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Expression of Tbx2 and Tbx3 in the developing hypothalamic–pituitary axis

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ABSTRACT

TBX2 and TBX3 are transcription factors that belong to the T-box family, members of which play important roles during mammalian embryogenesis. Mutations in T-box genes have been linked to several human genetic disorders and increasing evidence suggests that Tbx2 and Tbx3 may play a key role in cancer. The primary functions of Tbx2 and Tbx3 remain poorly defined, mainly because of their widespread expression in several tissues and their multiple potential roles in morphogenesis, organogenesis and cell-fate commitment. Here, we describe in detail the expression of Tbx2 and Tbx3 in the developing hypothalamic–pituitary axis. Localized transcripts can be detected during the early stages of pituitary commitment. Expression of Tbx2 is restricted to the infundibular region of the ventral diencephalon (VD) at all ages examined, whereas Tbx3 can be detected in both the VD and Rathke's pouch, the precursor of the anterior pituitary. Outside the developing hypophyseal organ novel sites of Tbx3 and Tbx2 expression include migrating branchiomotor (BM) and visceromotor (VM) neurons in the hindbrain, neuroepithelial cells of the developing tongue (Tbx3) as well as the developing blood vessel network (Tbx2).

1. Results and discussion

The T-box family members are ancient and evolutionary conserved transcription factors represented in all metazoans (Agulnik et al., 1996). They share a common DNA binding domain, the T-box (Kispert and Herrmann, 1993) and are involved in many aspects of vertebrate development and differentiation (Naiche et al., 2005). TBX2 and the highly related protein TBX3 are transcriptional repressors (Paxton et al., 2002; Carreira et al., 1998) implicated in human cancers such as pancreatic cancer (Mahlamaki et al., 2002), breast cancer (Jacobs et al., 2000) and melanomas (Vance et al., 2005). TBX2 can bind the histone H3 N-terminal tail and nucleosomal DNA and overexpression of TBX2 can lead to mitotic defects, suggesting a role for T-box factors in epigenetic reprogramming and control of mitotic checkpoints (Demay et al., 2007). During embryogenesis *Tbx2* and *Tbx3* have been associated with the Wnt/β-catenin (Renard et al., 2007; Yang et al., 2006; Fong et al., 2005), FGF (Firnberg and Neubuser, 2002) and BMP/Smad (Manning et al., 2006; Suzuki et al., 2004) signalling pathways. Expression of *Tbx2* and *Tbx3* is essential in the developing limb (King et al., 2006), heart (Stennard and Harvey R.P., 2005) and mammary gland (Jerome-Majewska et al., 2005; Rowley et al., 2004). Consistent with the important role of T-box factors during development, inactivation of either *Tbx2* (Harrelson et al., 2004) or *Tbx3* (Davenport et al., 2003) results in embryonic lethality, and mutations or chromosomal rearrangements of T-box genes have been implicated in several human developmental diseases (Packham et al., 2003). Despite the important roles of T-box factors in many aspects of embryonic development, their expression in the

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developing central nervous system remains poorly described. Recent evidence has postulated a prominent role for *Tbx2* in the developing ventral hypothalamus in chick (Manning et al., 2006). We sought to examine the expression of the *Tbx2* and *Tbx3* in the developing pituitary in the mouse. We compare their expression to that of known regulators of hypothalamic/pituitary development at different embryonic stages. We also describe new expression domains of *Tbx3* in migrating BM/VM motorneurons, in the sensory neuroepithelium of the developing tongue and in the developing central nervous system blood vessel network.

1.1. Tbx2 and Tbx3 expression during hypothalamic–pituitary development

The pituitary gland is an excellent model for studying organogenesis and cell commitment. The adult pituitary is composed of two different anatomical and functional units: the adenohypophysis which consists of the anterior (AP) and intermediate pituitary and originates from oral ectoderm, and the neurohypophysis which derives from neural ectoderm (Schwind, 1928; Rizzoti and Lovell-Badge, 2005). The AP arises from Rathke's pouch (RP), a dorsal invagination of the roof of the oral ectoderm (OE) towards the ventral diencephalon (VD), (Fig. 1A). The neurohypophysis originates from the infundibulum (INF; Fig. 1A–D), an evagination of the VD that is in direct contact with the dorsal part of RP (Fig. 1B and C). In the anterior pituitary, multiple signalling pathways converge to promote the generation of mature endocrine cells from a common primordium in a temporally and spatially specific fashion (reviewed in Rizzoti and Lovell-Badge, 2005). There are five endocrine cell types in the AP, all of which are characterized by the hormones they produce and secrete (Fig. 1D); lactotropes (L; producing prolactin, Prl), gonadotropes (G; producing luteinising

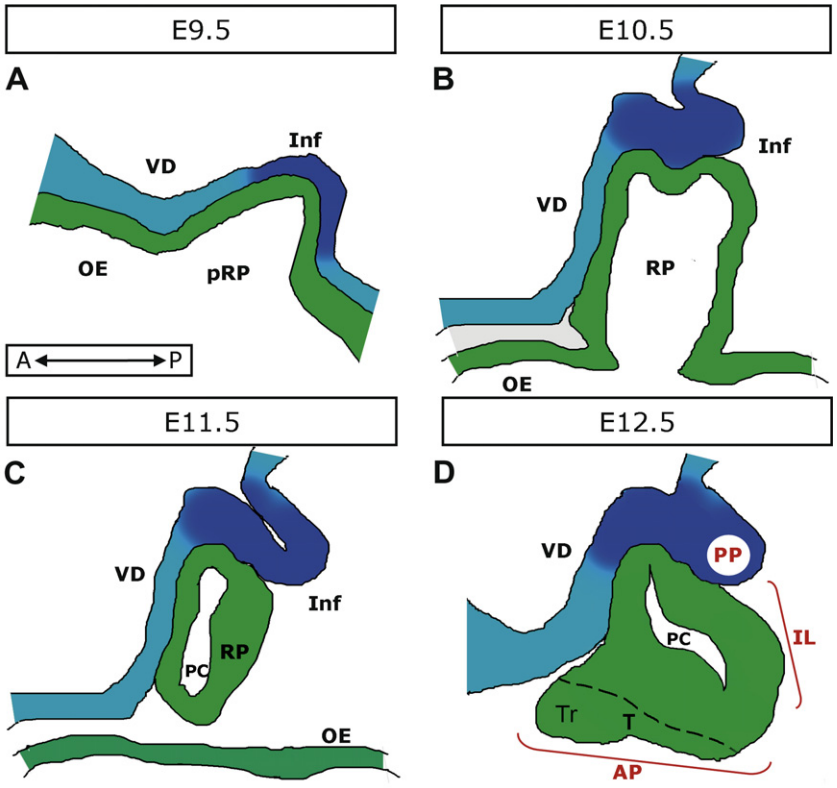

Fig. 1. Schematic diagram showing different stages of pituitary gland development. (A) A thickening of the oral ectoderm, the hypophyseal placode, starts to invaginate toward the ventral diencephalon at E9.5, creating the presumptive Rathke's pouch. (B) At E10.5, the pRP is enlarged and the VD initiates an evagination to generate the infundibulum. (C) The definitive RP is completely closed and isolated from the oral cavity around E11.5. (D) From the RP arises the anterior pituitary (adenohypophysis) and the infundibulum will give rise to the posterior pituitary (neurohypophysis). *Abbreviations:* AP, anterior pituitary; IL, intermediate lobe; Inf, infundibulum; OE, oral ectoderm; PC, pituitary cleft; PP, posterior pituitary; pRP, presumptive Rathke's pouch; RP, Rathke's pouch; T, thyrotrophs; Tr, rostral thyrotrophs; VD, ventral diencephalon.

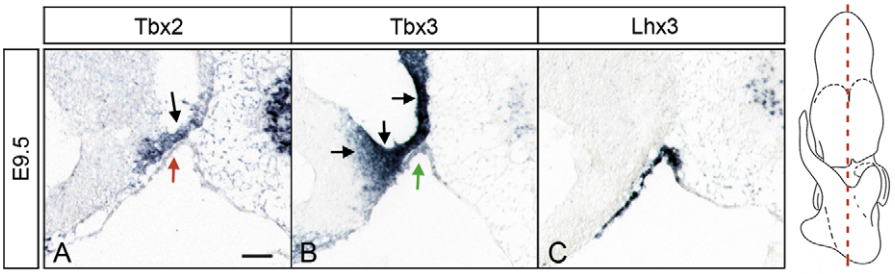

Fig. 2. Expression of *Tbx2* and *Tbx3* transcripts during early pituitary commitment. Sagittal sections of E9.5 embryos, hybridized with *Tbx2*, *Tbx3* and *Lhx3* antisense riboprobes. The scheme on the right shows the plane of section. (A) *Tbx2* is expressed in a small region of the VD floor (black arrow) and is excluded from the OE (red arrow). (B) *Tbx3* is strongly expressed across the VD and hypothalamus (black arrows); *Tbx3* is also expressed in the OE (green arrow) in the presumptive Rathke's pouch which can be identified by expression of the LIM homeodomain – encoding gene *Lhx3* (C). Scale bar 100 μm.

hormone, LH and follicle-stimulating hormone, FSH), thyrotropes (T; producing thyroid stimulating hormone, TSH), corticotropes (C; producing adenocorticotropic hormone, ACTH) and somatotropes (S; producing growth hormone, GH). A sixth cell type, the melanotropes (M; producing melanocyte stimulating hormone, MSH), is confined in the intermediate lobe of the AP. To date, the only T-box factor known to be expressed in the developing pituitary is *Tbx19* (Pulichino et al., 2003; Lamolet et al., 2001; Liu et

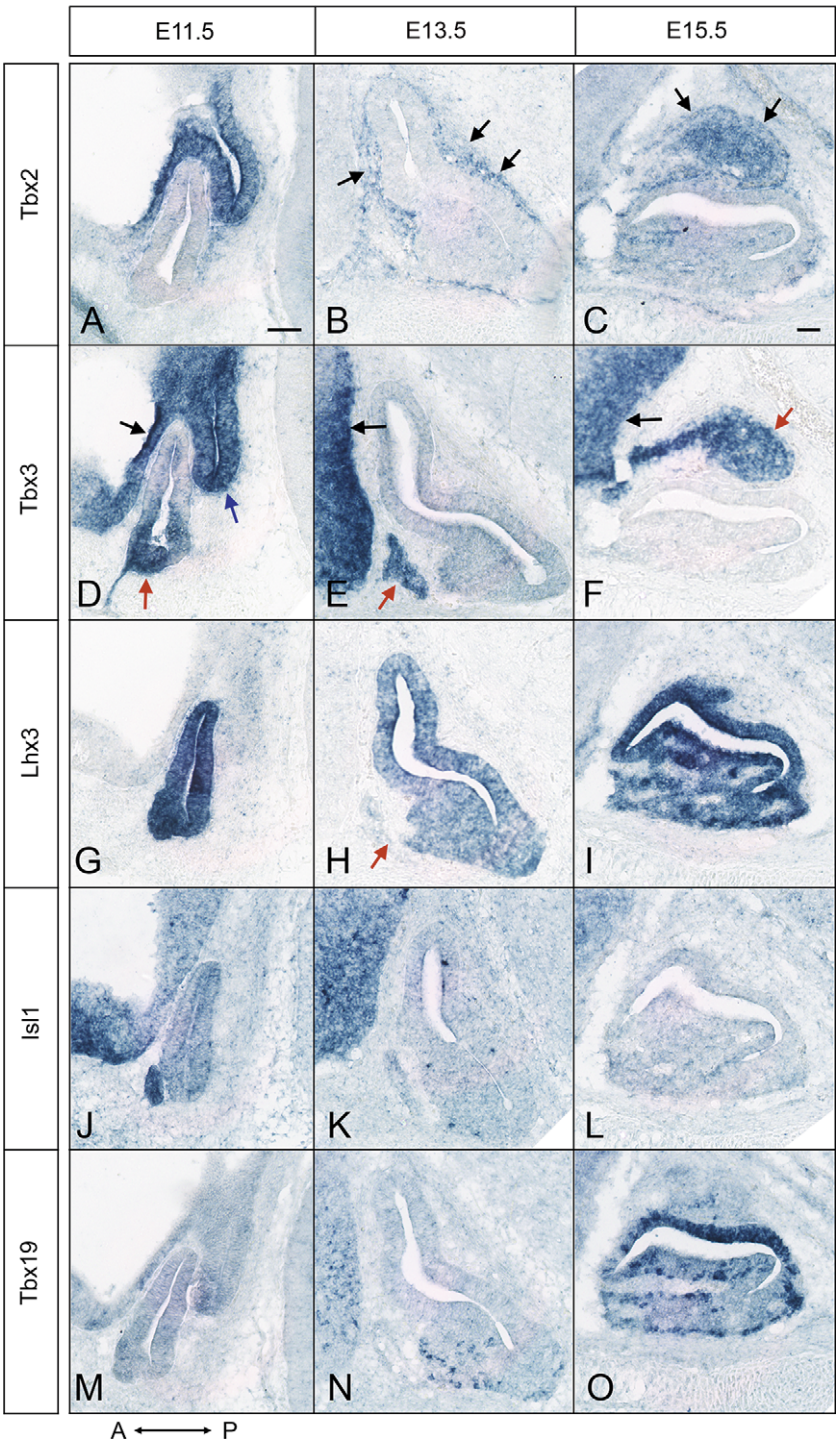

Fig. 3. Expression of *Tbx2* and *Tbx3* at early stages of pituitary development. Sagittal (A, D, G, J, M) and parasagittal (B, C, E, F, H, I, K, L, N, O) sections at E11.5, E13.5 and E15.5, hybridized with *Tbx2*, *Tbx3* antisense riboprobes and other markers of pituitary development (*Lhx3, Isl1, Tbx19*). Expression of *Tbx2* can be detected in the infundibulum at E11.5 (A). At E13.5 *Tbx2* can be observed in cells surrounding the pituitary gland (B, black arrows). At this level of parasagittal section the developing neural lobe is not present. At E15.5 *Tbx2* transcripts can be observed in the neural lobe (C, black arrows). *Tbx3* is expressed in the VD (D–F, black arrows) and infundibulum (D, blue arrow) from E11.5 to E15.5. At E11.5 its expression is also detected in the rostral tip of RP (D, red arrow) in a pattern that partially overlaps that of *Lhx3* (D and G) and *Isl1* (J). By E13.5 the expression domains of *Tbx3* and *Lhx3* in RP become mutually exclusive (E and H). At this stage, *Tbx3* is confined to a specific cell population at the rostral tip of the gland, (E, red arrow). At E15.5, expression of *Tbx3* in the anterior pituitary is no longer observed (F). In contrast, high levels of *Tbx3* can still be detected in the developing neurohypophysis (F, red arrow). At E13.5 and E15.5, expression of *Tbx19* (*T-Pit*) is excluded from the *Tbx3*-expressing domain in RP (E–F and M–O). Scale bar = 50 μm.

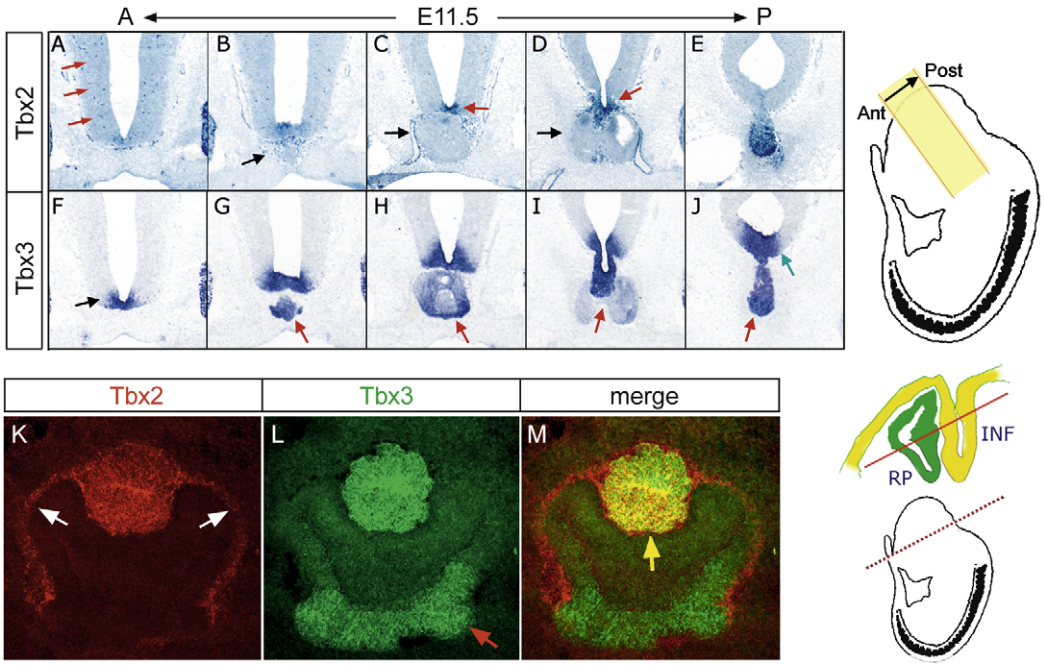

Fig. 4. Expression of *Tbx2* and *Tbx3* at E11.5. (A–J) Coronal sections of E11.5 embryos hybridized with *Tbx2* and *Tbx3* antisense riboprobes, showing the expression patterns of *Tbx2* and *Tbx3* in the developing pituitary. The scheme on the right indicates the plane of sections. (A–E) Expression of *Tbx2* is observed in the VD and the infundibulum. Expression is not observed in RP (B–D, black arrows). *Tbx2* transcripts are localized in a small region between the infundibulum and RP, from where the hypophyseal-portal system is arising (C and D, red arrows). (F–J) *Tbx3* is expressed both in the VD (black and green arrows) and in RP (red arrows). (K–M) Horizontal sections of E11.5 embryos hybridized with *Tbx2* and *Tbx3* antisense riboprobes. The schemes on the right indicate the plane of sections. (K and M) *Tbx2* transcripts are present in cells surrounding the developing pituitary gland (white arrows) and in the infundibulum. (L and M) *Tbx3* is expressed in the anteriormost region of Rathke's pouch (L, red arrow) and overlaps with *Tbx2* in the infundibulum (K–M, yellow arrow).

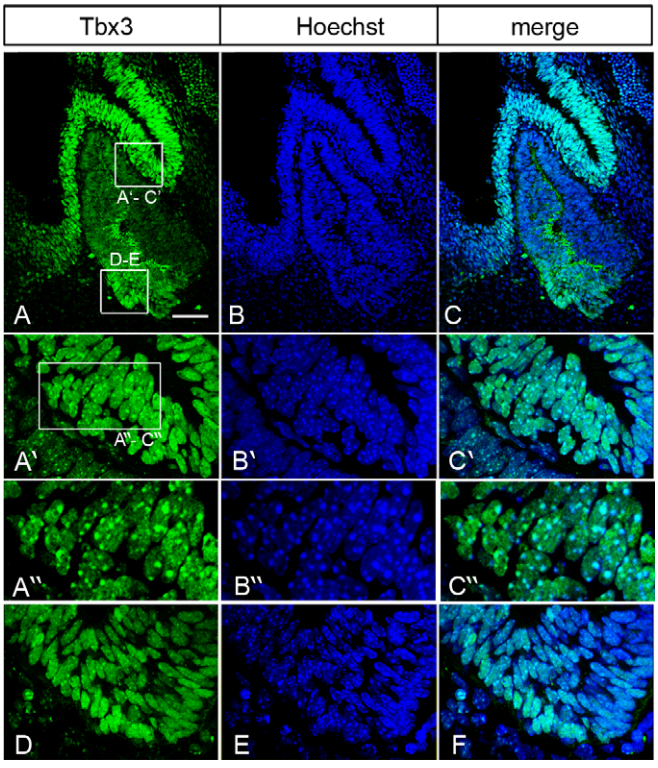

Fig. 5. (A–F) Confocal images from coronal sections of E11.5 embryos immunostained for TBX3. (A–C) Expression of TBX3 protein can be detected in the developing hypothalamic–pituitary axis in a pattern similar to that of the *Tbx3* mRNA. (A′–C′ and A″-C″) High magnification images showing co-localized with the intensely DAPI-staining pericentric heterochromatin foci. (D–F) Diffuse nuclear staining of TBX3 in the rostral region of the RP. Boxes in A and A′ indicate the position of high magnification images in A′–C′ and A″–C″, respectively. Scale bar = 50 μm.

al., 2001). Expression of *Tbx19* is detected at later stages of pituitary development (E15.5) and controls the differentiation of corticotropin and melanotropin-producing cells.

1.1.1. Tbx2 and Tbx3 localization during early hypothalamic–pituitary commitment

At E9.5, *Tbx2* is expressed in a small region of the VD (Fig. 2A, black arrow) but not in the underlying OE (Fig. 2A, red arrow). Unlike the developing chick where only *Tbx2* is expressed in the VD, in mouse expression of *Tbx3* overlaps that of *Tbx2* in the VD. However, *Tbx3* is expressed in a wider area of the VD encompassing the entire prospective infundibulum and extending into the posterior hypothalamus (Fig. 2B, black arrows). At E9.5 the presumptive RP is receiving inductive signals that will promote its shaping, branching and differentiation (Ericson et al., 1998; Rizzoti and Lovell-Badge, 2005). *Tbx3* transcripts can be observed in the invaginating OE from the early stages of pituitary organogenesis (Fig. 2B, green arrow). The expression domain of *Tbx3* in RP partly overlaps with that of the definitive marker of prospective RP, the LIM-homeobox encoding gene *Lhx3* (Fig. 2C).

1.1.2. Tbx2 and Tbx3 expression is developmentally regulated

From E10.5 onwards the overlying infundibulum begins to evaginate ventrally towards RP to become a distinct structure by E11.5. The definitive RP is fully invaginated at E10.5 and by E11.5 it is completely separated from the remaining OE (Fig. 1B–C). The lumen of the pouch persists as the pituitary cleft (Fig. 1B–C), separating the intermediate from the anterior lobe in the mature gland (Fig. 1D). The positional determination of the hormone secreting cell types, takes place within the future anterior and intermediate lobes of the pituitary, from E12.5 to E13.5. By E15.5, the different pituitary cell lineages are irreversibly committed to a specific terminal differentiation. Using a set of markers of pituitary development as positional landmarks, we analyzed the developmental

pattern of *Tbx2* and *Tbx3* expression throughout the main developmental window of the hypothalamic–pituitary axis (from E11.5 to E15.5) (Fig. 3). Expression of *Tbx2* can be detected in the infundibulum at E11.5 (Fig. 3A). At E13.5 *Tbx2* transcripts can be observed in a salt-and-pepper distribution in the periphery of the pituitary gland (Fig. 3B, black arrows). At E15.5 *Tbx2* signals persist in the neural lobe (Fig. 3C, black arrows). Consistent with the postulated role of *Tbx2* in regulating Shh expression, we could observe a mutually exclusive expression of the two genes in the VD (data not shown) (Manning et al., 2006; Nissim et al., 2007). *Tbx3* is expressed in the VD (Fig. 3D–F, black arrows) and infundibulum (Fig. 3D, blue arrow). At E11.5 expression can also be detected in the rostral tip of RP (Fig. 3D, red arrow) in a pattern that partially overlaps with that of *Lhx3* (Fig. 3D and G) and *Isl1* (Fig. 3J). By E13.5 the expression domains of *Tbx3* and *Lhx3* in RP become mutually exclusive (Fig. 3E and H). At this stage *Tbx3* is confined to a specific cell population at the rostral tip of the gland, which is known to give rise to the *Pit1*-independent thyrotrope lineage (Lin et al., 1994) (Fig. 3E, red arrow). At E15.5, when the main cell lineages of the pituitary are already committed, expression of *Tbx3* in the anterior pituitary is no longer observed (Fig. 3F). In contrast, high levels of *Tbx3* can still be detected in the developing neurohypophysis (Fig. 3F, red arrow). At E13.5 and E15.5, expression of *Tbx19* (*T-Pit*) is excluded from the *Tbx3*-expressing domain in RP (Fig. 3E–F and N–O).

To further assess the extent of *Tbx2* and *Tbx3* expression in the developing hypothalamic–pituitary axis, we examined coronal and transverse sections of E11.5 mouse embryos for the presence of *Tbx* transcripts. Expression of *Tbx2* can be observed in the presumptive pars neuralis (Fig. 4C–D, red arrows) and in the ventral diencephalon and infundibulum which is evaginating ventrally towards RP (Fig. 4A–E). Expression of *Tbx3* starts in the diencephalon close to the hypothalamic region (Fig. 4F, black arrow) and extends caudally spanning a large area of the VD floor (Fig. 4J, blue arrow). Transcripts can be detected in the developing RP (Fig. 4G and H, red arrows) as well as in the infundibular region (Fig. 4I–J, red arrows). Using fluorescent double in situ hybridization in transverse sections of E11.5 embryos, we demonstrate the overlapping expression of *Tbx2* (Fi. 4K) and *Tbx3* (Fig. 4L) in the infundibular region (Fig. 4M, yellow arrow). Expression of *Tbx2* can also be observed in cells surrounding RP. *Tbx3* transcripts can also be observed in the anterior region of RP (Fig. 4L, red arrow).

1.1.3. Expression of TBX3 protein

Studies to date have shown that TBX proteins may exhibit either nuclear or cytoplasmic expression patterns (Collavoli et al., 2003; Miyahara et al., 2004). In particular, we previously showed that TBX2 is localized in small subnuclear foci that do not correspond to telomeres, Cajal or PML bodies suggesting a role for TBX2 in nuclear organization (Bilican and Goding, 2006). We used an antibody raised against the N-terminal region of mouse TBX3 protein to examine the expression of TBX3 *in vivo*. At all stages examined, expression of TBX3 resembles that of *Tbx3* mRNA (Fig. 5). However, at the subcellular level, TBX3 protein shows an exclusive nuclear localization pattern in the infundibulum and in RP, (Fig. 5D–I). Interestingly, Tbx3 was co-localised with the intensely DAPI-staining pericentric heterochromatin foci (Fig. 5A′–C′ and A″–C″), in contrast to the staining pattern observed with Tbx2 in cultured cells where it was clearly excluded from pericentric heterochromatin (Bilican and Goding, 2006).

1.2. Novel sites of expression of Tbx2 and Tbx3 outside the hypothalamic–pituitary region

Outside the developing hypothalamic–pituitary region we found several novel sites of *Tbx2* and *Tbx3* expression. Papaioannou and co-workers have recently described an atypical inter-somitic blood vessel expression of *Tbx2* (Harrelson and Papaioannou, 2006). Using in situ hybridization we have detected *Tbx2* signals in the developing blood vessels of the CNS (Fig. 6A–E).

Tbx2 and *Tbx20* transcripts have recently been detected in migrating branchiomotor (BM) and visceromotor (VM) neurons and *Tbx20* in particular appears to be expressed in virtually all migrating BM/VM neurons at E11.5 (Song et al., 2006). BM neurons innervate branchial arch-derived muscles and VM cells innervate parasympathetic ganglia. During motor neuron development *Tbx20* appears to activate *Tbx2* expression, whereas it represses *Tbx2* expression in myocardial cells (Cai et al., 2005). Moreover, *Tbx2* has been also found in dorsal root ganglia (Harrelson and Papaioannou, 2006). Here, we show the expression of *Tbx2* (Fig. 7A, F and K) and *Tbx3* (Fig. 7B, G and L) in migrating BM/VM neurons at E11.5. *Tbx2* and *Tbx3* expression domains do not overlap completely with *Tbx20*, which appears to be more extensively expressed. We also compare these domains with the expression of *Isl1* (Fig. 7D, I and N) and *Mash1* (Fig. 7E, J and O). *Isl1* and *Tbx20* are both expressed in wider regions than *Tbx2* (Fig. 7A, F and K) or *Tbx3* (Fig. 7B, G and L). *Tbx2* and *Tbx3* are expressed in a complementary pattern to that of the proneural gene *Mash1* (Fig. 7E, J and O). *Tbx2*-positive and *Tbx3*-positive cells can also be detected in the V and VII ganglia (Fig. 7A′–E′). Finally, we can detect *Tbx2* and *Tbx3* mRNA in the mandibular process before the appearance of the tongue primordium at E10.5 (Fig. 7F′ and I′) and in the developing sensory neuroepithelium of the tongue at different developmental stages (Fig. 7G′–H′ and J′–K′).

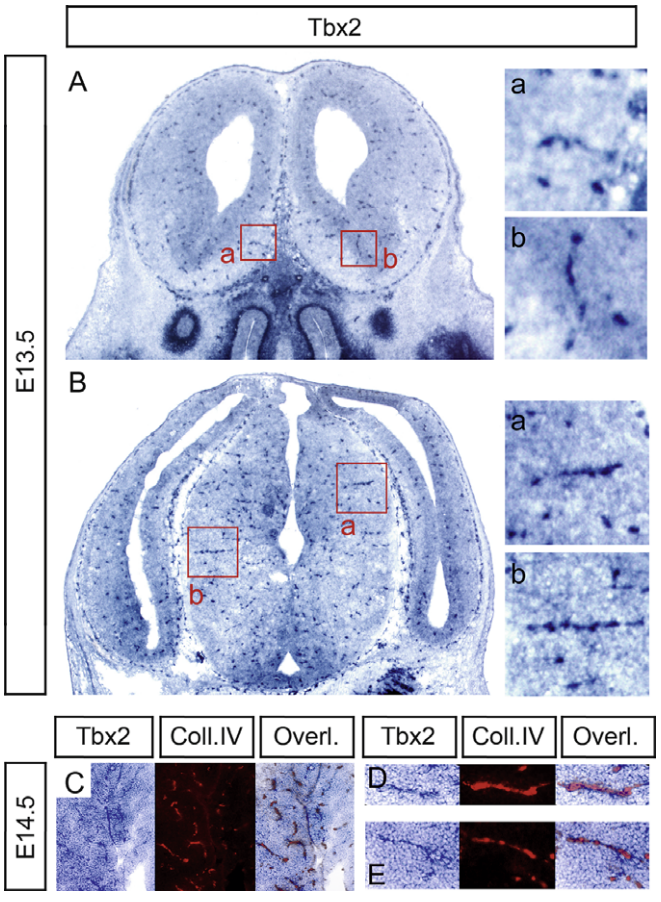

Fig. 6. *Tbx2* expression in the CNS. Coronal sections of E13.5 brains hybridized with *Tbx2* antisense riboprobe. Expression of *Tbx2* can be observed in putative blood vessels in the anterior (A) and posterior (B) forebrain. (C–E) The staining for Tbx2 mRNA (blue) overlaps with anti-Collagen IV, an early marker of blood vessels development (red). Boxes in A and B indicate the area of high magnification images shown in (a and b).

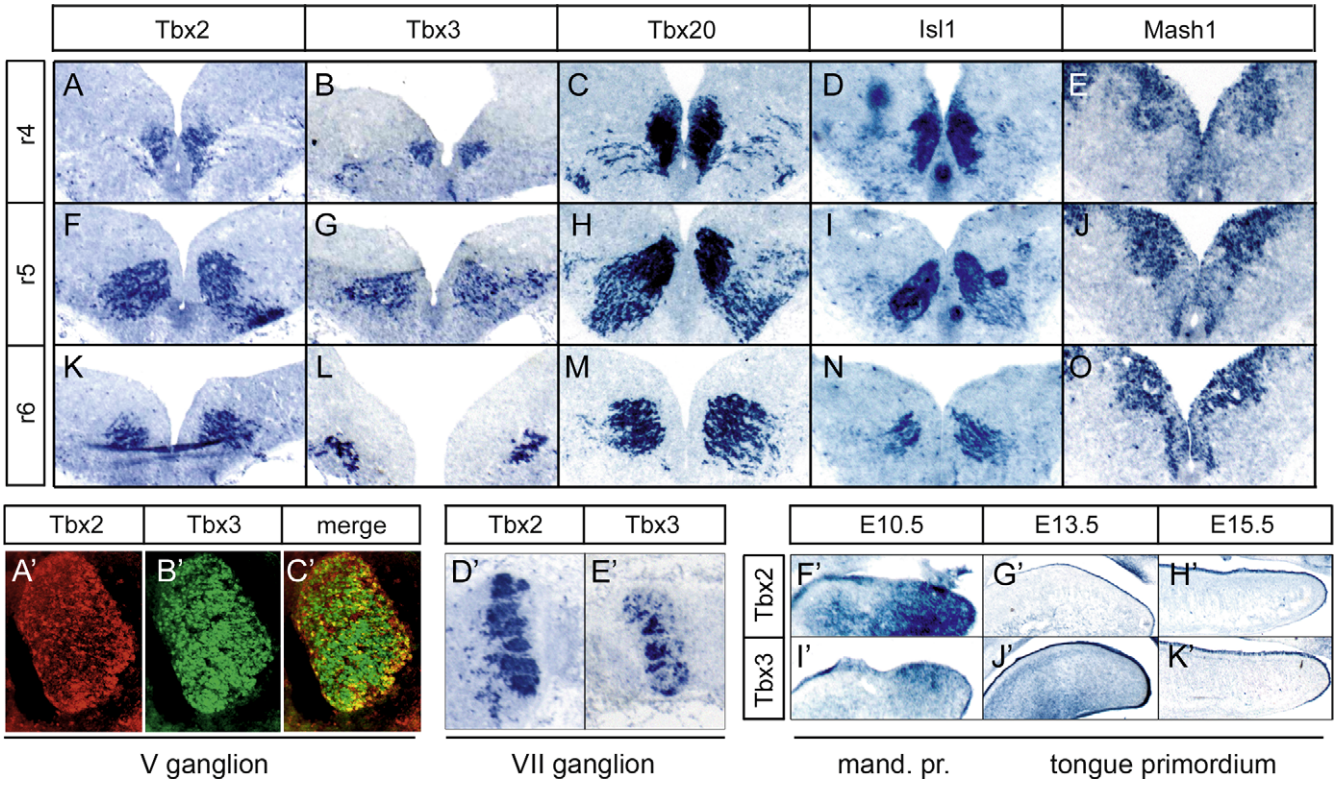

Fig. 7. Expression of *Tbx2* and *Tbx3* in BM/VM motorneurons, cranial ganglia and the developing tongue. (A–O) Coronal sections of E11.5 embryos showing expression of *Tbx2*, *Tbx3*, *Tbx20*, *Isl1* and *Mash1*. (A′–C′) Expression of *Tbx2* and *Tbx3* transcripts at E11.5 in the V cranial ganglia (trigeminal) and (D′–E′) in the VII cranial ganglia (geniculate). (F′–I′) Expression of *Tbx2* and *Tbx3* at E10.5 in the mandibular process (mand. pr.) before the appearance of the tongue primordium, in the tongue primordium at E13.5 (G′ and J′) and in the tongue at E15.5 (H′ and K′).

2. Experimental procedures

2.1. Mouse strains and embryo treatment

B6/CBA F1 mouse embryos at different stage of gestation, from embryonic day (E) 9.5 to E16.5, were used. The day of the vaginal plug detection was defined as E0.5. Embryos were collected and washed in ice cold DEPC-treated PBS then fixed by immersion in a 4% PFA solution in PBS for 20′ (for immunochemistry) or overnight (for in situ hybridization) at 4 °C. Embryos were cryo-protected by immersion in 20% w/v DEPC-treated sucrose solution in PBS. The embryos were embedded and frozen in OCT™ compound (R. A. Lamb, Eastbourne, UK) and stored at −80 °C.

2.2. In situ hybridization

cDNA clones used for preparation of RNA probes for in situ hybridization were as follows: *Tbx2*: IMAGE clone 3512836; *Tbx3*: IMAGE clone 4188081; *Tbx20* riboprobes were prepared from E10.5 mouse embryonic hearts. In situ hybridization was carried out as previously described (Fruttiger et al., 1999). For double in situ hybridization probes labeled with digoxygenin and FITC were used. Following hybridization, a POD-conjugated antibody (Roche GmbH, Mannheim, DE) was used to detect the FITC-labelled probe and the signal was developed using the TSA (tyramide, Perkin-Elmer, Waltham, USA) reagent, diluted with the appropriate fluorophore (Fluorescein, Cy3 or Texas-red). POD was subsequently killed by 3% H_2O_2 incubation in PBS for 30′ at room temperature. The DIG-labelled probe was detected with an anti-DIG antibody conjugated with horse radish peroxidase and the signal was detected using a different fluorophore.

2.3. Immunochemistry

The anti-TBX3 antibody used in this study is a mouse monoclonal raised against the N-terminal region of mouse TBX3 (Goding, unpublished). *Tbx3* was used at 1:250 dilution in PBS containing 0.1% Triton X and 10% heat-inactivated sheep serum. The primary antibody was detected with using a FITC-conjugated goat anti-mouse IgG (Perbio Pierce). Sections were counterstained with the nucleic acid fluorescent marker Hoechst 33342 (Invitrogen Ltd., Paisley, UK) for 1 min (10 mg/ml stock, diluted 1:1000 in 1× PBS, 0.1% Triton X-100). The sections were mounted under coverslips using Citifluor anti-fade reagent (City University, UK). The antibody anti-Collagen type IV (AbCam plc) was used at 1:75 concentration after in situ hybridization and detected by a secondary FITC-conjugated antibody 1:150 (Vector Labs).

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