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

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**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 rostrocaudal 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; Trainer 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 (Coulby 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 (Yavaron 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; Moisiewitsch, 2000). As shown in a variety of in vitro models, 5-HT has also been implicated in NCC migration (Moisiewitsch 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 development and evolution of the vertebrate mandibular arch.

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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 development 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 morpholin antisense oligonucleotides (MOs) were designed against the 5-HT2B mRNA (GenBank accession AY498911): 5-HT2B-MO1 (5’-GTTGCGAGGAATGGAGATTGTGCT-3’), which is complementary to nucleotides –28 to –4; and 5-HT2B-MO2 (5’-AAT-AAGAAGGTGAGAGTTGCACT-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’-CCAGGCCGTGCCCCGCAGTTGTT-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’-CAGGCCTGGCGCCGATCTT-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- to 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 MgCl2, 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), XDIld (Papalopulu and Kintner, 1993), XEphrinb2 (Smith et al., 1997), XHand2 (Smith et al., 2000), XMe11 (Van Campenhout et al., 2006), XStag (Mayor et al., 1995) and XSox9 (Spokony et al., 2002). Fluorescein (FLUO) (Roche) labeled antisense RNA probes were also generated for XSox9 and XHlx2 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.

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-branchial 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) (Sadaghihani 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...
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 \textit{GFP} or \textit{n-\beta-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 muscles 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).

\textbf{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 \textit{XSlug} and \textit{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 \textit{XSox9} and that of \textit{XHoxa2}, which is a second 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 \textit{Xbap} [the \textit{Xenopus} ortholog of \textit{bagpipe} in \textit{Drosophila} and of \textit{Bapx1} (\textit{Nkx3.2}) in vertebrates] to label the palatoquadrate
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-F), as was the expression of XMel1 in the third and fourth branchial arches (Fig. 3F).

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...
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’).

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%
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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 hyoangularis (Ha) and quadratoangularis (Qa) muscles (arrow in B″).

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

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

(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″).

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
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. S8-F-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 growth and development.
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, Xhap, 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 Xhap expression. In addition, we showed that the ectopic Xhap-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 muscles could lose the capacity to attach themselves to the dorsal part of the branchial basket because it has acquired ventral characteristics in 5-HT2B-overexpressing 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).

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 XMe11, appeared to be normal. Moreover, in 5-HT2B-overexpressing embryos, the expression of XHand2 was not duplicated in the first branchial arch.

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.

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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 Xhap 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 Xhap/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 Xhap 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 hoid NC 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 quardrate 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 Xhap mRNA expression in pre-differentiating NCCs, confirming a positive regulation of Xhap 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., 2009) (the present work). 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 Xhap 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 Xhap expression during first arch NCC patterning. However, although endothelin signaling is able to modulate both Xhap and XHand2 gene expression, 5-HT2B signaling seems to be required to maintain Xhap 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 vertebrae 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 vertebrae. 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/Xhap 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.

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