plate (Kimura et al., 2006) (Fig. 3A, green). N1 starts to be activated slightly later, from HH5 onwards, but only in the caudal (deuterencephalic) neural plate (Fig. 3B, red). We therefore studied the N2 enhancer in more detail. At stages HH4-4+, strong N2 activity is only detected around the node (future ventral CNS; Fig. 3A). Co-electroporation with DsRed driven by a ubiquitous promoter (β-actin), showed that the region with N2 activity was only a fraction of the whole electroporated area (not shown). These results suggest that the initial perinodal expression of *Sox2* is driven by the N2 enhancer.

The most caudal cells with N2 enhancer activity lie roughly at the prospective forebrain/midbrain boundary at stage HH4 (Fig. 3A). At this stage, the N1 enhancer has not yet been activated in

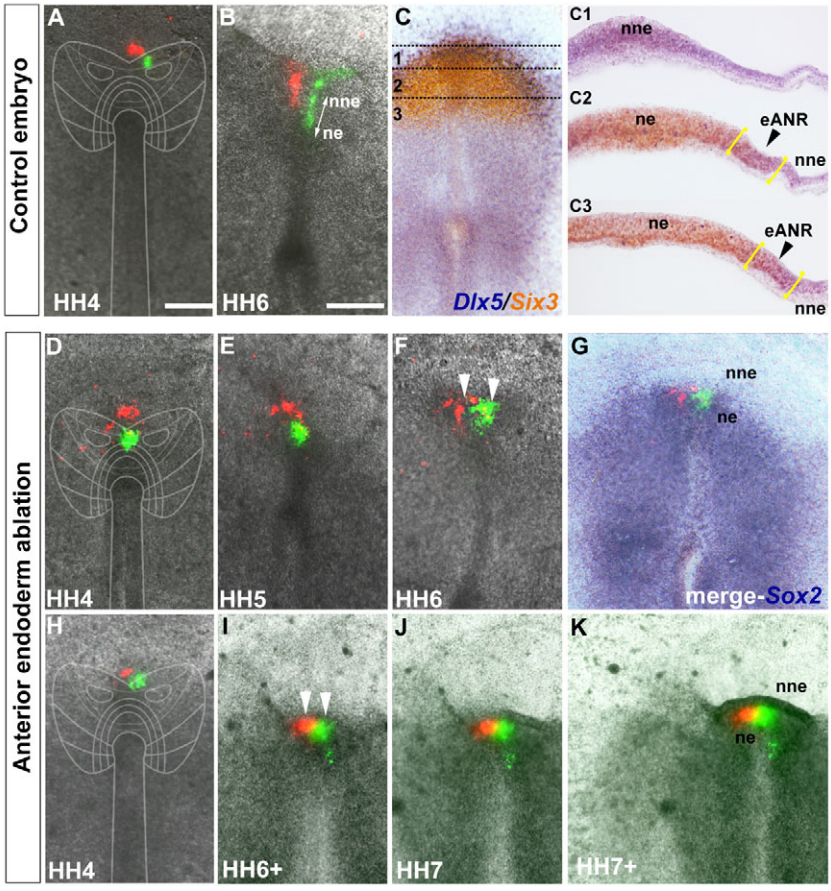

Fig. 4. The anterior endoderm is required for cell intercalation at the APT. (**A-K**) Combined fluorescent and bright-field images of chick embryos labelled at stage HH4 in control (A-C) and endoderm-ablated (D-K) specimens. Cells outside (red) and inside (green) the median part of the APT were labelled with DiI and DiO and cell behaviour was followed by time-lapse microscopy until HH6-7 (B,E-G,I-K). Red- and green-labelled cells show DV intercalation across the emergent ANR at HH6 in a control embryo (B); note that some of the intermixed eANR cells seem to express both *Dlx5* and *Six3* (C and sections C2,C3; brackets delimit the overlapping genes at the eANR domain). Endoderm-ablated embryos showed neither AP nor DV intercalation (D-K). The *Sox2* expression domain encompasses both green- and red-labelled cells (G). Scale bars: 250 μm (A for A,D,H; B for B,C,E-G,I-K). APT, anterior proneural transition zone; eANR, emergent ANR; ne, neural ectoderm; nne, non-neural ectoderm.

the caudal neural plate. At stage HH5, N2 activity expands, fully extending to the prospective midbrain-hindbrain boundary (Fig. 3C-E), corresponding to the caudal boundary of *Otx2* expression (Fig. 3C″). By stages HH7/8, all the primary prosencephalic subdivisions (including neural derivatives of the APT) express *Sox2*, coinciding with the domain of activity of N2 (Fig. 3D,E). Together, the combination of gene expression and cell-tracing analyses indicates that a sharp border between neural and non-neural territories at the rostral region of the neural plate can first be fully defined at about stages HH5-7.

Signals from the rostral endoderm promote initial APT specification

What drives intercalative and molecular sharpening of the APT? At stages HH4-5, the rostral ectoderm is underlain directly by ‘anterior endoderm’ (a tissue made up of cells from different sources, some derived from the original hypoblast of the early blastodisc and some corresponding to definitive anterior endoderm, derived from the primitive streak) (Bellairs et al., 1981; Kimura et al., 2006). The anterior endoderm has been implicated in anterior neural patterning in both mouse (Acampora et al., 2009; Camus et al., 2000; Martinez-Barbera and Beddington, 2001; Thomas and Beddington, 1996) and chick (Chapman et al., 2003; Knezevic and Mackem, 2001; Pera et al., 1999; Withington et al., 2001). Therefore, we explored the role of this tissue in the sharpening and neural induction of the APT. First, we repeatedly (see Materials and methods) ablated the anterior endoderm at neural plate stages (HH4/4+) and examined with time-lapse video microscopy the behaviour of ectodermal APT cells labelled with DiI and DiO. Successful removal of endoderm was confirmed by sectioning of the specimens after time-lapse recording (supplementary material Fig. S3, Movie 3). In contrast to what was observed in controls (Fig. 4A,B), no AP or DV intercalation occurred in ablated embryos (Fig. 4D-F,H-K). Outer- and inner-labelled APT cells appeared to be closely aggregated just within the border of the *Sox2*-positive ectoderm domain (Fig. 4G). This was also true at stage HH7, when the head fold forms (Fig. 4J,K). Notably, the outer APT cells, which normally would have largely dispersed into non-neural tissue lying outside the *Sox2* expression domain, were now included next to the inner APT cells within this field. This suggests an anomalous mechanism of cell recruitment and/or of anterior patterning at the rostral neural plate region devoid of underlying anterior endoderm. In addition, such data suggest that anterior centrifugal expansion of *Sox2* expression does not depend on the presence of rostral endoderm and might, in fact, overshoot its normal range in its absence. Moreover, the abnormality of the anterior plate border after endoderm ablation is confirmed by examining the expression of the rostral neural marker *Six3* (Fig. 5A,F,K) (Bovolenta et al., 1998), which was either completely absent or strongly diminished in the rostral neuroectoderm placed over the missing endoderm tissue (Fig. 5B,G). Simultaneously, the expression of *Bmp4* expanded centripetally into the prospective rostral neural plate (Fig. 5B,G′,L′; supplementary material Fig. S3C).

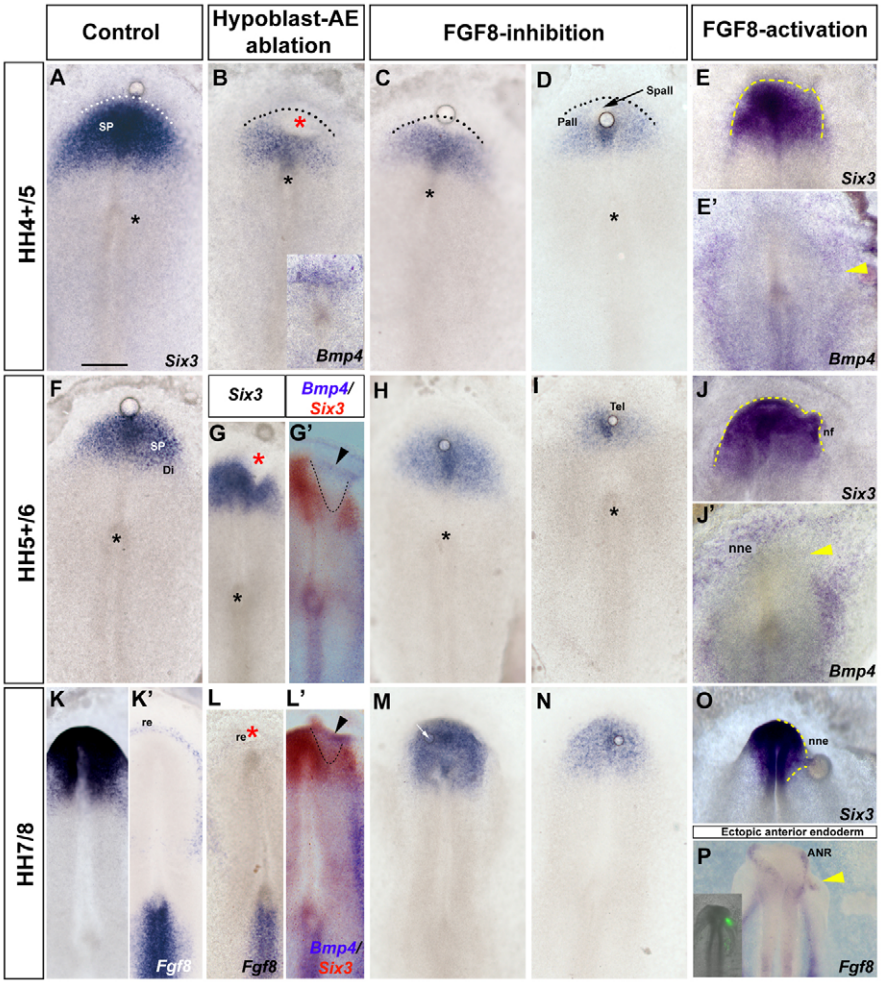

Fig. 5. Signals from the rostral endoderm promote APT sharpening. (**A-P**) Bright-field images of chick embryos grown in New cultures and subjected to different experimental manipulations: implants of beads soaked in PBS/DMSO (A,F), 2 mM SU5402 (C,H,M), 5 mM SU5402 (D,I,N) or FGF8 (E,E′,J,J′,O); ablations of the hypoblast and/or the anterior endoderm (AE) (B,G,G′,L,L′) and ectopic grafts of AE (P). Embryos were fixed at the stages indicated in the panels and hybridized with probes specific for *Six3*, *Fgf8* and *Bmp4*. Note that both AE ablation (B,G,G′,L′) and SU5402-bead implantation (C,D,H,I,M,N) inhibit expression of the neural marker *Six3* and expand the expression of the non-neural marker *Bmp4* (B,G′,L′), whereas implantation of FGF8 beads expands *Six3* (E,J,O) and inhibits *Bmp4* (E′,J′) expression. Note also that AE grafts expand *Fgf8* expression (P). Inset in P shows the position of the CFSE-labelled graft. Red asterisks mark the ablated tissue, black asterisks indicate the position of Hensen’s node. Yellow arrowheads indicate *Bmp4* inhibition (E′,J′) or *Fgf8* overexpression (P). Dotted white and black lines highlight the APT and dashed yellow lines highlight ectopic gene expression. Scale bar: 250 μm. APT, anterior proneural transition zone; ANR, anterior neural ridge; Di, diencephalon; nf, neural fold; nne, non-neural ectoderm; Pall, pallium; re, rostral endoderm; SP, secondary prosencephalon; Spall, subpallium; Tel, telencephalon.

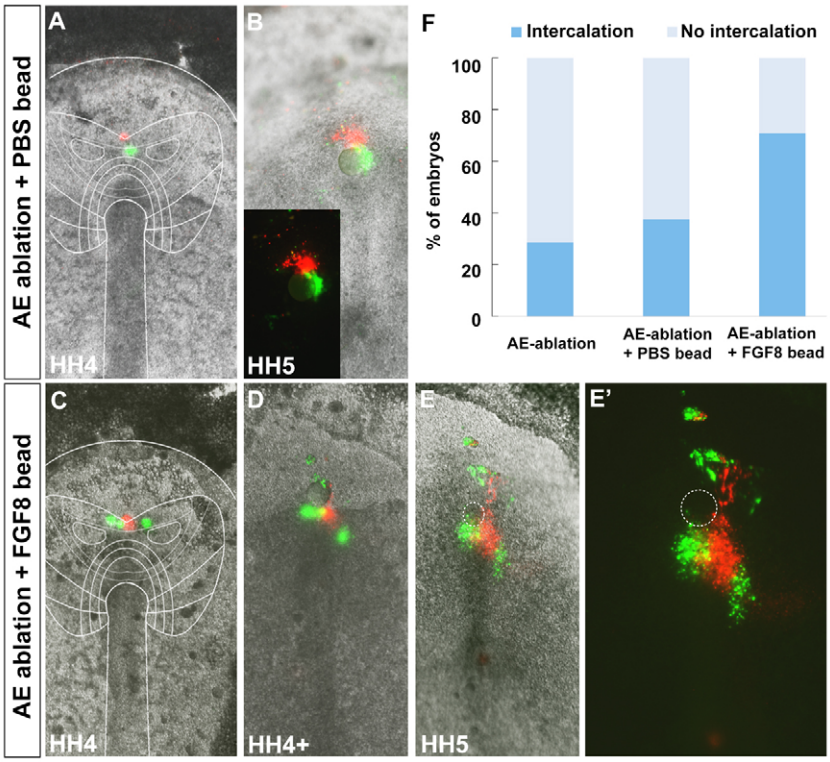

Fig. 6. Fgf8 activity promotes DV cell intercalation at the APT. (**A-E′**) Combined fluorescent and bright-field images of stage HH4 chick embryos implanted with control PBS- (A,B) or FGF8-loaded (C-E′) beads after endoderm ablation. Cells outside (red) and inside (green) the median part of the APT were labelled with DiI and DiO. Note that Fgf8-bead largely rescues cell intercalation (D-E′), which is absent in PBS treated endoderm ablated embryos (B). Dashed circle in E and E′ indicates position of bead. (**F**) Proportion of embryos with or without cell intercalation in each experimental condition.

The rostral endoderm normally expresses *Fgf8* (Ladher et al., 2005); this signal was undetectable in the operated embryos (Fig. 5L, red asterisk), confirming efficient ablation of the rostral endoderm and lack of regeneration. In order to test whether signals from the endoderm are sufficient to induce ectopic anterior neural border tissue, we placed small pieces of rostral endoderm under the non-neural ectoderm outside the APT (Fig. 5P; supplementary material S3A,B). This procedure induced an ectopic patch of ectodermal *Fgf8* expression (corresponding to ectopic ANR, which normally expresses FGF8) (supplementary material Fig. S3A) (Crossley et al., 2001; Shimamura and Rubenstein, 1997). These experiments suggest that the underlying endoderm is required to promote ectodermal cell movements that help to define the APT fate and to evoke expression of *Six3* in the anterior neural plate.

FGF8 itself is a good candidate for mediating, at least in part, this activity of the endoderm. *Fgf8* is expressed by both hypoblast and definitive endoderm cells (Albazerchi and Stern, 2007; Ladher et al., 2005; Streit and Stern, 1999). At HH4, expression appears as a semilunar stripe (at the anterior intestinal portal), which crosses the midline just under the APT (Fig. 5K′). We therefore explored the role of FGF signals in refinement of the APT border zone. To test whether FGF8 can induce an ectopic ANR, we implanted a heparin bead soaked in FGF8 at stages HH4-8 in the non-neural ectoderm just adjacent to the APT. An ectopic neural fold-like formation expressing *Six3* was induced around the bead carrying FGF8 (Fig. 5E,J,O). *Bmp4* expression was inhibited in the non-neural ectoderm exposed to FGF8 (Fig. 5E′,J′, compare control left side with experimental right side) and the expression of the markers *Six3*, *Bmp4* and *Fgf8* itself (ANR marker) was expanded outwards around the FGF bead. The effect was strongest when the bead was implanted at HH5+-8, that is, after the neural and non-neural subdomains of the APT become molecularly and clonally separated, giving rise to the eANR. Therefore, FGF under the studied conditions can force the adoption of a neural fate within an ectopic anterior ectoderm field expressing non-neural markers.

We then tested whether FGF signalling (from the anterior endoderm) is required for formation or maintenance of the ANR. A bead soaked in the FGFR inhibitor SU5402 was implanted next to the median APT at HH4 (midline, 250 µm anterior to the node, according to previous fate maps) (Fernandez-Garre et al., 2002) and the embryos were incubated until stages HH5-7. Beads soaked in DMSO (control) or SU5402 (1 mM) had no effect (Fig. 5A,F). With 2-5 mM SU5402, 96% of the embryos (n=26/27) showed a general reduction of *Six3* expression at the anterior neural plate, as well as a small halo devoid of *Six3* signal around the bead (n=10; Fig. 5C,D,H,I). Little or no inhibition of *Six3* expression was seen when the bead was inserted later (n=10 at HH5+/6; n=6 at HH6/7; Fig. 5K,M,N).

To explore whether FGF8 was sufficient to rescue the *dorsoventral intercalation* behaviour promoted by rostral endoderm, we implanted FGF8- or PBS-loaded heparin beads after endoderm ablation was carried out twice. Time-lapse recording of DiI and DiO labelled cells at the median APT (Fig. 6A,C) revealed efficient rescue of cell movement in a large proportion of FGF8-bead implanted embryos, whereas PBS-beads had no effect (Fig. 6).

Together, these experiments are consistent with the notion that FGF-related signals emanating from the hypoblast and/or rostral endoderm between stages HH4 and HH5 promote DV intercalatory clonal separation of prospective neural and non-neural parts of the APT, and contribute to correlative molecular re-patterning of the APT, thus sharpening the ANR border zone.

DISCUSSION

The anterior proneural transition zone or APT is a vaguely defined but distinct anterior boundary domain of the early neural plate (Fernandez-Garre et al., 2002), which encompasses the future dorsal region of the forebrain and the neighbouring non-neural ectoderm, inclusive of adenohypophysial and olfactory placodes (Houart et al., 2002; Houart et al., 1998; Puelles et al., 2005). Compared with other neural plate border regions, the APT is

singular in lacking neural crest derivatives. At about stages HH7/8, the inner part of the APT zone becomes elevated (crest-like) and starts to express FGF8; this region becomes an organizing centre, the anterior neural ridge (ANR), whose signals are important for further forebrain patterning, particularly of the telencephalon (Vieira et al., 2010; Wilson and Houart, 2004). The mechanisms that dissociate the neural versus non-neural fates of APT cells, i.e. that subdivide this zone into separate arc-shaped subregions (the inner neural one representing the emergent ANR), are not yet understood. Here, we have studied the initial events associated with the sharpening of the APT domain.

It is well known from work in different vertebrates that the early neural plate elongates through mediolateral cell intercalation, which generates movements of convergence and extension [e.g. *Xenopus* (Keller et al., 2000); chick (Lawson et al., 2001) and mouse (Ybot-Gonzalez et al., 2007)]. However, these studies have not attended to the APT, a singular region that changes shape considerably during its formation and subsequent remodelling. Ezin and collaborators (Ezin et al., 2009) recently studied cell movements across the neural border at prospective diencephalic and midbrain levels, investigated in relation to local neural crest formation. They described 'mediolateral convergence' movements in the intermediate parts of the proneural transition zone, consisting mainly of displacements away from the midline, confusingly described as being 'lateroventral'; these movements were accompanied by longitudinal 'extension' movements. However, the movements of cells in the APT were apparently not examined by these authors. Our results suggest that longitudinal or AP cell intercalation is indeed present early on at HH4, but diminishes thereafter, particularly at the median (most anterior) portion of the APT, where no such movement occurs even at HH4 (Fig. 2G). We also observed divergent (centrifugal) cell movements into prospective non-neural territory throughout the early APT at stages HH4 and HH4+, in addition to the previously described convergent movements directed centripetally into the neural plate. This combination of movements, plus correlative differential adhesive properties, is likely to contribute to the formation of the characteristic ANR fold that subsequently develops within the APT boundary region. Thus, the predominant pattern of clonal dispersion movements preceding the formation of the anterior neural folds is one of radial divergence from the nascent neural/non-neural boundary.

We found that median APT cells labelled at stage HH4 were strictly constrained in their intercalations to the midline (no longitudinal or AP dispersion). They spread significantly both ventrally (centripetally) into the neural plate and dorsally (centrifugally) away from it, the latter advancing into adenohypophysial non-neural territory, thus undergoing separation along the future dorsoventral axis. This singularity explains why the median part of the APT was difficult to map with precision at stage HH4 (Fernandez-Garre et al., 2002; Sanchez-Arrones et al., 2009).

Our finding that all along the APT dorsoventral intercalation stops at the nascent border by HH5 suggests that the APT sharpens into neural and non-neural subpopulations at this stage. This coincides with the moment when this region starts to express the neural marker *Sox2*. Longitudinal intercalation apparently takes place before this bidirectional DV sharpening occurs, whereas dorsoventral intercalation seems to be temporally correlated with the disaggregation of APT cells with neural and non-neural fates.

The longitudinal intercalation followed by dorsoventral intercalation observed at the lateral proneural transition zone in the chick at stage HH4 is comparable to the convergence-extension movements described in the *Xenopus* neural plate (Hirose and Jacobson, 1979; Jacobson and Hirose, 1981). By contrast, the median APT pattern of strong and exclusive dorsoventral intercalation has not been described previously in any species. This differential cell behaviour clearly correlates topographically with the formation in the median region of the adenohypophysial primordium, separating the bilateral olfactory placodes (Creuzet, 2009).

We also show that concomitant with these intercalation movements, during early neurula stages (HH4-4+-5), the expression of neural markers in the rostral neural plate expands from ventral areas (close to the node) to dorsal ones (prospective roof plate, or APT). Before stage 5, neural (*Sox2*) and non-neural markers (*Msx1*, *Dlx5*, *Gata3* and *Bmp4*) are partly co-expressed in the APT, consistent with the mixed neural and non-neural fate of its cells (Streit, 2007). Although neural *Six3* expression is later detected selectively at the ANR primordium and the terminal midline of the forebrain, it seems that there exist transiently APT cells that co-express *Dlx5*-*Gata3* and *Six3* during the final sharpening of this zone at HH5. The ulterior separation of the domains of expression of these markers at stages HH5 and HH6 mirrors the separation of neural and non-neural cellular subclones at the APT (Fig. 7). The *Msx1*-*Dlx5*-*Gata3*-positive subregion next to the late neural plate border has recently been shown to have an elevated rate of apoptosis (Gibson et al., 2011), which might contribute to sharpen the boundary between neural and non-neural cells at these stages. After stage HH7, it is possible to identify molecularly sub-regions of the neural border (supplementary material Fig. S2L,M).

Our results also point to cooperation between planar and vertical signals in setting up and refining the anterior proneural border. The expression of *Sox2* spreads as a wave away from Hensen's node along the neuroepithelium, suggesting that planar signals might be involved. We observed that the underlying endoderm (comprising both hypoblast and definitive rostral endoderm) influences vertically the range of *Sox2* expansion and the intercalatory character of the APT cell population. The expression pattern of *Ganf*, described by Fernandez-Garre et al. (Fernandez-Garre et al., 2002) as centred across the anterior neural border, might also obey directly anterior endoderm signalling. The N2 enhancer of *Sox2* (Uchikawa et al., 2003) itself might integrate these planar and vertical signals. Its activity spreads in a wave-like manner away from the node just like *Sox2* transcripts at the same stage. Moreover, its sequence contains putative binding sites for factors that could integrate signals responsible for the neural/non-neural segregation. For example, *Msx1* and *Gata3* binding sites are present within the conserved N2-Sox2 regulatory sequence (ERC browser, from –3012 to –2479) (Uchikawa et al., 2003). In the neural plate, ectopic BMP signalling inhibits *Sox2* expression at a relatively late stage (Linker and Stern, 2004). Signals from the rostral endoderm might act by modulating these activities at the APT and thus controlling the timing of N2-enhancer activation and further downstream effects (Albazerchi and Stern, 2007; Martinez-Barbera and Beddington, 2001; Thomas and Beddington, 1996).

Removal of the rostral endoderm under the APT at the primitive streak stage interferes with cell intercalation at the boundary, misplaces the *Sox2* border slightly and causes loss of *Six3* expression, a characteristic anterior neural marker, whereas the expression of peripheral *Bmp4* results expanded into the anterior neural plate. *Six3* knockout mouse embryos lack *Fgf8* and *Foxg1* expression at the ANR and have a grossly abnormal forebrain pattern without secondary prosencephalon (stunted in front of the

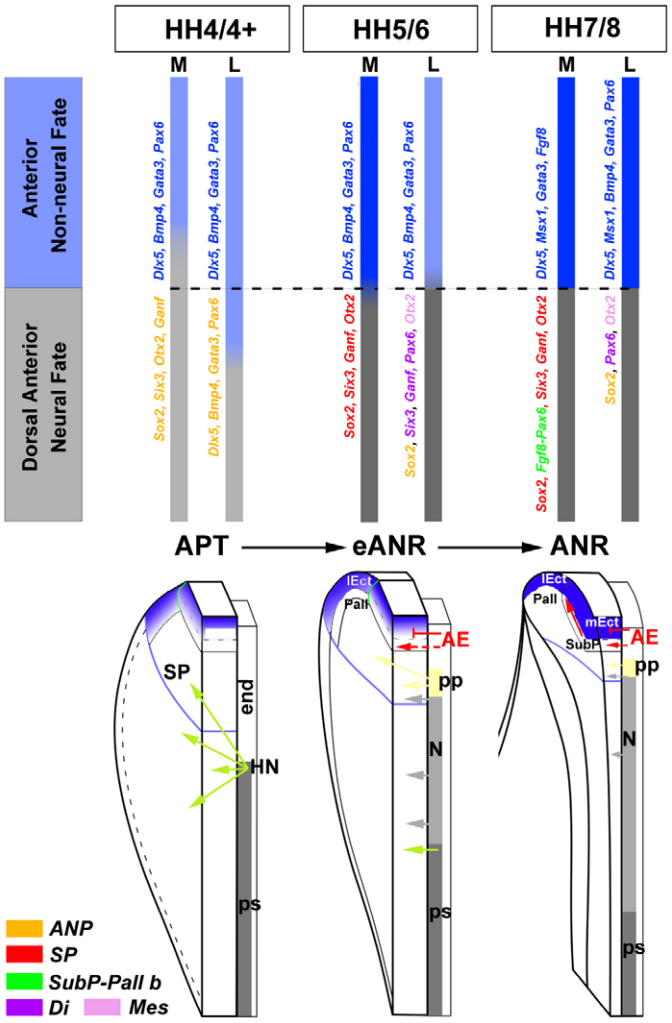

Fig. 7. Schematic of proposed changes leading to ANR establishment. The bar diagram (top) illustrates the sequential changes in neural and non-neural gene expression analysed in the course of this study. The schemata at the bottom represent the rostralmost (M, median) and more caudal (L, lateral) parts of the neural border of the prospective forebrain domain (secondary prosencephalon or SP) between stages HH4 and HH8. Three stages are considered with regard to the status of the neural-non neural border. At HH4, the peripheral APT is still rather imprecisely defined as a neural primordium at molecular and cellular levels (dashed lines; compare upper bar diagram), whereas the ventral (perinodal) neural domain is already fully specified by planar nodal signalling (HN) and ulterior notochordal effects (N). At stages HH5/6, we distinguish instead of the APT the emergent ANR (eANR), consequent to culminated DV intercalatory movements (separating centrifugally non-neural APT derivatives); the eANR becomes neurally induced under the vertical influence of AE-derived *FGF8* signalling (among other possible signals), probably with a median to lateral progression (less distance from the node medially). This specification is completed at HH7/8, when we have the definitive ANR; this becomes elevated, starts to express *Fgf8* and acquires specific organizing properties. AE, anterior endoderm; ANR, anterior neural ridge; APT, anterior proneural transition zone; Di, diencephalon; eANR; emergent anterior neural ridge; end, endoderm; HN, Hensen's node; lANR, lateral anterior neural ridge; lEct, lateral ectoderm; mEct, median ectoderm; Mes, mesencephalon; N, notochord; Pall, pallium; pp, prechordal plate; ps, primitive streak; SP, secondary prosencephalon; SubP, subpallium.

diencephalon) (Lagutin et al., 2003). By contrast, ablation of the prechordal plate leads to lack of *Six3* expression only at the basal hypothalamus but there is no effect on the telencephalic subpallium (Camus et al., 2000). These observations suggest that nodal and prechordal effects are not sufficient to evoke telencephalic *Six3* and *Fgf8* expression, which depends upon additional signals. Consistent with earlier findings (Chapman et al., 2003; Knezevic and Mackem, 2001; Pera et al., 1999; Withington et al., 2001), we suggest that these signals emanate first from the hypoblast (Albazerchi and Stern, 2007) and later from its remnants mixed with the rostral endoderm, being supplemented finally by ANR signalling (Fig. 7).

Our results imply that FGF-related signals are the vertical influence of the endoderm on APT patterning leading to eANR formation. An ectopic FGF8 source creates an extra border, and FGF8-loaded beads rescue the lack of dorsoventral intercalation observed after endodermal ablation, whereas the FGFR inhibitor SU5402 inhibits expression of neural markers at the APT. However, the SU5402 concentration required for this effect is very high, more than 100-fold levels previously found to inhibit the early steps of neural induction (Albazerchi and Stern, 2007; Sheng et al., 2003; Streit et al., 2000). These findings, therefore, suggest that some other factor operating through a pathway common to FGFs might be the endogenous agent responsible for the anterior regionalising effects of the endodermal layer. We therefore do not exclude the possibility that other factors might contribute to sharpen the neural border, defining differentially the neural and non-neural territories. For instance, *Hesx1* and *Tcf3* are expressed in the anterior endoderm and their early interaction within the local network apparently allows them to maintain anterior forebrain identity in the mouse (Andoniadou et al., 2011).

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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