Anterior neural plate regionalization in *cripto* null mutant mouse embryos in the absence of node and primitive streak

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Abstract

The relation between the role of the organizer at the gastrula stage and the activity of earlier signals in the specification, maintenance, and regionalization of the developing brain anlage is still controversial. Mouse embryos homozygous for null mutation in the *cripto* gene die at about 9.0 days postcoitum (d.p.c.) and fail to gastrulate and to form the node (the primary organizer). Here, we study the presence and the distribution of anterior neural plate molecular domains in *cripto* null mutants. We demonstrate that, in *cripto* /H11002/H11002 embryos, the main prosencephalic and mesencephalic regions are present and that they assume the correct topological organization. The identity of the anterior neural domains is maintained in mutant embryos at 8.5 d.p.c., as well as in mutant explants dissected at 8.5 d.p.c. and cultured in vitro for 24 h. Our data imply the existence of a stable neural regionalization of anterior character inside the *cripto* /H11002/H11002 embryos, despite the failure in both the gastrulation process and node formation. These results suggest that, in mouse embryos, the specification of the anterior neural identities can be maintained without an absolute requirement for the embryonic mesoderm and the node.

Keywords: Node; AVE; cripto; Anterior neural plate; Regionalization; Maintenance

Introduction

Classical embryological experiments have identified the existence of a specific population of cells in the gastrulae of the amphibian (dorsal blastopore lip), the zebrafish (dorsal embryonic shield), the bird (Hensen’s node), and the mouse (node) that act to organize the body plan (reviewed by Lemaire and Kodjabachian, 1996). The organizer has been defined as a specialized region of the vertebrate embryo, that can act as an independent unit controlled by self-sustaining signals, and that is able to recruit surrounding ectoderm cells to become neural, giving them a complete anterior–posterior (A/P) pattern. Heterotopic transplantation experiments have highlighted that the mouse node is able to induce a secondary axis but, unlike the Xenopus organizer, the mouse node is not sufficient to induce duplication of the most anterior structures (Beddington, 1994). These findings indicate that the murine node might not possess all the properties for a complete A/P regionalization of the neural axis.

Evidence that signals from outside the classical organizer could be required for induction of the anterior neural plate (ANP) was first produced in mouse by physical ablation of the anterior visceral endoderm (AVE) (Thomas and Beddington, 1996) and confirmed by the comparative analysis of *Lim1*, *nodal*, and *Otx2* knock-out and chimeric embryos (Acampora et al., 1995; Conlon et al., 1994; Rhinn et al., 1998; Shawlot and Behringer, 1995; Shawlot et al., 1999; Varlet et al., 1997). Collectively, the results of these experiments indicate that the mouse node does not initiate the
anterior pattern, and that a head organizer activity might reside in the AVE (reviewed by Beddington and Robertson, 1998, 1999). A two-step model for mouse anterior neural development proposes that initial specification of anterior neural identity requires the AVE, while the subsequent maintenance and refinement of the previously induced identities involve signals emitted by the node and its derivatives (Shawlot et al., 1999; Thomas and Beddington, 1996). Recent studies supporting this model also point to a role of node derivatives in maintaining and stabilizing the anterior neural specification. Camus and coworkers (2000) have demonstrated that, in the mouse embryo, the removal of the rostral segment of the anterior midline, containing the prechordal mesoderm, causes head truncation accompanied by the loss of several forebrain markers. Moreover, the analysis of HNF3β/Foxa2 conditional mutants has shown that, in the absence of the axial mesendoderm, specification of the ANP occurs, but is labile (Hallonet et al., 2002). However, results have also been produced that do not fit with the above model. Anterior neural induction does not occur in Wnt3−/− embryos, which lack the primitive streak and the node, despite apparently normal AVE development (Liu et al., 1999). Furthermore, heterotopic transplantation experiments show that the AVE alone fails to induce ectopic neural tissue (Tam and Steiner, 1999). Both findings argue against a role of the AVE as the embryonic head organizer. On the other hand, HNF3β null mutants lack the node and the axial mesendoderm, but, surprisingly, the patterning along A/P axis is only slightly affected (Ang and Rossant, 1994; Klingensmith et al., 1999; Weinstein et al., 1994).

To summarize the above, the specific role of the AVE and the node during anterior neural development remains a contentious issue. Our work intends to contribute to the debate, by analyzing in detail the anterior neural patterning in cripto−/− embryos. cript0 null mutants represent the only mouse mutant model to combine the lack of primitive streak and node and the distal mislocalization of a still functional AVE (Beddington, 1998; Ding et al., 1998), thus providing a powerful tool to gain insight into the specification, regionalization, and stabilization of the ANP in the mouse embryo. cripto is the founding member of the family of EGF-CFC genes which code for extracellular factors that, in conjunction with nodal, a ligand of the transforming growth factor beta (Tgf-β) family, are involved in the molecular control of several early decisions during vertebrate embryonic development (Minchiotti et al., 2002; Reissmann et al., 2001; Shen and Schier, 2000; Yan et al., 2002). The mouse cripto gene is expressed in the whole epiblast before gastrulation and, successively, in the forming mesoderm and in the developing heart (Ding et al., 1998; Dono et al., 1993; Minchiotti et al., 2000). Targeted inactivation of cripto causes early embryonic lethality, at approximately 9.0 days postcoitum (d.p.c.), affecting the definition of A/P polarity, embryonic mesoderm formation, and cardiac development (Ding et al., 1998; Xu et al., 1999). Here, we determine the expression pattern of diagnostic anterior neural markers in both cripto−/− embryos and in vitro cultured explants, focusing our attention on the definition and the maintenance of the spatial relationships among the different expression domains. Our analysis elucidates the molecular identity of cripto−/− anterior neuroectoderm and indicates that the correct specification and location of the presumptive anterior neural territories may occur and be maintained even in the absence of the node and its derivatives.

Materials and methods

Production of mutant embryos

The analysis of cripto inactivation was performed in a mixed genetic background composed of 25% 129SvJ, 25% Black Swiss, and 50% C57Bl16. Mutant homozygotes were obtained by crossing heterozygous mice. Noon of the day on which the vaginal plug was detected was considered as 0.5 d.p.c. in the timing of the embryo collection. For the genotyping of pups and embryos, DNA was extracted from the tail tips and yolk sac, respectively, and then analyzed by means of PCR as previously described (Xu et al., 1999).

Embryo dissection and explant culture

Timed pregnant cripto−/− mice were killed by cervical dislocation, and embryos at 8.5 d.p.c. were dissected from
decidual tissue in chilled Hank’s medium (Gibco-Life Technologies). For explant in vitro culture, wt embryos were opened and treated as previously described (Echevarria et al., 2001). The cripto\textsuperscript{−/−} embryos showed a sac shape in which it was only possible to recognize an embryonic (lower) and an extraembryonic (upper) region (Fig. 1A). These embryos were first cut apically so as to remove the most external part of the extraembryonic region, and then opened longitudinally along two opposite axes, starting from the extraembryonic and moving toward the embryonic region (Fig. 1A). The result was a symmetric bow-tie-shaped explant in which the embryonic region was in the center and the extraembryonic at the periphery of both sides of the explant (Fig. 1B, C, and D). Both wt and mutant explants were cultured in vitro for 24–30 h as previously described (Echevarria et al., 2001). Both embryos and in vitro cultured explants were fixed for a time varying from 3 h to overnight in 4% paraformaldehyde in PBS at 4°C, washed in PBT (0.1% Tween 20 in PBS), dehydrated through ascending methanol, and stored at −20°C.

**In situ hybridization on sections**

RNA probes were synthesized from linearized plasmid in the presence of 35S-CTP (Amersham) (Simeone, 1999). In situ hybridization (ISH) experiments on sections were performed as previously described (Simeone, 1999). Autoradiography was performed by using Kodak NTB2 emulsion, with exposure times from 15 to 20 days. After developing, sections were counterstained with hematoxylin and then mounted in DEPEX. Serially labeled sections were examined and photographed in both dark- and bright-fields, using a Zeiss compound microscope/Polaroid camera with Kodak Elite II film.

**Whole-mount in situ hybridization (WISH)**

RNA antisense probes were synthesized from linearized plasmid in the presence of Digoxigenin-UTP (Boehringer Manheim) or Fluorescein-UTP (Boehringer Manheim). Embryos were hybridized at 68°C in 50% formamide, 5× SSC,
Results

cripto<sup>−/−</sup> embryos essentially consist of anterior neuroectoderm

We first analyzed the expression profile of the anterior neural marker Otx2 during the development of cripto<sup>−/−</sup> mutants, using ISH on both sections and whole embryos (Figs. 2, 3A and A'). At 6.7 d.p.c., wt and mutant embryos showed comparable Otx2 expression in the whole embryonic ectoderm (Fig. 2A, B, A', and B'), while at 7.5 d.p.c., Otx2 transcripts were restricted to the anterior side of wt embryos (Fig. 2C and D), but persisted in almost the entire embryonic region of cripto<sup>−/−</sup> mutants (Fig. 2C' and D'; Ding et al., 1998). At 8.5 d.p.c., the Otx2 expression, which at this stage identifies forebrain and midbrain regions, was maintained throughout the cripto<sup>−/−</sup> embryos (Fig. 3A and A'; Table 1). To verify if the Otx2 expression, observed in 8.5 d.p.c. cripto<sup>−/−</sup> embryos, actually reflected a neural phenotype rather than the presence of undifferentiated ectoderm (as in the wt embryo at the onset of gastrulation), we also examined the expression of the pan-neural marker Sox2. We found Sox2 transcripts in almost all the embryonic cells of cripto<sup>−/−</sup> mutants (Fig. 3B'; Table 1). The comparison between Otx2 and Sox2 transcripts distribution indicated that the two expression domains were almost completely overlapping and that most Otx2-expressing cells also expressed Sox2, demonstrating their neural character. Thus, we conclude that, at 8.5 d.p.c., cripto<sup>−/−</sup> embryos principally consist of neuroectodermal cells, most of which express the anterior marker Otx2. The widespread Otx2 expression suggests the presence of putative forebrain and midbrain territories.

cripto<sup>−/−</sup> embryos express forebrain markers

To determine the identity of the neural tissue present in cripto<sup>−/−</sup> embryos, we analyzed the expression at 8.5 d.p.c. of specific diagnostic markers (Table 1). We first investigated the pattern of the forebrain genes Bfl (which identifies most of the telencephalon; data not shown), Six3, and Nkx2.1 (which both mark the hypothalamus and optic stalk primordium; Fig. 4A and B) (Hatini et al., 1994; Oliver et

Table 1
Summary of the whole-mount in situ hybridization experiments on wt and cripto<sup>−/−</sup> samples at 8.5 d.p.c.

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* indicates the number of positive samples (embryos in the second lane and explants in the third lane) out of the number of total samples analyzed. For each marker, the positive embryos show always comparable expression.

pH 5, 0.1% Tween 20, 50 μg/ml heparin, 50 μg/ml salmon sperm DNA, using a probe concentration of 100–500 ng/ml. Embryos were washed three times for 1 h each wash at 68°C in 50% formamide, 4× SSC, pH 5, 1% SDS, and then three times for 1 h each at 68°C in 50% formamide, 2× SSC, pH 5. Hybridization was detected with alkaline-phosphatase-coupled anti-Digoxigenin antibodies (Boehringer Manheim), followed by a reaction with nitroblue tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt (BCIP) (Boehringer Manheim), developing a red color and thereafter, for the alkaline-phosphatase-coupled anti-fluorescein antibodies (Boehringer Manheim), followed by a reaction with 2-(4 Iodophenyl)-3-(4nitrophenyl)-5-phenyl-tetrazolium chloride (INT) and BCIP (Boehringer Manheim), developing a blue color and thereafter, for the double WISH, with alkaline-phosphatase-coupled anti-fluorescein antibodies (Boehringer Manheim), followed by a reaction with 2-(4 Iodophenyl)-3-(4nitrophenyl)-5-phenyl-tetrazolium chloride (INT) and BCIP (Boehringer Manheim), developing a red color. Stained embryos were examined and photographed by using a Leica MZ12 dissection microscope. All the images were processed in Adobe Photoshop 5.0 (Adobe System Inc., Mountain View, CA). The following probes were used: Bfl (Hatini et al., 1994), Fgf8 (Crossley and Martin, 1995), Irx3 (Bosse et al., 1997), Gbx2 (Wasserman et al., 1997), Nkx2.1 (Pabst et al., 2000), Nkx2.2 (Shimamura et al., 1995), Otx2 (Simeone et al., 1992), Pax2 (Rowitch and McMahon, 1995), Pax6 (Walther and Gruss, 1991), Shh (Echelard et al., 1993), Six3 (Oliver et al., 1995), Sox2 (Collignon et al., 1996), and Wnt1 (Joyner, 1996).
Based on the above results, we went on to analyze the expression of midbrain and isthmic genes (Fig. 5; Table 1). All the genes examined were also expressed in more posterior domains along the axis of the wt embryo. However, we only considered the expression domains inside the ANP, because the other structures are absent in cripto−/− embryos (Ding et al., 1998; Xu et al., 1999). Our analysis showed Irx3 expression, which marks caudal diencephalon, mesencephalon, and rhombencephalon (Fig. 5A; Bosse et al., 1997), in a large proximal region of cripto null mutant embryos (Fig. 5A’). We also detected Wnt1 transcripts, which identify at this stage the dorsal midbrain and isthmus (Fig. 5B; Wurst and Bally-Cuif, 2001), and Fgf8 transcripts, which mark the isthmus and the anterior neural ridge (ANR) (Fig. 5C; Crossley and Martin, 1995), in a circumferential ring of proximal cells in cripto−/− embryos (Fig. 5B’ and C’). No Fgf8 expression was detected at the distal embryonic tip of cripto−/− mutants (Fig. 5C’), suggesting the absence of the ANR primordium (Fig. 5C). Instead, Pax2 expression was found in two different domains in cripto−/− embryos: one small domain at the very distal tip of the embryos, probably indicating the optic stalk primordium, and a second domain located approximately at the boundary between the embryonic and extraembryonic regions, which coincides with the mid–hindbrain expression in wt embryos (Fig. 5D and D’). Finally, the Gbx2 expression, which defines the isthmus and anterior hindbrain (Fig. 5E; Wassarman et al., 1997), was restricted to the most proximal side of the mutant embryos (Fig. 5E’). We did not find in the embryonic region of cripto null mutants any Shh expression (data not shown), which marks the prosencephalic and the mesencephalic basal plate of wt embryos (Echelard et al., 1993).

In conclusion, all the telencephalic, diencephalic, mesencephalic, and isthmic territories investigated are specified in the cripto−/− embryos, with the exception of the ANR. Moreover, neural markers that in wt embryos define the more rostral regions, are positioned more distally in cripto−/− mutants, while markers expressed in the more posterior regions of wt embryos are located more proximally in the mutants. Collectively, these data indicate the presence of a complex neural regionalization of anterior character inside the cripto−/− embryos at 8.5 d.p.c.

**In vitro cultured cripto−/− explants maintain the anterior neural regionalization**

Cripto−/− embryos die in utero, at about 9.0 d.p.c. (Xu et al., 1999), preventing us from addressing the question if and how anterior neural expression might be further maintained. To investigate whether neural signals acting in cripto−/− embryos might specify a stable regionalization inside the anterior neuroectoderm, we used cultured embryo explants. It has been shown that, under the opportune conditions, in vitro development of anterior neural tube dissected at early embryonic stages can mimic development in utero (Echevarria et al., 2001). Furthermore, it has been proposed that in vitro embryo culture can prolong the survival time of the brain anlage in early lethal mutations (Echevarria et al., 2001).

We dissected cripto−/− mutants as well as anterior neural tube from control embryos at 8.5 d.p.c. and cultured them in vitro for 24–30 h (Figs. 1 and 6). During this period, neither wt nor cripto−/− explants showed appreciable tissue degeneration, and both explants maintained their morphological structure (Figs. 1 and 6). Cripto−/− explants were heterogeneous in size and shape, as the embryos from which they were derived (Figs. 1 and 6). However, in the bow-tie-shaped cripto−/− explants, the distal embryonic region was distinguishable in the center, whereas the extraembryonic one was at both sides. The reciprocal extension of embryonic and extraembryonic regions was variable (Figs. 1 and 6).

We used the explants to investigate the expression profile of most of the genes analyzed in 8.5-d.p.c. embryos (Fig. 6; Table 1). The expression of the more anterior genes, such as Six3 and Otx2, was detected in the middle region of the explants approximately (Fig. 6A and B), corresponding to the distal part of the 8.5-d.p.c. cripto null mutants (Figs. 3A and 4A’). In perfect agreement, the expression of the more posterior markers, such as Wnt1, Fgf8, and Gbx2, were observed at 8.5 d.p.c. in the proximal region of the cripto−/− embryos (Fig. 5B’, C’, and E’) was located in the corresponding more external part of the explants (Fig. 6D, E, and F). In
conclusion, cripto−/− explants expressed all the diagnostic markers analyzed in the same topological order identified in 8.5-d.p.c. embryos. These results indicate that cripto−/− embryos maintain anterior neural patterning, even after 24 h of in vitro culture.

Topological relationships among the different anterior neural domains are conserved in cripto−/− embryos

One fundamental point to be raised is the definition of the spatial relationships among the different anterior neural gene expression patterns in cripto−/− embryos. For this purpose, we performed double WISH experiments using the following pairs of genes, Otx2/Gbx2 (Fig. 7A, A', D, and D'), Fgf8/Wnt1 (Fig. 7B and B'), and Six3/Irx3 (Fig. 7C, C', E, and E') on both wt and mutant 8.5-d.p.c. embryos as well as on explants cultured in vitro for 24 h.

In wt embryos, Otx2 and Gbx2 expression domains were demonstrated to be juxtaposed (Fig. 7A and D), showing mutual repressing activity (Broccoli et al., 1999; Garda et al., 2001; Millet et al., 1999). The boundary between the two expression domains was shown to define the formation of the isthmic organizer and the source of the Fgf8 signal (Fig. 7B; Garda et al., 2001; Li and Joyner, 2001; Martinez-Barbera et al., 2001). The region rostral to the isthmus, the caudal midbrain, in which Gbx2 was absent, also expressed Wnt1 (Fig. 7B; Millet et al., 1999). The double Otx2/Gbx2 WISH showed that adjacent relation was maintained between these two expression domains in all the cripto mutant

Fig. 3. Comparison between Otx2 and Sox2 expression in cripto−/− and wt embryos at 8.5 d.p.c. by means of whole-mount in situ hybridization. (B) A dorsal view, whereas all the other pictures are lateral views. (A) Otx2 expression in the forebrain and midbrain of wt embryo and (A') in almost all the cripto−/− embryonic region. (B) Pan-neural Sox2 expression in wt embryo. (B') Sox2 expression throughout the whole cripto−/− embryonic region. Abbreviations: fb, forebrain; mb, midbrain. Black lines indicate the boundaries between the different anterior neural regions.

Fig. 4. Forebrain markers are expressed in cripto−/− embryos. Whole-mount in situ hybridization analysis of wt and cripto−/− embryos at 8.5–9.0 d.p.c. All the pictures are lateral views. (A) Six3 expression in the forebrain of wt embryo and (A') at the distal tip of cripto−/− embryo. (B) Nkx2.1 expression in the ventral forebrain of control embryo and (B') at the distal tip of cripto−/− embryo. (C) Nkx2.2 expression in the medial anterior neural plate and along the hindbrain and spinal cord of wt embryo and (C') distally in cripto−/− embryo. (D) Pax6 expression identifying the forebrain, hindbrain and spinal cord of wt embryo and (D') a sharp band close to the distal tip of cripto−/− embryo. Abbreviations: fb, forebrain; hb, hindbrain; hy, hypothalamus; os, optic stalk; sc, spinal cord. Black lines indicate the boundaries between the different anterior neural regions.
embryos (a total of three) and explants (a total of three) analyzed (Fig. 7A’ and D’). Similarly, the Wnt1 and Fgf8 genes, which at this stage define two adjacent narrow stripes corresponding to the isthmic organizer (Fig. 7B), were expressed with a similar pattern in the proximal regions of cripto mutant embryos (four out of five) (Fig. 7B’). Moreover, we examined the relative expression of the Six3 and Irx3 genes, which define the first one the anterior forebrain and the second one the caudal diencephalon and the mesencephalon. The nested domains of the Six3 and Irx3 expressions were also conserved in all the cripto−/− embryos (a total of three) and explants (a total of three) analyzed (Fig. 7C, C’, E, and E’).

We can thus conclude that the relative position of the different anterior neural territories is conserved in both cripto null mutant embryos and explants. All our data show that a precise A/P polarity is detectable inside the neuroectoderm present in cripto−/− mutants, even if rotated at about 90° anticlockwise in relation to the wt and, thus, oriented along the embryonic proximodistal (P/D) axis (Fig. 8). The spatial distribution of the different markers in consecutive ring-like domains, along the P/D axis of cripto−/− embryos, corresponding to concentric domains in the explants indicates that neural plate regionalization in cripto mutant embryos develops with a radial distribution rather than with an A/P bilateral symmetry (Fig. 8).

Discussion

Different phases can be distinguished during early brain development, these being the initial induction of the ANP, the successive maintenance of its cephalic character, and the progressive refinement and embellishment of the previously induced identities. Our aim was to investigate the role of the mouse node and AVE in the maintenance of the ANP identities, taking advantage of the peculiar phenotypic characteristics of cripto−/− mutants (absence of the node and distal mislocalization of the AVE).

cripto−/− embryos maintain a regionalized anterior neural plate

During embryogenesis, Otx2 gene expression is mainly restricted to forebrain and midbrain regions (Simeone et al., 1992). The Otx2 gene is also widely expressed in the ectoderm layer of 7.5-d.p.c. cripto−/− embryos (Ding et al., 1998), thus suggesting, but not actually proving, that anterior neuroectoderm specification occurs in cripto mutants. In fact, since Otx2 is also expressed in the whole epiblast of wt pregastrula embryo, the Otx2 transcripts detected in 7.5-d.p.c. cripto−/− embryos might simply indicate the persistence of an undifferentiated ectoderm due to the failure of gastrulation. Here, we report that Otx2 in the embryonic
Fig. 6. *cripto*−/− explants maintain anterior neural regionalization. Whole-mount in situ hybridization analysis of 8.5- to 9-d.p.c. *cripto*−/− and wt anterior neural tube explants, after 24 h of in vitro culture. (A) *Six3* expression in the central region of *cripto*−/− explant and (A′) in the anterior forebrain of wt anterior neural tube explant. (B) *Otx2* expression in the middle of *cripto*−/− explant and (B′) in the forebrain and midbrain of wt explant. (C) *Irx3* expression in the medial region of *cripto*−/− explant and (C′) in the caudal diencephalon, mesencephalon, and anterior hindbrain of wt explant. (D) *Wnt1* expression at the boundary between the embryonic and extraembryonic region of *cripto*−/− explant and (D′) in the dorsal midbrain and at the midbrain–hindbrain boundary of wt explant. (E) *Fgf8* expression at the boundary between the embryonic and extraembryonic region of *cripto* null mutant explant and (E′) in the ANR and the isthmic primordium of wt explant. (F) *Gb2* expression at the extremities of the embryonic region of *cripto*−/− explant and (F′) in the isthmus and anterior hindbrain of wt neural tube explant. (G) Schematic representation of the expression pattern observed in *cripto*−/− and (G′) control explants.
Fig. 7. Topological conservation of the anterior neural domains in *cripto*<sup>−/−</sup> embryos and explants. *Otx2* and *Gbx2* expression domains are close to each other in correspondence of the wt isthmus (A, D) and are still adjacent in *cripto*<sup>−/−</sup> embryo (A') and explant (D'). *Fgf8* and *Wnt1* expression, identifying two adjacent narrow stripes in the isthmic region of the wt embryo (B) and showing a similar pattern in *cripto*<sup>−/−</sup> mutant (B'). *Six3* and *Irx3* expression domains in wt embryo (C) and explant (E), identifying the first the ventral forebrain and the second the caudal diencephalon, the mesencephalon, and the anterior hindbrain. Conservation of the topological relationships between *Six3* and *Irx3* expression domains in *cripto*<sup>−/−</sup> embryo (C') and explant (E'). Arrowheads label the limit between the expression patterns.
ectoderm of cripto−/− mutants shows a similar expression of the pan-neural marker Sox2, thus demonstrating neural identity. Furthermore, we used both single and double WISHs to reveal a coordinated spatiotemporal expression of all the diagnostic markers analyzed within the neural tissue of cripto−/− embryos, identifying forebrain, midbrain, and anterior hindbrain territories. Mutually repressive interactions that occur in wt embryos between the Otx2 and Gbx2 or between the Six3 and Irx3 genes appear also conserved in cripto−/− mutants, since sharp boundaries of these expression patterns are clearly equivalent to the boundaries observed in control embryos. Several papers have demonstrated that the development of delimited edges between different domains is based on the onset of such molecular repressive interactions (Garda et al., 2001; Kobayashi et al., 2002). Finally, Wnt1 and Fgf8 expression in cripto−/− embryos, as in wt embryos, is located at the deduced boundary between Otx2 and Gbx2 expression domains. We conclude that topological relationships among the different anterior gene expression domains, and the relative neural territories, are surprisingly normal in cripto mutant embryos, in spite of their extreme degeneration and the absence of both the node and primitive streak.

However, in cripto−/− embryos, the anterior neural markers are expressed distally, while more posterior neural genes are expressed proximally (Fig. 8), thus indicating that the A/P axis of the anterior neuroectoderm in cripto−/− mutants is oriented along the embryonic P/D axis. In addition, the specification and maintenance of anterior neural identities at the distal tip of cripto−/− embryos suggest that the head organizing activity is located distally in these mutants. There might, of course, be additional proximal influence from the boundary between the neural ectoderm and the extraembryonic tissues.

We used in vitro embryo culture to investigate the nature (labile or stable) of the anterior regionalization observed in cripto−/− mutants. Both wt neural tube and cripto−/− explants preserve tissue integrity and morphology during the time of in vitro culture and both show normal reciprocal localization of the expression domains of several regional markers. Therefore, cripto−/− explants, even after 24 h of in vitro culture, maintain anterior neural patterning.

**Anterior neuroectoderm of cripto null mutants develops domains that can be characterized as the isthmic organizer and as the ZLI but not as the ANR**

The refinement of anterior neural patterning is due to secondary organizers, located at discrete transverse domains of the developing neural primordium and secreting morphogenetic signals that refine the identity of the neighbouring neuroepithelial cells (Echevarría et al., unpublished observations; Martínez and Simeone, 1999). The most studied secondary organizer is the isthmic organizer (Martínez et al., 1991), which is located at the midbrain–hindbrain boundary. In this region, Fgf8 gene is expressed in correspondence with the contact area between the Otx2 and Gbx2 expression domains (Garda et al., 2001). Fgf8 protein has been demonstrated as being the effector molecule for the morphogenetic activities of the isthmus, controlling midbrain and anterior hindbrain regionalization (Crossley et al., 1996; Martínez et al., 1999). There is evidence for an additional secondary organizer, the ANR, which has been implicated in the prosencephalon regionalization. The ANR
also expresses and acts through Fgf8 (Shimamura and Rubenstein, 1997). Moreover, a third neuroepithelial region, the zona limitans intrathalamica (ZLI), situated in the middle of the diencephalic alar plate and related to the alar Shh expression (Rubenstein et al., 1994, 1998), has recently been proposed as an organizing center (Echevarria et al., unpublished observations). In the mouse, the ZLI presumptive domain has been identified in the neural plate by Fgf8 inductive experiments, even earlier than the onset of Shh expression (Shimamura and Rubenstein, 1997). From a recent work by the Shimamura group, it can be deduced that, at least in the chick embryo, the ZLI is specified in the contacting domains of Six3 and Irx3 expression (Kobayashi et al., 2002).

Our data demonstrate that cripto null mutants develop the molecular pattern that characterizes the isthmic organizer (Martinez, 2001; Wassarman et al., 1997; Wurst and Bally-Cuif, 2001). In fact, cripto−/− embryos express Fgf8 in a sharp band at the boundary between Otx2 (forebrain and midbrain) and Gbx2 (anterior hindbrain) domains, caudally to the Wnt1 expression. On the other hand, cripto null mutants do not show any additional Fgf8 expression, suggesting the absence of the ANR, which in control embryos arises at the junction between the most rostral part of the neural plate and the non-neural ectoderm. We hypothesize that the absence of the ANR in cripto−/− embryos might be due to the failure in the differentiation of non-neural ectoderm at the distal embryonic tip, which has been shown to express the pan-neural marker Sox2. Finally, although cripto−/− embryos fail to express Shh (Ding et al., 1998; and data not shown), they maintain the normal close and sharp-bounded expression domains of Six3 and Irx3 genes, suggesting that the ZLI presumptive domain might normally develop in cripto−/− mutants. The absence of Shh expression in cripto−/− embryos could be due to the lack of unknown additional mechanisms, including time.

The node and its derivatives might be dispensable for the maintenance of a regionalized anterior neural plate

There has been a long-standing controversy on the different roles to attribute to the node (and its derivatives) and the AVE in the ANP development. In fact, most of the genes that have been implicated in the formation of the ANP, such as Lim1, Hesxl, Otx2, and nodal, are expressed in both AVE and node derivatives (reviewed in Beddington and Robertson, 1998, 1999). cripto expression has been located in the forming mesoderm and primitive streak but has never been detected in the VE. The intrinsic value of cripto null mutants is, therefore, that, unlike the other null mutants that lack the node and/or its derivatives, cripto−/− embryos present a presumably normal VE. cripto−/− embryos, thus, provide an appropriate mouse mutant model to investigate whether and to which extent the AVE can mediate both the ANP regionalization and maintenance of anterior neural identity. Our molecular analysis of cripto−/− phenotype shows for the first time that cripto mutant embryos express diencephalic- and isthmic-specific markers. Interestingly, we have observed that all the genetic expression domains appeared in a topologically ordered manner, just as we can detect in the neural tube of control embryos. This cripto−/− anterior neural patterning is conserved after 24 h of in vitro culture. We propose that the persistence of ANP regionalization in the absence of the node and its derivatives may be the result of AVE morphogenetic activity. Since the AVE has been shown to fail to move anteriorwards, rather it persists at the distal tip of cripto−/− embryos (Ding et al., 1998), we therefore speculate that the signals emitted by the AVE provide neuroectodermal cells with the positional information necessary for the ANP regionalization. Therefore, even from its ectopic distal location, the AVE might induce a concentric regionalization in the mutant “neural sac.” However, we cannot exclude the possibility of an organizer activity in the proximal epiblast or at the boundary between the epiblast and the extraembryonic regions (see Foley et al., 2000; Streit et al., 2000), despite the absence of both morphological and molecular evidence of primitive streak and node (Ding et al., 1998; and unpublished results). Thus, a proximal organizer might cooperate with the AVE to develop the proximodistal regionalization pattern of the ANP in cripto mutants.

We draw attention to two further aspects of our work. First, the detection of the radial symmetry in cripto mutants, which fail to gastrulate, let us speculate that the phenomenon of gastrulation might be an evolutionary requirement for A/P axis and bilateral symmetry development in a previously radially organized embryo. Second, the presence of the Bf1, Six3, Pax6, Nkx2.1, and Nkx2.2 transcripts in the distal region of cripto mutants indicates that expression of anterior neural markers does not strictly require ANR or Shh expression in the ZLI. Since previous analysis on gene-inducing abilities of the ANR and of Shh has instead shown their involvement in the control of Bf1 and of Nkx2.1 and Nkx2.2 expression, respectively (Barth and Wilson, 1995; Pabst et al., 2000; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997), our data point to the existence of additional and still unknown mechanisms that regulate gene expression patterns inside the developing CNS.

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