Development 137, 2927-2937 (2010) doi:10.1242/dev.041079

Serotonin 2B receptor signaling is required for craniofacial morphogenesis and jaw joint formation in *Xenopus*

Elisa Reisoli[1], Stefania De Lucchini[2], Irma Nardi[1] and Michela Ori[1,*]

SUMMARY

Serotonin (5-HT) is a neuromodulator that plays many different roles in adult and embryonic life. Among the 5-HT receptors, 5-HT2B is one of the key mediators of 5-HT functions during development. We used *Xenopus laevis* as a model system to further investigate the role of 5-HT2B in embryogenesis, focusing on craniofacial development. By means of gene gain- and loss-of-function approaches and tissue transplantation assays, we demonstrated that 5-HT2B modulates, in a cell-autonomous manner, postmigratory skeletogenic cranial neural crest cell (NCC) behavior without altering early steps of cranial NCC development and migration. 5-HT2B overexpression induced the formation of an ectopic visceral skeletal element and altered the dorsoventral patterning of the branchial arches. Loss-of-function experiments revealed that 5-HT2B signaling is necessary for jaw joint formation and for shaping the mandibular arch skeletal elements. In particular, 5-HT2B signaling is required to define and sustain the *Xbap* expression necessary for jaw joint formation. To shed light on the molecular identity of the transduction pathway acting downstream of 5-HT2B, we analyzed the function of phospholipase C beta 3 (PLC) in *Xenopus* development and showed that PLC is the effector of 5-HT2B during craniofacial development. Our results unveiled an unsuspected role of 5-HT2B in craniofacial development and contribute to our understanding of the interactive network of patterning signals that is involved in the development and evolution of the vertebrate mandibular arch.

KEY WORDS: 5-HT2B, Xbap, Bapx1, Nkx3.2, Craniofacial morphogenesis, Jaw joint, Neural crest cells, *Xenopus*, Serotonin

INTRODUCTION

Craniofacial morphogenesis is a complex developmental process requiring multiple and coordinated embryological events. The visceral skeleton of all jawed vertebrate embryos is organized into a rostrocaudal bilateral series of pharyngeal arches. These arches are colonized by cranial neural crest cells (NCCs), which migrate along precise pathways from the mid-hindbrain segments of the neural tube. The NCC components of the branchial arches give rise to different skeletal elements that undergo profound changes during evolution (Sauka-Spengler and Bronner-Fraser, 2008). Genetic studies in several vertebrate model organisms have revealed that the distinct features that are acquired by cranial NCC-derived skeletal elements of each arch depend on a nested expression pattern of the homeotic genes belonging to the Hox family of transcription factors. The first arch, the mandibular arch, is an exception as it is devoid of Hox gene expression (Rijli et al., 1998; Pasqualetti et al., 2000; Trainor and Krumlauf, 2000; Baltzinger et al., 2005). In addition, signals from the endoderm and the ectoderm, which overlie the branchial arches, are crucial for correct craniofacial development and for the specification of the first branchial arch (Couly et al., 2002; Miller et al., 2003; Brito et al., 2008; Nair et al., 2007; Sato et al., 2008).

Although many elements in the gene regulatory network implicated in craniofacial morphogenesis are known, direct interactions and hierarchical relationships are still unclear. Defining the network that orchestrates such a complex series of events is particularly important as developmental mistakes may occur, leading to NCC-related developmental defects, such as craniofacial abnormalities, which account for one-third of all congenital human birth defects.

Serotonin (5-HT) is a well-known neurotransmitter that affects a wide variety of central and peripheral functions (reviewed by Berger et al., 2009). In addition to its function as a neuromodulator, it is now clear that 5-HT also has an important role as a growth and differentiation factor during development (Lauder, 1993; Gaspar et al., 2003; Fukumoto et al., 2005). During mammalian embryogenesis 5-HT is supplied by the maternal blood (Yavarone et al., 1993; Côté et al., 2007) and in *Xenopus* embryos 5-HT is present as a maternal pool in the eggs (Fukumoto et al., 2005). 5-HT is first produced by serotonergic neurons at tailbud stages (Van Mier et al., 1986). A specific population of neuroepithelial cells in *Xenopus* gills has also been shown to contain 5-HT (Saltys et al., 2006) and 5-HT uptake sites have been described in mammalian branchial arches and cranial NCCs (Shuey et al., 1993; Hansson et al., 1999; Narboux-Neme et al., 2008).

5-HT may also play a role in mouse craniofacial morphogenesis, as malformations are caused by exposure of cultured mouse embryos to selective 5-HT re-uptake inhibitors, such as fluoxetine (Prozac) and 5-HT receptor antagonists (Shuey et al., 1992; Choi et al., 1997; Moiseiwitsch, 2000). As shown in a variety of in vitro models, 5-HT has also been implicated in NCC migration (Moiseiwitsch and Lauder, 1995). Most of the biological actions of 5-HT are mediated by G-coupled receptors and, among these, the 5-HT2B receptor seems to be particularly important in mediating the effects of 5-HT in embryonic development (Choi et al., 1997). The 5-HT2B receptor is, in fact, an important regulator of cardiac morphogenesis, as in mice its genetic ablation leads to partial embryonic and postnatal lethality due to abnormal heart

[1]Unità di Biologia Cellulare e dello Sviluppo, Dipartimento di Biologia, Università di Pisa, S.S.12 Abetone e Brennero 4, 56127 Pisa, Italy. [2]Scuola Normale Superiore di Pisa, Piazza dei Cavalieri 7, Pisa (PI) 56126, Italy.

*Author for correspondence (mori@biologia.unipi.it)

Accepted 16 June 2010

development (Nebigil et al., 2000a). In previous work, we have demonstrated that signaling through 5-HT2B is also involved in eye morphogenesis in *Xenopus* embryos (De Lucchini et al., 2005; Reisoli et al., 2008). Expression of 5-HT2B in the craniofacial region of mice, as well as the potent teratogenic activity of its antagonists in cultured mouse embryos, suggest that this receptor might play a role in craniofacial development (Choi et al., 1997; Bhasin et al., 2004b). In spite of this evidence, a direct role for 5-HT2B in craniofacial morphogenesis has still to be defined.

Here we show that 5-HT2B is required for morphogenesis of the first branchial arch in *Xenopus laevis*. Our results provide new insight into the complex interactive network of extrinsic factors that regulates mandibular arch development and evolution.

MATERIALS AND METHODS

Embryo manipulation and microinjection

Xenopus laevis embryos were obtained by hormone-induced laying and were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). Capped mRNAs were synthesized in vitro from template cDNAs as previously reported: *5-HT2B* (Reisoli et al., 2008), *Nuclear*-β-*galactosidase* (*n*-β-*gal*), *Green fluorescent protein* (*GFP*) (Pasqualetti et al., 2000) and *Red fluorescent protein* (*RFP*), using the SP6 mMESSAGE mMACHINE Kit (Ambion, AM1340).

Two non-overlapping morpholino antisense oligonucleotides (MOs) were designed against the *5-HT2B* mRNA (GenBank accession AJ549811): 5-HT2B-MO1 (5′-GTGCCAGGGAATGGAGATTGTGTCT-3′), which is complementary to nucleotides –28 to –4; and 5-HT2B-MO2 (5′-AAT-AAGAAGGGTTGAGAGTTGGCAT-3′), which is complementary to the first 25 nucleotides of the coding region (the ATG complementary sequence is underlined).

An MO was designed against *Phospholipase C beta 3* (PLC-MO) (GenBank accession NM001087700): PLC-MO (5′-CCAGGCCGTG-CCCCCGCCATGTTT-3′), which is complementary to nucleotides –4 to +20. A five-mismatch MO, based on the PLC-MO sequence, was used as a control (5′-CgAGcCCGTGCCgCCGCgATcTTT-3′; lowercase indicates mismatched nucleotides) (Gene Tools).

MOs (5-HT2B-MO1, 5-HT2B-MO2, PLC-MO, PLC mismatch MO, 20 ng/embryo) or capped mRNAs (*5-HT2B*, 500 pg/embryo; *GFP*, *RFP*, *n*-β-*gal*, 300 pg/embryo each) were injected into one blastomere of 2- or 4-cell stage embryos in 3% Ficoll 400 (Fluka, 46327) in 0.1× Marc's Modified Ringer's Solution (MMR 1×: 0.1 M NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.5). We performed functional rescue experiments by co-injecting the 5-HT2B-MO1 (20 ng/embryo) with *5-HT2B* (250 pg/embryo), *n*-β-*gal* and *GFP* mRNAs. After injection, embryos were transferred in 0.1× MMR and incubated at 18°C until the desired developmental stage.

The injected side of embryos was visualized by the presence of GFP. Alternatively, embryos were fixed and stained to reveal n-β-gal activity using a red substrate (Biosynth, B-7200).

In situ hybridization (ISH) experiments

Digoxigenin (DIG) (Roche) labeled antisense RNA probes were generated for *5-HT2B* (De Lucchini et al., 2003), *Xbap* (Newmann et al., 1997), *XHoxa2* (Pasqualetti et al., 2000), *XDll4* (Papalopulu and Kintner, 1993), *XEphrinB2* (Smith et al., 1997), *XHand2* (Smith et al., 2000), *XMel1* (Van Campenhout et al., 2006), *XSlug* (Mayor et al., 1995) and *XSox9* (Spokony et al., 2002). Fluorescein (FLUO) (Roche) labeled antisense RNA probes were also generated for *XSox9* and *XHand2* in order to perform double ISH experiments. Whole-mount ISH was performed as described (Harland, 1991). Double ISH experiments were performed as described (Andreazzoli et al., 1999). We first revealed the DIG probe signal with BM Purple (Roche, 11442074001) and then the FLUO probe signal was visualized using BCIP substrate (Roche, 1383221). After color development, embryos were post-fixed and bleached under fluorescent light to remove the pigment. Control experiments were performed with sense probes. For histological examination, whole-mount ISH processed embryos were embedded in a gelatin-albumin solution as described (Levin, 2004) and then sectioned at 50 μm using a Leica VT1000S vibratome.

For ISH on cryosections (12 μm), embryos were fixed in 4% paraformaldehyde (PFA) in PBS, cryoprotected with 20% sucrose in PBS and embedded in Tissue-Tek O.C.T. compound (Sakura, 4583). ISH was performed according to Strähle et al. (Strähle et al., 1994) with minor modifications. NBT/BCIP substrate was used to detect the hybridization signal.

Skeletal and muscle staining

Stage 49 embryos, selected for asymmetrical distribution of GFP, were fixed overnight in 4% PFA in PBS and processed for Alcian Blue staining as described (Pasqualetti et al., 2000). Specimens were dissected by removing the skin and cutting the ethmoidal plate on the midline or the quadrate-ceratohyal junction and then flat-mounted on slides. For double staining, muscle-specific 12/101 monoclonal antibody (1:10, Developmental Studies Hybridoma Bank) and anti-GFP polyclonal antibody (1:2000, Molecular Probes) were used following a standard protocol. The immunohistochemistry procedure always preceded the Alcian Blue staining.

Cranial neural crest cell transplantation assay

Embryos overexpressing *5-HT2B* plus *GFP* or *RFP* mRNAs were maintained in 0.1× MMR until stage 15 and selected for the presence of GFP or RFP. NCC transplantation assays were performed as described (Borchers et al., 2000) with minor modifications.

RESULTS

5-HT2B overexpression results in abnormal craniofacial skeleton development and skeletomuscular connectivity

Although *5-HT2B* gene expression is hard to detect, in this paper we provide evidence for the presence of the *5-HT2B* mRNA in the branchial arches of *Xenopus* tailbud embryos and, in particular, in the mandibular arch in the region surrounding the oral cavity of stage 37 embryos (see Fig. S1A-B′ in the supplementary material). A very similar gene expression profile has been described for *5-HT2B* (*Htr2b*) in mouse embryos (Choi et al., 1997; Nebigil and Maroteaux, 2001).

In order to analyze a possible role of 5-HT2B in craniofacial development, we performed a gain-of-function analysis by microinjecting the in vitro transcribed *Xenopus 5-HT2B* mRNA into one blastomere of 2- to 4-cell stage embryos. To reveal the injected side of the embryo, the *5-HT2B* mRNA was injected in combination with *GFP* and *n*-β-*gal* mRNAs. The injected embryos were raised to stage 49 and then stained for cartilage analysis.

Most of the craniofacial skeletal elements originate from cranial NCCs that migrate from the dorsal neural tube in three distinct streams termed mandibular, hyoid and branchial. These streams colonize the first, second, and third and fourth pharyngeal arches, respectively, where they differentiate into visceral skeletal derivatives. The first arch crest (mandibular) gives rise to the upper (quadrate, Q), lower (Meckel's, M) jaw and the ethmoid-trabecular plate, whereas the second (hyoid) arch crest gives rise to the ceratohyal (C) cartilage and the third and fourth (branchial) arch NCCs contribute to the gill (G) cartilages (Fig. 1C) (Sadaghiani and Thiebaud, 1987).

In our experiments, *5-HT2B* overexpression gave rise to morphogenetic cranial skeletal defects in 68% (*n*=453) of injected tadpoles. The phenotype was characterized by the presence of an ectopic cartilage of well-defined shape and position. In 90% of the affected embryos, the ectopic cartilage was fused to the anterior lateral margin of the ceratohyal cartilage and pointed posteriorly towards the ear (Fig. 1B,D,E); in 55% of these injected embryos, the ectopic cartilage was fused to the dorsal lateral margin of the branchial basket (Fig. 1B,F,G). In the remaining 10% of affected

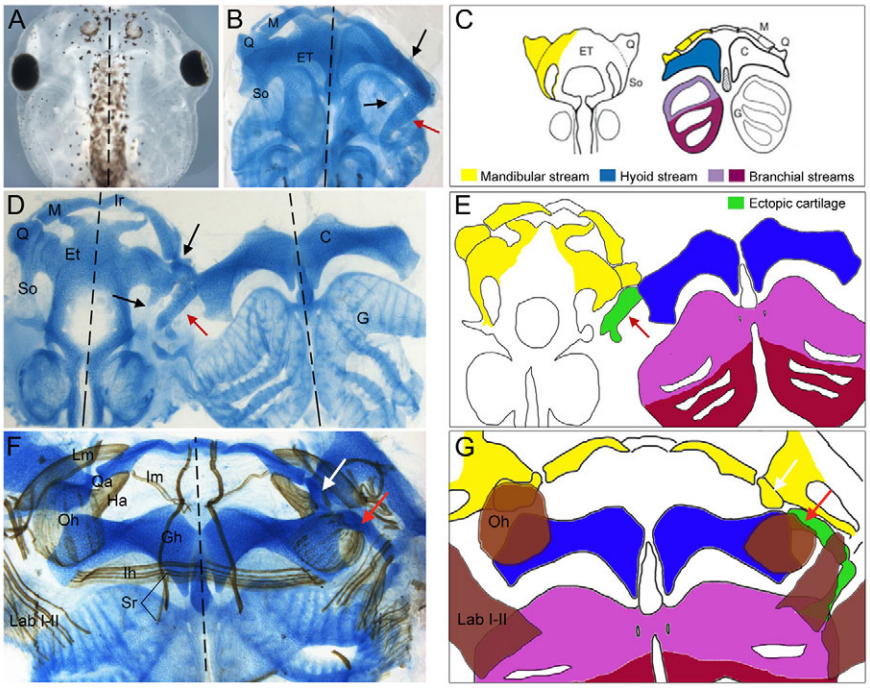

Fig. 1. Skeletal and muscular alteration in 5-HT2B-overexpressing tadpoles (stage 49). (**A**) Dorsal view of a 5-HT2B-overexpressing embryo. (**B**,**D**) Alcian Blue-stained whole-mount (B) and flat-mount (D) skeleton of injected embryos. Red arrows indicate the ectopic cartilage and black arrows indicate the reduction of the quadrate (Q) and subocular (So) cartilages. (**C**,**E**) Scheme of the contribution of cranial NCC streams to the neurocranium and pharyngeal skeleton of *Xenopus laevis* as reported by Pasqualetti et al. (Pasqualetti et al., 2000) (C) and in 5-HT2B-overexpressing embryos (E). (**F**) Flat-mount of an injected embryo double stained for cartilage (blue) and muscles (brown). (**G**) Scheme of muscular alterations of the 5-HT2B-overexpressing embryo shown in F. On the injected side, the orbitohyoideus muscle (Oh) is anchored to the ectopic cartilage (red arrow in F,G) instead of to the reduced quadrate (white arrow in F,G). Note the disorganization of the levatores arcuum branchialum muscles I-II (Lab I-II) on the injected side. C, ceratohyal; Et, ethmoidal plate; G, genohyoideus; Ha, hyoangularis; Ih, interhyoid muscle; Im, intermandibular; Ir, infrarostral; M, Meckel's; Qa, quadratoangularis; Lm, levator mandibulae; Sr, subarcuales rectus.

tadpoles the ectopic cartilage was detached from the ceratohyal cartilage (data not shown). Moreover, in all the affected embryos, the quadrate and subocular (So) cartilages, both of which derive from the mandibular stream NCCs, were reduced in size (Fig. 1B,D-G). By contrast, Meckel's cartilage, the other mandibular NCC derivative, was normal (Fig. 1D-G). Control embryos injected only with *GFP* or *n*-β-*gal* mRNAs developed a normal skeleton (data not shown).

We then addressed the question of whether such skeletal alterations were associated with altered muscular connectivity. In wild-type tadpoles, the first arch-specific muscles comprise the complex of the levator mandibulae (Lm) and the intermandibular (Im) muscles. The second arch-specific muscles include the interhyoideus (Ih) and the jaw-opening muscles, such as the orbitohyoideus (Oh), which inserts on both the ceratohyal and quadrate cartilages, the hyoangularis (Ha) and the quadratoangularis (Qa) (Hanken et al., 1997; Pasqualetti et al., 2000). In the branchial region, a broad muscle band, the levatores arcuum branchialum I-IV (Lab), covers the branchial basket dorsoventrally (Ziermann and Olsson, 2007).

The simultaneous visualization of the visceral skeleton and muscles revealed that the ectopic cartilage and the reduction of the quadrate were specifically associated with an altered orbitohyoideus (Oh) muscle connectivity. In fact, in 84% (n=95) of affected embryos this muscle had a correct insertion on the ceratohyal, whereas it was partially or completely anchored to the ectopic cartilage instead of to the reduced quadrate (Fig. 1F,G). In the same group of embryos, 83% also showed altered branchial muscle connectivity. In particular, the levatores arcuum branchialum I-II did not reach the dorsal part of the branchial basket correctly. By contrast, the muscles of the most posterior part of the branchial basket (levatores arcuum branchialum III-IV) were almost normal (Fig. 1F,G; see Fig. S2A,B in the supplementary material).

5-HT2B gain-of-function results in ectopic *Xbap* and *XHand2* expression

To shed light on the origin of the ectopic cartilage formation we looked at the various phases of NCC development. We carried out whole-mount ISH with molecular markers for pre-migratory and migrating NCCs. Using *XSlug* and *XEphrinB2* as markers for pre-migratory NCCs and for rhombomeres 2 and 4 (r2, r4), respectively, we did not observe any difference in the corresponding mRNA expression patterns between the wild-type and the injected side of embryos (Fig. 2A,B).

At stage 26, when the migration process is complete, the mRNA expression patterns of the skeletogenic NCC marker *XSox9* and that of *XHoxa2*, which is a second and more posterior branchial arch marker, were unaffected (Fig. 2C-D′). Altogether, these data suggested that 5-HT2B overexpression does not alter the specification or migration of NCCs.

Thus, we looked at pre-differentiating cranial NCCs (stages 35-37), using *Xbap* [the *Xenopus* ortholog of *bagpipe* in *Drosophila* and of *Bapx1* (*Nkx3.2*) in vertebrates] to label the palatoquadrate

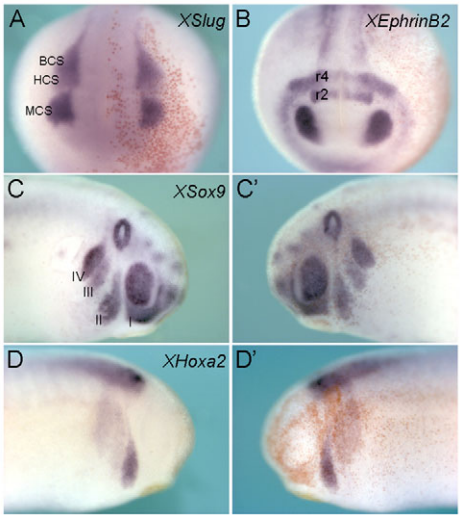

Fig. 2. 5-HT2B overexpression does not influence NCC specification and migration. The injected side, visualized by n-β-gal staining (red), is always on the right. (**A**,**B**) Frontal view of a neurula stage *Xenopus* embryo hybridized with *XSlug* (A) and *XEphrinB2* (B) probes. (**C-D'**) Lateral view of tailbuds hybridized with *XSox9* (C,C′) and *XHoxa2* (D,D′) probes. MCS, mandibular crest stream (branchial arch I); HCS, hyoid crest stream (branchial arch II); BCS, branchial crest stream (branchial arches III-IV).

cartilage, near to the point of articulation to Meckel's cartilage and in the caudal region of Meckel's cartilage itself. We also used *XHoxa2* to identify the second and most posterior branchial arches, *XHand2* to visualize the ventral part of all branchial arches, and *XMel1* to visualize the third and fourth branchial arches. In 55% (n=291) of injected embryos, the *Xbap* mRNA was ectopically expressed and resembled a mirror-image duplication of the wild-type *Xbap* mRNA expression site (Fig. 3A-B). By double in situ labeling, we showed that the ectopic *Xbap* signal in the first branchial arch was contained within a subdomain of the *XSox9* expression site located in a dorsal position with respect to its wild-type counterpart (Fig. 3B). These findings showed that, even though the 5-HT2B was widely overexpressed in the first arch, it was able to induce ectopic *Xbap* expression only in a subpopulation of *XSox9*-positive skeletogenic precursors.

XHand2 is a key transcription factor regulating craniofacial morphogenesis in vertebrates. In *Xenopus*, it is specifically expressed in the ventral part of all branchial arches (Smith et al., 2000). In 5-HT2B-overexpressing embryos, *XHand2* was ectopically expressed in the dorsal part of posterior branchial arches, to which the ectopic cartilage was often fused. Interestingly, *XHand2* was not ectopically expressed in the first branchial arch (Fig. 3C-D). The expression domains of *Xbap* and *XHand2* did not overlap in the first branchial arch (Fig. 3D). In the same group of embryos, pre-differentiating second arch NCCs, expressing *XHoxa2* mRNA, were unaffected (Fig. 3E-E″), as was the expression of *XMel1* in the third and fourth branchial arches (Fig. 3F,F′).

5-HT2B acts on NCCs in a cell-autonomous manner

To assess whether the overexpressed 5-HT2B receptor acts in a cell-autonomous manner in cranial NCCs, we performed a homochronic and homotopic NCC transplantation assay (Fig. 4A). *5-HT2B*, *GFP* and *n*-β-*gal* mRNAs were co-injected into one blastomere of 2-cell stage *Xenopus* embryos, which were raised until the neurula stage. Then we transplanted the overexpressing 5-HT2B pre-migratory cranial NCCs into wild-type embryos at the

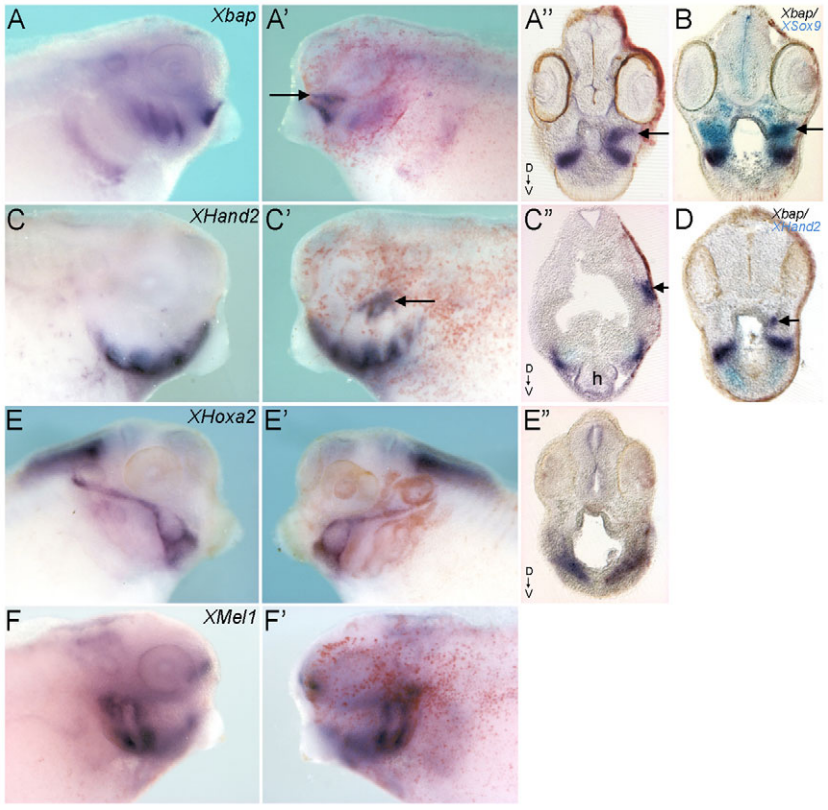

Fig. 3. 5-HT2B overexpression results in ectopic *Xbap* and *XHand2* mRNA expression. The injected side, visualized by n-β-gal staining (red), is always on the right. Lateral view (A,A′,C,C′,E,E′,F,F′) and vibratome sections (A″,B,C″,D,E″) of stage 37 *Xenopus* embryos. (**A-A″**) *Xbap* mRNA ectopic expression in the mandibular arch (arrow in A′,A″). (**B**) Double ISH showing *Xbap* ectopic expression (dark blue) in a subdomain (arrow) of the *XSox9*-expressing cells (light blue). (**C-C″**) *XHand2* ectopic expression in the dorsal domain of the posterior branchial arches (arrow in C′,C″). (**D**) Double ISH showing *Xbap* (dark blue) and *XHand2* (light blue). On the injected side, only *Xbap* is ectopically expressed in the mandibular arch (arrow). (**E-E″**) *XHoxa2* expression in the second branchial arch is normal. (**F**,**F′**) *XMel1* expression in third and fourth branchial arches is normal. h, heart.

same developmental stage (Fig. 4A). In this type of assay, 5-HT2B-overexpressing transplanted NCCs should develop in a wild-type context.

We performed three independent experiments in which we transplanted a total of 48 embryos. Among them, we selected 37 embryos on the basis of the correct transplantation of all the NCCs that made up the four migratory streams. We thus compared the distribution and position of transplanted NCCs with the expression of the pan-cranial NCC marker *XDll4* in wild-type embryos at the same developmental stage (Fig. 4B,C).

After migration, the transplanted NCCs were visualized in red by n-β-gal staining. In these embryos the endodermal, ectodermal and mesodermal components of the pharyngeal pouches appeared to be devoid of n-β-gal staining (Fig. 4D,E). This confirmed that they derived only from the wild-type host embryo tissues and that we had correctly transplanted the cranial NCCs.

To visualize the transplanted NCC-derived skeletal elements, stage 49 transplanted embryos (*n*=34) were simultaneously stained with anti-GFP antibody and Alcian Blue. We found that 91% of transplanted embryos (31/34) developed a visceral skeleton that presented ectopic cartilage and the same phenotype described for *5-HT2B* widespread overexpression (compare Fig. 1D with Fig. 4F). As a control and further confirmation of these results, we performed a complementary experiment, transplanting wild-type pre-migratory cranial NCCs, labeled by injection of mRNA of the vital tracer *RFP*, into host embryos overexpressing *5-HT2B* plus *GFP* mRNAs. In this assay, wild-type cranial NCCs should develop in an environment overexpressing 5-HT2B. In two rounds of experiments we analyzed 22 transplanted embryos, following the NCCs at migratory and postmigratory stages under fluorescent light (Fig. 5A-C). No alterations were observed in these developing embryos. The transplanted wild-type NCC-derived skeletal elements, visualized at stage 49 (*n*=22) by Alcian Blue, showed no skeletal defects (Fig. 5C′).

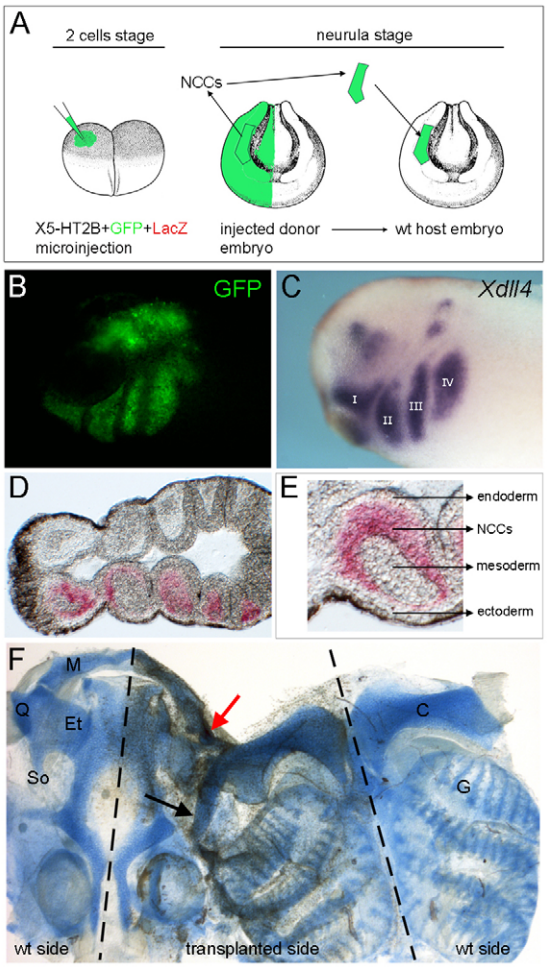

Fig. 4. 5-HT2B influences cranial NCC development in a cell-autonomous manner. (**A**) Scheme of the cranial NCC transplantation assay. (**B**,**C**) Lateral view of stage 30 transplanted and wild-type embryos, respectively. (B) GFP fluorescence in transplanted cranial NCCs. (C) Cranial NCCs visualized by the *XDll4* probe in a wild-type embryo. (**D**,**E**) Horizontal vibratome sections of *Xenopus* tadpole. The red staining identifies transplanted NCCs. (E) Magnification of the second pharyngeal arch of a transplanted embryo stained for n-β-gal. (**F**) Flat-mount preparation of a stage 49 transplanted embryo double stained for cartilage (blue) and GFP immunoreactivity (brown). Skeletal elements derived from the transplanted cranial NCCs are visualized by the presence of GFP immunoreactivity. Note the reduced quadrate (red arrow) and the presence of ectopic cartilage (black arrow).

Depletion of 5-HT2B alters first branchial arch morphogenesis leading to the loss of the jaw joint

To verify whether 5-HT2B plays a physiological role in craniofacial development, we used a morpholino (MO) approach to specifically knock down *5-HT2B* gene function.

Injection of 5-HT2B-MO1 into one blastomere of 2-cell stage *Xenopus* embryos resulted in altered morphogenesis of the skeletal elements originating from the mandibular NCC stream in 37%

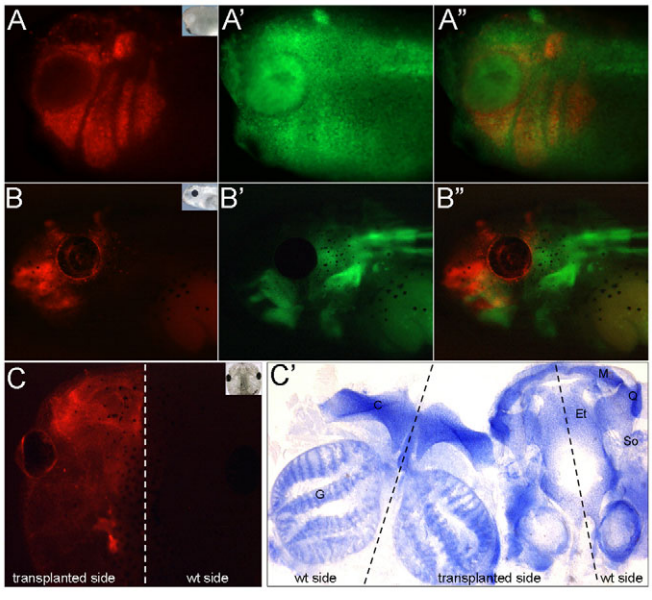

Fig. 5. Wild-type NCCs transplanted in host embryos overexpressing 5-HT2B develop a normal skeleton. (**A-B″**) Lateral view of stage 30 (A-A″) and 42 (B-B″) *Xenopus* embryos. (A,B) RFP fluorescence in transplanted cranial NCCs. (A′,B′) GFP fluorescence in the host embryo. (A″,B″) Merge of A,A′ and B,B′, respectively. (**C**) Dorsal view of a stage 49 transplanted embryo. Cranial NCC derivatives are visualized by RFP fluorescence. (**C′**) Flat-mount preparation of the embryo in C, stained with Alcian Blue. The skeleton appears normal.

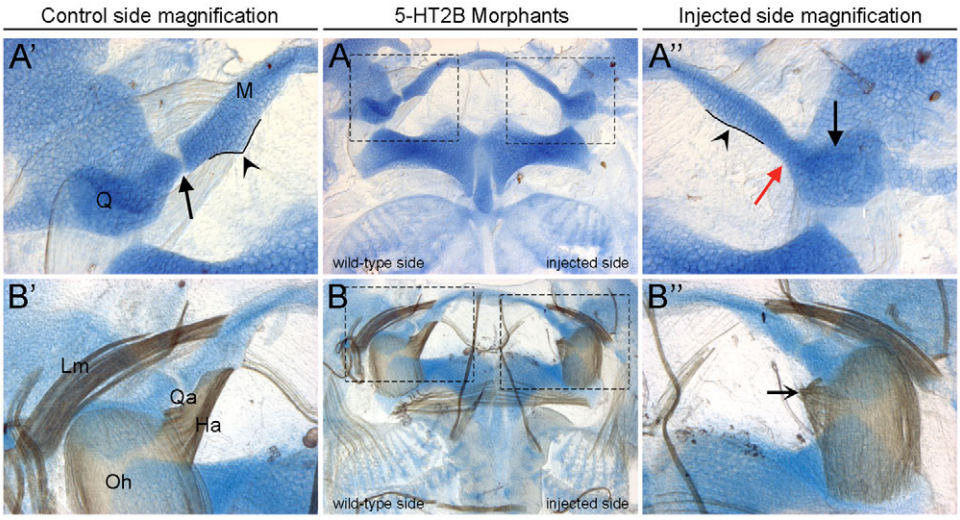

Fig. 6. Skeletal and muscular connectivity alterations in 5-HT2B morphants. (**A**) Flat-mount preparation of an Alcian Blue-stained 5-HT2B-MO-injected *Xenopus* embryo. (**A′**,**A″**) Magnification of the jaw joint region of the control (A′) and injected (A″) side of the embryo in A. Note the lack of the jaw joint (red arrow in A″) compared with the uninjected side (arrow in A′), the reduction of the quadrate (Q; black arrow in A″), and the absence of the ventral cartilaginous muscular process of the Meckel's cartilage, which is present on the control side (arrowhead in A″ and A′, respectively). (**B**) Flat-mount preparation of a 5-HT2B-MO-injected embryo double stained for cartilage and muscle. (**B′**,**B″**) Magnification of the jaw joint region of the uninjected (B′) and injected (B″) sides of the embryo in B. Note the abnormal development of the hyangularis (Ha) and quadratoangularis (Qa) muscles (arrow in B″).

(*n*=436) of the injected embryos (Fig. 6A-A″). Most of the affected embryos showed a thinner Meckel's cartilage that lacked the cartilaginous muscular process on its ventral side. This skeletal phenotype was associated with an alteration in the connectivity of the hyoangularis (Ha) and quadratoangularis (Qa) muscles. Normally, these muscles originate from the ceratohyal and quadrate cartilages, respectively, and both insert on the ventral side of Meckel's cartilage (Fig. 6B,B′). In 5-HT2B morphants, these muscles were correctly anchored to the ceratohyal and quadrate cartilages, respectively, but they were shortened and they failed to reach Meckel's cartilage (Fig. 6B,B″), leading to a functional impairment of the mouth opening. In 26% of the injected embryos, Meckel's cartilage was fused to the quadrate, leading to the loss of the jaw joint (Fig. 6A-A″ and Table 1).

In fish and chick embryos, the downregulation of *Bapx1* expression is associated with the loss of the jaw joint (Miller et al., 2003; Wilson and Tucker, 2004; Nair et al., 2007). ISH analyses showed that in pre-differentiating NCCs, *Xbap* expression was strongly reduced in 61% of morphants (*n*=228), in accordance with the observed morphological modifications of the first branchial arch-derived skeletal elements that were missing the jaw joint (Fig. 7A-A‴). By contrast, expression of *XHoxa2* and *XHand2* was not altered in 5-HT2B-depleted embryos (Fig. 7B-B″,C-C″).

Finally, we found that expression of the pan-skeletogenic NCC marker *XSox9* was unaffected in NCCs of injected embryos in postmigratory stages, indicating that *5-HT2B* functional abrogation does not influence the migration of NCCs (Fig. 7D,D′).

The low frequency of affected embryos (37% of the injected embryos and just 70% of those displaying loss of the jaw joint) could be due to a low efficacy of the MO. However, these results might also be expected, considering the late role of 5-HT2B in NCC development and therefore the need to knock down its function after NCC migration. To confirm the specificity of 5-HT2B-MO1, we injected a second, non-overlapping MO: 5-HT2B-MO2. Although injection of 5-HT2B-MO2 possibly caused non-specific phenotypes, such as a generalized reduction of all the craniofacial skeletal elements, it also reproduced the loss of the jaw joint in 25% of the morphants (*n*=395) (Table 1). Moreover, co-injection of the *5-HT2B* mRNA together with 5-HT2B-MO1 significantly rescued both the skeletal and molecular phenotypes. Ninety percent (*n*=300) of the injected embryos developed a

Table 1. 5-HT2B and PLC are required for jaw joint formation

Treatment	Skeletal alteration in the first branchial arch region (%)	Joint loss (%)	Severe skeletal reduction in the four branchial arches (%)
5-HT2B-MO1 20 ng	37 (163/436)	26 (114/436)	5 (22/436)
5-HT2B-MO2 20 ng	36 (144/395)	25 (99/395)	30 (118/395)
5-HT2B-MO1 20 ng + *5HT2B* mRNA	10 (29/300)	5 (16/300)	3 (9/300)
PLC-MO 20 ng	21 (41/204)	12 (24/204)	9 (18/204)
PLC-control-MO 20 ng	0 (0/87)	0 (0/87)	0 (0/87)
5-HT2B-MO1 10 ng	0 (0/77)	0 (0/77)	0 (0/77)
PLC-MO 10 ng	2 (2/98)	2 (2/98)	2 (2/98)
5-HT2B-MO1 10 ng + PLC-MO 10 ng	25 (53/208)	13 (27/208)	1 (2/208)

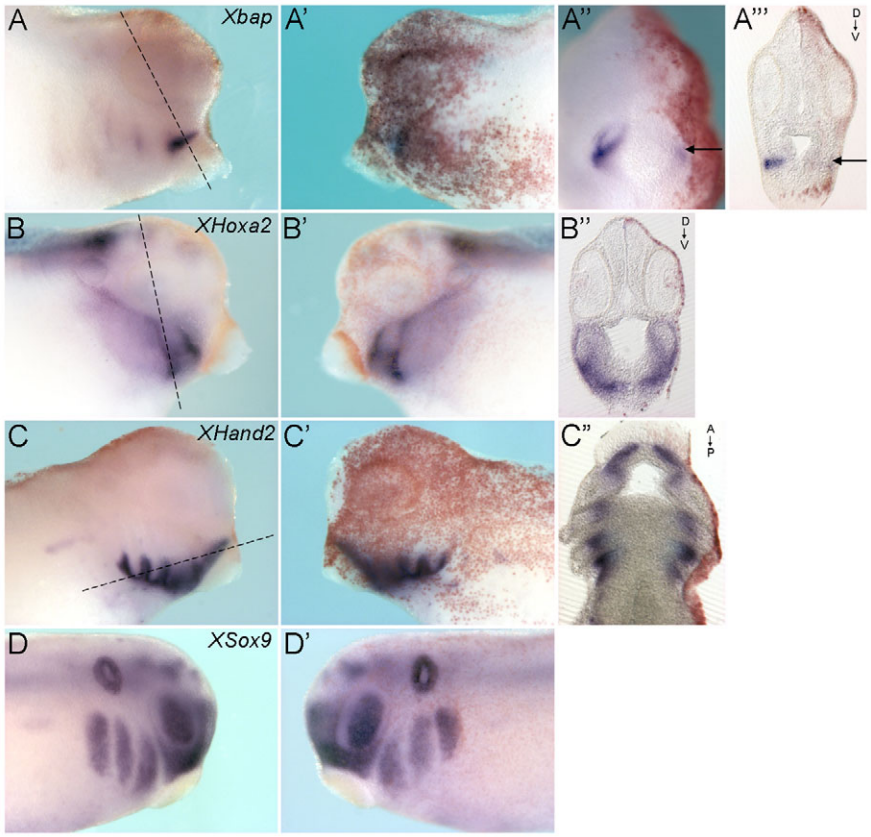

Fig. 7. 5-HT2B loss of function results in the downregulation of *Xbap* expression in postmigratory NCCs. The injected side, visualized by n-β-gal staining (red), is always on the right. (**A**,**A′**,**B**,**B′**,**C**,**C′**) Lateral view of stage 37 *Xenopus* embryos hybridized with *Xbap* (A,A′), *XHoxa2* (B,B′) and *XHand2* (C,C′) probes. (**A″**) Frontal view of the embryo in A. (**A‴**,**B″**,**C″**) Vibratome coronal sections across the line in A, B and C, respectively. (A-A‴) The *Xbap* expression is reduced on the injected side of stage 37 5-HT2B morphants (arrows in A″, A‴) as compared with the control side. *XHoxa2* (B-B″) and *XHand2* (C-C″) expression is unaffected. (**D**,**D′**) Lateral view of a whole-mount stage 28 5-HT2B morphant hybridized with *XSox9* probe.

normal skeleton and 78% (*n*=52) presented a normal level of *Xbap* mRNA expression, demonstrating the specificity of the morpholino approach (Table 1 and see Fig. S3 in the supplementary material).

Phospholipase C beta 3 acts downstream of 5-HT2B to regulate *Xbap* expression and jaw joint formation

To shed light on the molecular identity of the transduction pathway acting downstream of 5-HT2B, we performed a loss-of-function study of the principal 5-HT2B effector phospholipase C beta 3 (PLC) (Raymond et al., 2001). We injected a specific MO (PLC-MO) into one side of early-cleaving embryos and analyzed the expression of two known PLC signaling target genes: *Xbap* and *XHand2* (Walker et al., 2007).

In stage 37 PLC-MO-injected embryos, both markers were strongly downregulated in the mandibular arch (*Xbap* downregulation by 63%, *n*=113; *XHand2* downregulation by 49%, *n*=93) (see Fig. S4A-B″ in the supplementary material), and *XHand2* mRNA expression was also reduced in posterior branchial arches (see Fig. S4B-B″ in the supplementary material). The head skeleton of PLC morphants presented loss of the jaw joint in 12% of injected embryos and a reduction in size of other skeletal elements, such as Meckel's cartilage, the ceratoyal and the gills (Fig. 8A-B″ and Table 1). In particular, the distal part of Meckel's cartilage was often absent in the injected embryos (Fig. 8B-B″). The injection of a mismatched PLC MO did not cause alterations at a molecular or morphological level in the embryos (see Fig. S4C-E in the supplementary material).

To demonstrate an epistatic interaction between 5-HT2B and PLC, we used two different approaches. First, we injected subthreshold levels of 5-HT2B-MO and PLC-MO individually and together in the same injection session. Whereas the injection of a low dose (10-12 ng) of each MO resulted in normal embryos (*n*=77 and *n*=98, respectively), the co-injection of 10 ng of 5-HT2B-MO plus 10 ng per embryo of PLC-MO gave rise to loss of the jaw joint in 13% of embryos (*n*=208) (Fig. 8C-E). These data suggested that 5-HT2B-MO and PLC-MO display synergistic effects. Second, to demonstrate that PLC is a direct downstream effector of 5-HT2B we performed a rescue experiment by injecting *5-HT2B* mRNA plus PLC-MO into the same embryo. Upon overexpression of 5-HT2B, 57% of embryos presented ectopic cartilage (*n*=157). By contrast, co-injection of *5-HT2B* mRNA plus PLC-MO resulted in just 21% of embryos presenting the ectopic cartilage (*n*=83), demonstrating the efficacy of the functional rescue (Fig. 8F-H). Note that in the rescue experiments the embryos carrying the ectopic cartilage showed a mild phenotype with respect to those overexpressing *5-HT2B* mRNA (compare Fig. 8G with 8H). Taken together, these data showed that PLC is the effector of 5-HT2B signaling in cranial NCC development.

DISCUSSION

Despite the crucial role played by intrinsic factors expressed in cranial NCCs in directing normal pharyngeal arch morphogenesis, it is now becoming apparent that the correct development of craniofacial structures is strongly influenced by the integration of this information with extrinsic factors and by the interactions of NCCs with their environment. In such a complex scenario, this study provides novel insights into the molecular mechanisms underlying craniofacial development. Using gain- and loss-of-function approaches in *Xenopus* embryos we demonstrated in vivo that 5-HT2B receptor signaling is required during craniofacial

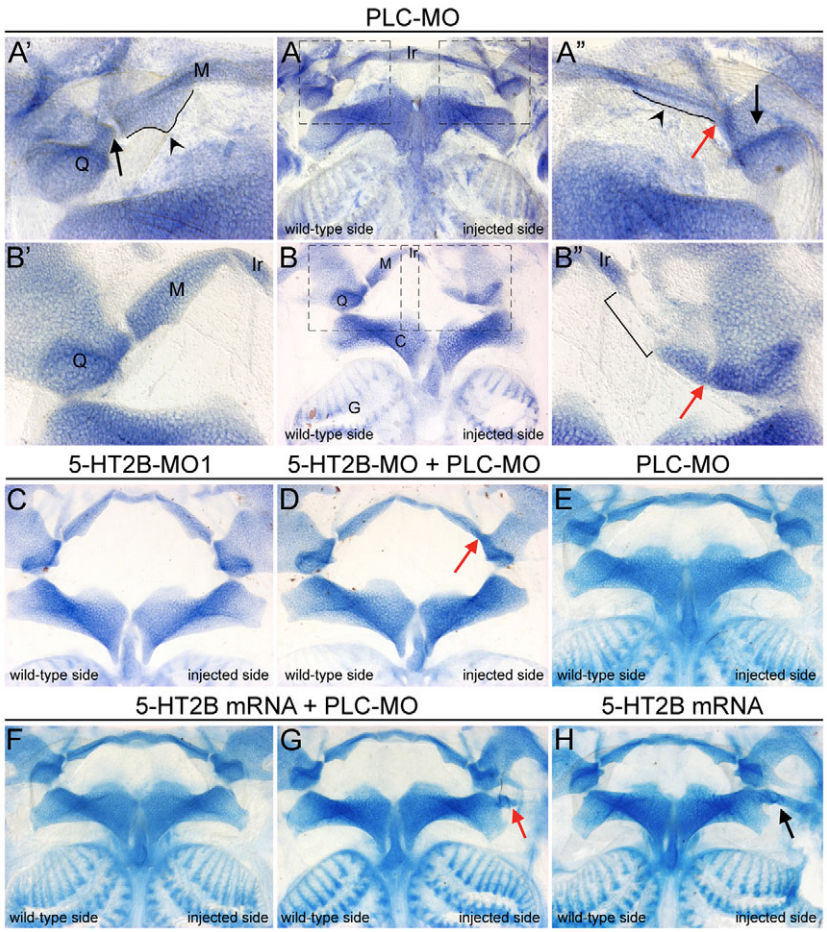

Fig. 8. Phospholipase C beta 3 acts downstream of 5-HT2B to regulate the jaw joint formation. Flat-mount preparations of Alcian Blue-stained injected *Xenopus* embryos. (**A-B″**) PLC-MO morphants. (A′,A″,B′,B″) Magnification of the jaw joint region of the embryos in A,B. Note the lack of the jaw joint (red arrow in A″,B″) and of the ventral cartilaginous muscular process of the Meckel's (arrowhead in A″), as compared with the control side (arrowhead in A′). (B″) A strong phenotype PLC morphant presenting the loss of the jaw joint (arrow) and of the distal portion of the Meckel's (squared bracket). (**C**,**E**) Embryos injected with subthreshold levels of either 5-HT2B-MO (C) or PLC-MO (E) develop a normal visceral skeleton. (**D**) The co-injection of subthreshold levels of 5-HT2B-MO plus PLC-MO causes loss of the jaw joint (red arrow). (**F**,**G**) The co-injection of PLC-MO together with *5-HT2B* mRNA results in 79% normal embryos (F) and 21% presenting the ectopic cartilage (G). (**H**) In 5-HT2B overexpression embryos, the ectopic cartilage is always bigger than that originating in the 5-HT2B mRNA+PLC-MO injected embryos (compare red arrow in G to black arrow in H).

skeletal development, as it influences the behavior of NCCs in the postmigratory first branchial arch and the formation of the jaw joint.

Widespread 5-HT2B overexpression resulted in a morphological change in the craniofacial skeleton and in skeletomuscular connectivity. This was characterized by the formation of an ectopic cartilaginous element and by a reduction in the quadrate and subocular cartilages. By contrast, Meckel's cartilage, the other mandibular NCC derivative, appeared normal. The skeletal phenotype of 5-HT2B-overexpressing embryos is, in fact, associated with an alteration in orbitohyoideus muscle connectivity.

In 5-HT2B-overexpressing embryos, the orbitohyoideus muscle maintained a correct insertion on the ceratohyal but it was anchored partially or completely to the ectopic cartilage instead of to the reduced quadrate. Taking into account that the connective tissues of cranial muscles that are derived from specific rhombomeric levels are exclusively attached to skeletal regions of the same origin (Köntges and Lumsden, 1996; Pasqualetti et al., 2000), such a change in the connectivity of the orbitohyoideus muscle suggested that the ectopic cartilage derives, at least in part, from the first branchial arch NCCs.

This is supported by the finding that in 5-HT2B-overexpressing embryos, *Xbap*, which is expressed in the precursors of the jaw joint region in the first branchial arch, was ectopically expressed, resembling a mirror-image duplication of the wild-type *Xbap* expression. In addition, we showed that the ectopic *Xbap*-expressing cells belong to a subpopulation of skeletogenic *XSox9*-positive NCCs of the first branchial arch, located dorsally to the *XHand2* expression domain. This suggests that the ectopic cartilage arises, at least in part, from the altered morphogenesis of a subpopulation of first arch skeletogenic NCCs. 5-HT2B overexpression also caused a change in the skeletomuscular connectivity of the branchial basket. In some embryos, in fact, the ectopic cartilage was fused posteriorly to the branchial basket skeleton. In these cases the levatores arcuum branchialum I-II muscles, which normally cover the gills dorsoventrally, did not reach the dorsal part of the branchial basket correctly. This phenotype may correlate with the ectopic expression of *XHand2* in the dorsal part of branchial arches III and IV. In mammals, *Hand2* is expressed in the ventral part of branchial arches.

Our data suggest that the overexpression of 5-HT2B also influences the morphogenesis and/or patterning of the posterior arch NCCs by altering their dorsoventral positional information. Branchial NCC precursors, which contribute to the dorsal part of the branchial basket, could acquire ventral characteristics in 5-HT2B-overexpressing embryos. This could contribute to the formation of the posterior part of the ectopic cartilage when it is fused to the branchial basket. Accordingly, the levatores arcuum branchialum muscles could lose the capacity to attach themselves to the dorsal part of the branchial basket because it has acquired ventral features. Note that the expression of other molecular markers labeling the third and fourth branchial arches, such as *XMel1*, appeared to be normal. Moreover, in 5-HT2B-overexpressing embryos, the expression of *XHand2* was not duplicated in the first branchial arch.

Why is the overexpression of 5-HT2B able to induce the ectopic expression of *XHand2* in the posterior branchial arches but not in the mandibular arch? One explanation is that the anterior and posterior arches, which are differentially instructed by anterior-posterior positional information, could interpret and integrate the ectopic activation of 5-HT2B signaling differently, resulting in a difference in the regulation of *XHand2* transcription in the mandibular towards the more posterior branchial arches. In this regard, studies on the promoter region of the mouse *Hand2* gene have revealed the presence of a specific enhancer that drives the expression of *Hand2* in the first and second branchial arches, suggesting a differential regulation of *Hand2* transcription between the first two arches and those more posterior (Charité et al., 2001).

It is also worth noting that the expression domains of *Xbap* and *XHand2* do not overlap in the first branchial arch. 5-HT2B overexpression might therefore influence just a subpopulation of mandibular NCCs located dorsally with respect to the *XHand2* expression domain. Note that 5-HT2B misexpression did not alter *XHoxa2* expression in the second branchial arch. Since Hoxa2 is able to restrict *Xbap/bapx1* expression in the first branchial arch by repressing its transcription in the second branchial arch (Pasqualetti et al., 2000; Miller et al., 2004; Balzinger et al., 2005), we believe that 5-HT2B overexpression positively modulates *Xbap* expression only in the Hox-free first branchial arch. Since there is not a clear molecular alteration of the second branchial arch skeletal precursors, it is difficult to assess whether the second branchial arch NCCs contribute to the formation of the ectopic cartilage. We can only hypothesize that hyoid NCC precursors might participate in the formation of the ectopic cartilage when fused to the ceratohyale.

The misexpression of 5-HT2B did not interfere with the induction or migration of NCCs or with the rhombomeric organization of the hindbrain. On the whole, these data would seem to indicate that enhanced 5-HT2B signaling specifically influences the behavior of postmigratory NCCs. This underlines the high plasticity of the NCCs at postmigratory stages, as already suggested in previous work (Pasqualetti et al., 2000).

We next addressed the question of whether 5-HT2B signaling could directly alter NCC behavior or whether it could modify their environment and surrounding tissues by producing NCC-instructive signals. Using transplant assays, we followed the development of 5-HT2B-overexpressing NCCs that had been introduced into a wild-type context, or, alternatively, we analyzed the fate of wild-type NCCs that had been transplanted into a host embryo overexpressing 5-HT2B. The data demonstrated that 5-HT2B activity in NCCs is sufficient, in a cell-autonomous manner, to generate the ectopic cartilage. 5-HT2B signaling is therefore able to alter the intrinsic properties of NCCs by directly, or indirectly, altering the ability of NCCs to respond to the correct patterning signals.

Enhanced 5-HT2B signaling is able to influence visceral arch morphogenesis, but does it have a physiological role in craniofacial development? We showed that *5-HT2B* mRNA is present in the branchial arches of late tailbud *Xenopus* embryos, and it is known that it is also expressed in the branchial arches of the mouse embryo at ~E9 (Choi et al., 1997; Lauder et al., 2000). There are no data regarding the development of the first arch in *5-HT2B* knockout embryos, probably because the most penetrating phenotype of *5-HT2B* mutant mice leads to early embryonic death in utero (Nebigil et al., 2000a).

We used a morpholino approach to specifically knock down *5-HT2B* function in *Xenopus* embryos. In all 5-HT2B morphants, NCCs appeared to be induced normally and were able to migrate to the appropriate location, confirming that 5-HT2B signaling was not required in the early phases of NCC development. However, in 5-HT2B morphants we observed specific skeletal defects that were successfully rescued by co-injecting the 5-HT2B MO and *5-HT2B* mRNA. A hypomorphic quadrate was fused with Meckel's cartilage into a single element, leading to the loss of the jaw joint. Moreover, Meckel's cartilage was altered in shape due to the lack of the cartilaginous muscular process normally located on its ventral aspect. This process is necessary for the attachment of the hyoangularis and quadratoangularis muscles and its absence resulted in the abnormal development of such muscles, which, even though still present, failed to reach their target cartilage. The consequence of both the skeletal and muscular abnormalities was a critical functional impairment of the mouth opening. This phenotype closely resembles that reported after *bapx1* functional abrogation in zebrafish embryos (Miller et al., 2003).

The development of the jaw joint between the quadrate and the proximal part of Meckel's cartilage has, in fact, been shown to require Bapx1 function both in zebrafish and chicken embryos (Miller et al., 2003; Wilson and Tucker, 2004; Nair et al., 2007). In line with this, in our experiments 5-HT2B morphants showed a strong reduction of *Xbap* mRNA expression in pre-differentiating NCCs, confirming a positive regulation of *Xbap* expression by 5-HT2B signaling. To date, the only signaling pathway known to be able to positively control *Bapx1* expression is endothelin 1 (Edn1), a small secreted peptide that induces signaling from its cognate receptor, endothelin receptor type A (Ednra), coupled to G proteins of the Gq/G11 family (Ivey et al., 2003). In zebrafish *edn1* (*sucker*) mutants, Meckel's cartilage is fused to the palatoquadrate and the expression of *bapx1* is lost (Miller et al., 2003). This means that the abrogation of Ednra results in the loss of the jaw joint in zebrafish morphants (Nair et al., 2007). Endothelin signaling appears conserved among vertebrates and it has been shown that PLC (Plcb3) is the effector of the Edn1 signal transduction pathway in zebrafish (Walker et al., 2007).

Neither endothelin nor 5-HT2B signaling is necessary in early steps of cranial NCC specification or migration; however, they are both required in postmigratory NCCs in order to drive correct morphogenesis of the first arch (Nair et al., 2007; Ruest and Clouthier, 2009) (the present work). Moreover, like Ednra, 5-HT2B is a Gq-coupled receptor that stimulates PLC (Raymond et al., 2001). Although the 5-HT2B receptor is able to activate various signal transduction pathways (Raymond et al., 2001; Millan et al., 2008), we showed that in developing cranial NCCs PLC activation could represent the 5-HT2B effector and that this transduction pathway is crucial for the development of NCC-derived skeletogenic elements of the mandibular arch.

Since there are currently no data on the function of PLC in the *Xenopus* embryo, we performed a functional analysis of the *Xenopus* ortholog of the *Phospholipase C beta 3* gene. The injection of an MO directed against *PLC* resulted in the loss of the jaw joint and, in some cases, also the loss of the distal part of Meckel's cartilage with variable penetrance. In agreement with this, PLC morphants showed a strong reduction in *Xbap* expression and reduced expression of *XHand2* mRNA in all the pharyngeal arches. Double injection of subthreshold levels of 5-HT2B and PLC MOs demonstrated that the two genes function synergistically. Moreover, the abrogation of the activity of PLC is sufficient to block the effects of 5-HT2B overexpression, demonstrating that PLC is the downstream effector of 5-HT2B signaling.

It is tempting to speculate that endothelin receptor and serotonergic receptor 2B signaling cooperate in reinforcing the PLC pathway and in modulating *Xbap* expression during first arch NCC patterning. However, although endothelin signaling is able to modulate both *Xbap* and *XHand2* gene expression, 5-HT2B signaling seems to be required to maintain *Xbap* expression in the domain of the mandibular arch that gives rise to the jaw joint. In 5-HT2B morphants, in fact, *XHand2* mRNA expression is unaltered and, consequently, the distal part of Meckel's cartilage develops normally. As the regulatory network involved in the specific expression of *Bapx1* in vertebrates is conserved (Newman et al., 1997; Tribioli et al., 1997; Miller et al., 2003; Tucker et al., 2004; Wilson and Tucker, 2004), perhaps 5-HT2B signaling controls *Bapx1* expression in mammalian embryos as well. This aspect, to be verified and further explored, could be of interest particularly in the light of the possible involvement of *BAPX1* in human birth defects, such as those of the oculo-articular-vertebral spectrum, which involve alterations in the first and second branchial arch derivatives (Fischer et al., 2006), and considering the wide spectrum of serotonergic drugs available and commonly used in therapy including during pregnancy.

In conclusion, the main finding of our work is that 5-HT2B receptor signaling is both sufficient and necessary to modulate the shape and functionality of distinct elements of the jaw, including the jaw joint, which is the major evolutionary novelty of vertebrates. We have also provided the first evidence that 5-HT2B might share a transduction cascade with other signaling pathways, via PLC, that is able to define and sustain the *Bapx1/Xbap* gene expression domain in order to shape the mandibular arch skeletal elements. Further comparative studies in fish, frog, chick and mammals could provide a more complete picture of the evolution of the molecular network that acts in mandibular arch morphogenesis.

Acknowledgements

We thank E. J. Bellefroid for *Xenopus Mel1* cDNA; S. J. Smith and T. J. Mohun for *Xenopus Hand2* cDNA; Marina Mione for the RFP construct; Fiorenza De Bernardi, University of Milan, Italy and Luca Zacchigna and Stefano Piccolo, University of Padua, Italy, for kindly hosting in their respective labs E.R. during the move of our laboratory; M. Fabbri, G. De Matienzo and E. Landi for technical assistance; S. Di Maria for frog care; and Paola Casini, Gaia Gestri and Simona Casarosa for helpful comments on the manuscript. This work was financed by grants from the Scuola Normale Superiore of Pisa (Italy) project 2009-10 'Serotonin signaling role during nervous system and neural crest development: embryological and biotechnological approaches' and from MIUR, Rome, Italy, PRIN 2008 project 'Molecular and evolutionary aspects of the neuronal differentiation'.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.041079/-/DC1

References

Andreazzoli, M., Gestri, G., Angeloni, D., Menna, E. and Barsacchi, G. (1999). Role of Xrx1 in Xenopus eye and anterior brain development. *Development* **126**, 2451-2460.

Baltzinger, M., Ori, M., Pasqualetti, M., Nardi, I. and Rijli, F. M. (2005). Hoxa2 knockdown in Xenopus results in hyoid to mandibular homeosis. *Dev. Dyn.* **234**, 858-867.

Berger, M., Gray, J. A. and Roth, B. L. (2009). The expanded biology of serotonin. *Annu. Rev. Med.* **60**, 355-366.

Bhasin, N., La Mantia, A. S. and Lauder, J. M. (2004b). Opposing regulation of cell proliferation by retinoic acid and the serotonin2B receptor in the mouse frontonasal mass. *Anat. Embryol.* **208**, 135-143.

Borchers, A., Epperlein, H. H. and Wedlich, D. (2000). An assay system to study migratory behavior of cranial neural crest cells in Xenopus. *Dev. Genes Evol.* **210**, 217-222.

Brito, J. M., Teillet, M. A. and Le Douarin, N. M. (2008). Induction of mirror-image supernumerary jaws in chicken mandibular mesenchyme by Sonic Hedgehog-producing cells. *Development* **135**, 2311-2319.

Charité, J., McFadden, D. G., Merlo, G., Levi, G., Clouthier, D. E., Yanagisawa, M., Richardson, J. A. and Olson, E. N. (2001). Role of Dlx6 in regulation of an endothelin-1-dependent, dHAND branchial arch enhancer. *Genes Dev.* **15**, 3039-3049.

Choi, D. S., Ward, S. J., Messaddeq, N., Launay, J. M. and Maroteaux, L. (1997). 5-HT2B receptor-mediated serotonin morphogenetic functions in mouse cranial neural crest and myocardiac cells. *Development* **124**, 1745-1755.

Côté, F., Fligny, C., Bayard, E., Launay, J. M., Gershon, M. D., Mallet, J. and Vodjdani, G. (2007). Maternal serotonin is crucial for murine embryonic development. *Proc. Natl. Acad. Sci. USA.* **104**, 329-334.

Couly, G., Creuzet, S., Bennaceur, S., Vincent, C. and Le Douarin, N. M. (2002). Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head. *Development* **129**, 1061-1073.

De Lucchini, S., Ori, M., Cardini, M., Marracci, S. and Nardi, I. (2003). Expression of 5-HT2B and 5-HT2C receptor genes is associated with proliferative regions of Xenopus developing brain and eye. *Brain Res. Mol. Brain Res.* **115**, 196-201.

De Lucchini, S., Ori, M., Cremisi, F., Cardini, M. and Nardi, I. (2005). 5-HT2B-mediated serotonin signaling is required for eye morphogenesis in Xenopus. *Mol. Cell. Neurosci.* **29**, 299-312.

Fischer, S., Lüdecke, H. J., Wieczorek, D., Böhringer, S., Gillessen-Kaesbach, G. and Horsthemke, B. (2006). Histone acetylation dependent allelic expression imbalance of BAPX1 in patients with the oculo-auriculo-vertebral spectrum. *Hum. Mol. Genet.* **15**, 581-587.

Fukumoto, T., Kema, I. P. and Levin, M. (2005). Serotonin signaling is a very early step in patterning of the left-right axis in chick and frog embryos. *Curr. Biol.* **15**, 794-803.

Gaspar, P., Cases, O. and Maroteaux, L. (2003). The developmental role of serotonin: news from mouse molecular genetics. *Nat. Rev. Neurosci.* **4**, 1002-1012.

Hanken, J., Klymkowsky, M. W., Alley, K. E. and Jennings, D. H. (1997). Jaw muscle development as evidence for embryonic repatterning in direct-developing frogs. *Proc. Biol. Sci.* **264**, 1349-1354.

Hansson, S. R., Mezey, E. and Hoffman, B. J. (1999). Serotonin transporter messenger RNA expression in neural crest-derived structures and sensory pathways of the developing rat embryo. *Neuroscience* **89**, 243-265.

Harland, R. M. (1991). *In situ* hybridization, an improved whole mount method for *Xenopus* embryos. *Methods Cell Biol.* **36**, 685-695.

Ivey, K., Tyson, B., Ukidwe, P., McFadden, D. G., Levi, G., Olson, E. N., Srivastava, D. and Wilkie, T. M. (2003). Galphaq and Galpha11 proteins mediate endothelin-1 signaling in neural crest-derived pharyngeal arch mesenchyme. *Dev. Biol.* **15**, 230-237.

Köntges, G. and Lumsden, A. (1996). Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. *Development* **122**, 3229-3242.

Lauder, J. M. (1993). Neurotransmitters as growth regulatory signals: role of receptors and second messengers. *Trends Neurosci.* **16**, 233-240.

Lauder, J. M., Wilkie, M. B., Wu, C. and Singh, S. (2000). Expression of 5-HT(2A), 5-HT(2B) and 5-HT(2C) receptors in the mouse embryo. *Int. J. Dev. Neurosci.* **18**, 653-662.

Levin, M. (2004). A novel immunohistochemical method for evaluation of antibody specificity and detection of labile targets in biological tissue. *J. Biochem. Biophys. Methods* **58**, 85-96.

Mayor, R., Morgan, R. and Sargent, M. G. (1995). Induction of the prospective neural crest of Xenopus. *Development* **121**, 767-777.

Millan, M. J., Marin, P., Bockaert, J. and la Cour, C. M. (2008). Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. *Trends Pharmacol. Sci.* **29**, 454-464.

Miller, C. T., Yelon, D., Stainier, D. Y. and Kimmel, C. B. (2003). Two endothelin 1 effectors, hand2 and bapx1, pattern ventral pharyngeal cartilage and the jaw joint. *Development* **130**, 1353-1365.

Miller, C. T., Maves, L. and Kimmel, C. B. (2004). moz regulates Hox expression and pharyngeal segmental identity in zebrafish. *Development* **131**, 2443-2461.

Moiseiwitsch, J. R. (2000). The role of serotonin and neurotransmitters during craniofacial development. *Crit. Rev. Oral Biol. Med.* **11**, 230-239.

Moiseiwitsch, J. R. and Lauder, J. M. (1995). Serotonin regulates mouse cranial neural crest migration. *Proc. Natl. Acad. Sci. USA* **92**, 7182-7186.

Nair, S., Li, W., Cornell, R. and Schilling, T. F. (2007). Requirements for Endothelin type-A receptors and Endothelin-1 signaling in the facial ectoderm for the patterning of skeletogenic neural crest cells in zebrafish. *Development* **134**, 335-345.

Narboux-Nême, N., Pavone, L. M., Avallone, L., Zhuang, X. and Gaspar, P. (2008). Serotonin transporter transgenic (SERTcre) mouse line reveals developmental targets of serotonin specific reuptake inhibitors (SSRIs). *Neuropharmacology* **55**, 994-1005.

Nebigil, C. G. and Maroteaux, L. (2001). A novel role for serotonin in heart. *Trends Cardiovasc. Med.* **11**, 329-335.

Nebigil, C. G., Choi, D. S., Dierich, A., Hickel, P., Le Meur, M., Messaddeq, N., Launay, J. M. and Maroteaux, L. (2000a). Serotonin 2B receptor is required for heart development. *Proc. Natl. Acad. Sci. USA.* **97**, 9508-9513.
Newman, C. S., Grow, M. W., Cleaver, O., Chia, F. and Krieg, P. (1997). Xbap, a vertebrate gene related to bagpipe, is expressed in developing craniofacial structures and in anterior gut muscle. *Dev. Biol.* **181**, 223-233.
Nieuwkoop, P. D. and Faber, J. (1956). *Normal Table of Xenopus laevis (Daudin), a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis.* Amsterdam: North-Holland.
Papalopulu, N. and Kintner, C. (1993). *Xenopus Distal-less* related homeobox genes are expressed in the developing forebrain and are induced by planar signals. *Development* **117**, 961-975.
Pasqualetti, M., Ori, M., Nardi, I. and Rijli, F. M. (2000). Ectopic Hoxa2 induction after neural crest migration results in homeosis of jaw elements in Xenopus. *Development* **127**, 5367-5378.
Raymond, J. R., Mukhin, Y. V., Gelasco, A., Turner, J., Collinsworth, G., Gettys, T. W., Grewal, J. S. and Garnovskaya, M. N. (2001). Multiplicity of mechanisms of serotonin receptor signal transduction. *Pharmacol. Ther.* **92**, 179-212.
Reisoli, E., De Lucchini, S., Anelli, T., Biagioni, S., Nardi, I. and Ori, M. (2008). Overexpression of 5-HT2B receptor results in retinal dysplasia and defective ocular morphogenesis in Xenopus embryos. *Brain Res.* **9**, 32-39.
Rijli, F. M., Gavalas, A. and Chambon, P. (1998). Segmentation and specification in the branchial region of the head: the role of the Hox selector genes. *Int. J. Dev. Biol.* **42**, 393-401.
Ruest, L. B. and Clouthier, D. E. (2009). Elucidating timing and function of endothelin-A receptor signaling during craniofacial development using neural crest cell-specific gene deletion and receptor antagonism. *Dev. Biol.* **328**, 94-108.
Sadaghiani, B. and Thiébaud, C. H. (1987). Neural crest development in the Xenopus laevis embryo, studied by interspecific transplantation and scanning electron microscopy. *Dev. Biol.* **124**, 91-110.
Saltys, H. A., Jonz, M. G. and Nurse, C. A. (2006). Comparative study of gill neuroepithelial cells and their innervation in teleosts and Xenopus tadpoles. *Cell Tissue Res.* **323**, 1-10.
Sato, T., Kurihara, Y., Asai, R., Kawamura, Y., Tonami, K., Uchijima, Y., Heude, E., Ekker, M., Levi, G. and Kurihara, H. (2008). An endothelin-1 switch specifies maxillomandibular identity. *Proc. Natl. Acad. Sci. USA* **105**, 18806-18811.
Sauka-Spengler, T. and Bronner-Fraser, M. (2008). A gene regulatory network orchestrates neural crest formation. *Nat. Rev. Mol. Cell Biol.* **9**, 557-568.
Shuey, D. L., Sadler, T. W. and Lauder, J. M. (1992). Serotonin as a regulator of craniofacial morphogenesis: site specific malformations following exposure to serotonin uptake inhibitors. *Teratology* **46**, 367-378.
Shuey, D. L., Sadler, T. W., Tamir, H. and Lauder, J. M. (1993). Serotonin and morphogenesis. Transient expression of serotonin uptake and binding protein during craniofacial morphogenesis in the mouse. *Anat. Embryol.* **187**, 75-85.
Smith, A., Robinson, V., Patel, K. and Wilkinson, D. G. (1997). The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. *Curr. Biol.* **7**, 561-570.
Smith, S. J., Kotecha, S., Towers, N. and Mohun, T. J. (2000). Xenopus Hand2 expression marks anterior vascular progenitors but not the developing heart. *Dev. Dyn.* **219**, 575-581.
Spokony, R. F., Aoki, Y., Saint-Germain, N., Magner-Fink, E. and Saint-Jeannet, J. P. (2002). The transcription factor Sox9 is required for cranial neural crest development in Xenopus. *Development* **129**, 421-432.
Strähle, U., Blader, P., Adam, J. and Ingham, P. W. (1994). A simple and efficient procedure for non-isotopic in situ hybridization to sectioned material. *Trends Genet.* **10**, 75-76.
Trainor, P. and Krumlauf, R. (2000). Patterning the cranial neural crest: hindbrain segmentation and Hox gene plasticity. *Nat. Rev. Neurosci.* **1**, 116-124.
Tribioli, C., Frasch, M. and Lufkin, T. (1997). Bapx1: an evolutionary conserved homologue of the Drosophila bagpipe homeobox gene is expressed in splanchnic mesoderm and the embryonic skeleton. *Mech. Dev.* **65**, 145-162.
Tucker, A. S., Watson, R. P., Lettice, L. A., Yamada, G. and Hill, R. E. (2004). Bapx1 regulates patterning in the middle ear: altered regulatory role in the transition from the proximal jaw during vertebrate evolution. *Development* **131**, 1235-1245.
Van Campenhout, C., Nichane, M., Antoniou, A., Pendeville, H., Bronchain, O. J., Marine, J. C., Mazabraud, A., Voz, M. L. and Bellefroid, E. J. (2006). Evi1 is specifically expressed in the distal tubule and duct of the Xenopus pronephros and plays a role in its formation. *Dev. Biol.* **294**, 203-219.
Van Mier, P., Joosten, H. W., Van Rheden, R. and Ten Donkelaar, H. J. (1986). The development of serotonergic raphespinal projections in Xenopus laevis. *Int. J. Dev. Neurosci.* **4**, 465-475.
Walker, M. B., Miller, C. T., Swartz, M. E., Eberhart, J. K. and Kimmel, C. B. (2007). Phospholipase C, beta 3 is required for Endothelin1 regulation of pharyngeal arch patterning in zebrafish. *Dev. Biol.* **304**, 194-207.
Wilson, J. and Tucker, A. S. (2004). Fgf and Bmp signals repress the expression of Bapx1 in the mandibular mesenchyme and control the position of the developing jaw joint. *Dev. Biol.* **266**, 138-150.
Yavarone, M. S., Shuey, D. L., Sadler, T. W. and Lauder, J. M. (1993). Serotonin uptake in the ectoplacental cone and placenta of the mouse. *Placenta* **14**, 149-161.
Ziermann, J. M. and Olsson, L. (2007). Patterns of spatial and temporal cranial muscle development in the African clawed frog *Xenopus laevis* (Anura: pipidae). *J. Morphol.* **268**, 791-804.