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Published online: 18 Jan 2017.

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Serotonin signaling contribution to an evolutionary success: the jaw joint of vertebrates

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Abstract

Serotonin (5-HT) is an ancient molecule that appeared very early during evolution, and it is present in different phyla. The 5-HT signaling system includes several G-coupled receptors and it is widely conserved in vertebrates. 5-HT is implicated in an astonishing number of biological processes and it has a key role as a morphogen in several complex networks during development before it can act as a neurotransmitter. Recent advances on how serotonin signaling can influence early development and its role in vertebrate morphogenesis come from mice and *Xenopus*. The emergence of jawed vertebrates (gnathostomes) from jawless vertebrates (agnathans) represents a major event in the evolution of vertebrates. The acquisition of a jaw is assumed to have occurred after the split between gnathostomes and jawless vertebrates. A crucial question concerns what changes were introduced in the developmental patterning programme to obtain a jaw joint that is one of the most innovative inventions in the history of vertebrates. Molecular and developmental studies performed in *Xenopus* revealed for the first time that serotonin, through the 5-HT2B receptor signaling, is both sufficient and necessary to modulate the shape and functionality of the jaw, including the jaw joint. Accordingly, serotonin can be added to the complex interactive network of extrinsic factors that regulates mandibular arch development, thus contributing to one of the major vertebrate successes in evolution.

Keywords: *5-HT2B, craniofacial morphogenesis, jaw joint, serotonin, gnathostomes evolution*

Introduction

The emergence of jawed vertebrates (gnathostomes) from jawless vertebrates (agnathans) represents a major event in the evolution of vertebrates. This transition was accompanied by many morphological and functional innovations such as jaws, paired appendages and an adaptive immune system. In particular, shaping the first branchial arch into distinct elements of the jaws articulated by a jaw joint allowed the acquisition of a buccal skeleton: this was the major evolutionary novelty of vertebrates that shifted from a lifestyle of passive filter feeding to one of active predation (Gans & Northcutt 1983). This event is in large part responsible for the high variation and diversification within gnathostomes, characterised by elaborate and diverse oral structures while remaining adherent to a common basic plan.

There are still living jawless vertebrates, called cyclostomes, including lampreys and hagfishes, and recent molecular phylogenetic data support monophyly of this group (Kuraku 2008). Even if cyclostomes do not have a jaw, the mandibular arch can be identified as such in both lamprey and hagfish and it exhibits a differentiated and specific morphology. Thus the acquisition of a jaw is assumed to have taken place in the lineage of gnathostomes after the split between gnathostomes and cyclostomes (reviewed by Kuratani & Ota 2008).

The vertebrate skull is divided into two components, the neurocranium and viscerocranium (Portman 1969). The viscerocranium comprises anteroposteriorly segmented structures, the pharyngeal arch (PA) skeletons, which are derived from the neural crest cells (NCCs), a migratory and multipotent cell population

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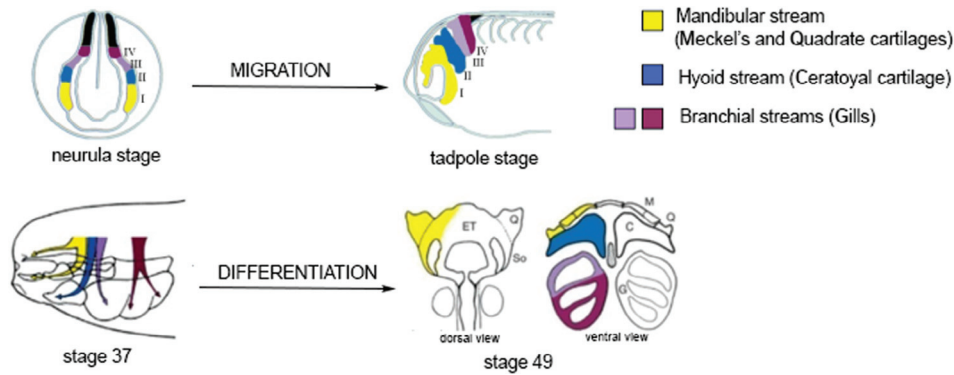
Figure 1. Craniofacial skeleton development in *Xenopus laevis*. Cranial neural crest cells at the neurula stage are subdivided into four streams (see colour code) that migrate into branchial pouches. At the late tailbud stage NCCs start to differentiate, to give rise to the primordial facial skeleton in larval stages. C = ceratohyal; Et = ethmoidal plate; G = gills; M = Meckel's; Q = quadrate; So = subocular cartilage. Modified from Sadaghiani and Thiébaud (1987).

unique to vertebrates (reviewed in Nikitina et al. 2009). Skeletogenic neural crest-derived cells, originating at the border of the developing midbrain and hindbrain, migrate in discrete streams into the pharyngeal pouches, bulging paired segments that are located along the ventral side of the developing embryo (Figure 1). In the first vertebrates the pharyngeal skeleton was simple, consisting of unjointed cartilaginous rods, while in the gnathostome lineage these rods were modified to form distinct cartilages connected by joint tissue. In particular, in the rostralmost PA or mandibular arch articulating dorsal and ventral cartilages form the palatoquadrate and Meckel's elements of the jaws. How the gill bars of the first vertebrates gave rise to the jointed gnathostome jaws is one of the major questions in vertebrate evolution. In the last few years, developmental studies have provided insights into this vertebrate novelty at both molecular and cellular levels.

Patterning the branchial arches

Anterior-posterior patterning

The patterning of the pharyngeal arches involves the progressive partitioning of neural crest-derived skeletal precursors into distinct subpopulations along the anterior–posterior (AP) and dorsal–ventral (DV) axes through the action of several molecules and signaling pathways. It is generally accepted that the morphological AP differentiation of each arch is provided by the nested expression pattern of Hox genes (termed the Hox code) (Minoux & Rijli 2010). Interestingly, while the nested expression patterns of Hox genes are critical for the AP identity of the hyoid and branchial arches, the mandibular arch is specified by the absence of Hox transcripts. This developmental state is known as the "Hox code-default state". Extensive

studies in mice and *Xenopus laevis* (Daudin, 1802) have led to the theory that the Hox gene expression (primarily from the Hoxa cluster) acts on a ground state patterning programme of mandibular type, which exists within all arches (reviewed in Minoux & Rijli 2010). A differential Hox gene expression would then provide patterning cues to this "mandibular code" in order to drive the differentiation of the posterior arches. As an example, target mutagenesis in the mouse and morpholino-induced knockdown in *Xenopus* have shown a crucial role of Hoxa2 for the hyoid arch patterning: in both cases embryos display a homeotic anterior transformation phenotype, i.e. the hyoid derivatives are missing and have been replaced by a set of mandibular arch-like derivatives (Rijli et al. 1993; Baltzinger et al. 2005). Conversely, in both *Xenopus* and chick embryos, ectopic Hoxa2 expression in the mandibular arch suppresses jaw formation and yields a reverse homeotic transformation of mandibular elements towards hyoid morphology, confirming a role of Hoxa2 as a key determinant of hyoid identity (Grammatopoulos et al. 2000; Pasqualetti et al. 2000). Indeed, targeted deletion of the Hoxa cluster in mice leads to mandibular arch-like structures also in the posterior arches, further supporting the hypothesis of a "mandibular code" retained by all the arches (Minoux et al. 2009). Modern agnathans are thought to have a structure of Hox gene clusters similar to that of gnathostomes. Interestingly, molecular studies in lamprey indicate that the branchial Hox code is partially conserved, including a Hox-free first arch. Thus, as in gnathostomes, the mandibular arch of lampreys is specified by the Hox code default state, suggesting that Hox genes have not been involved in the evolution of the jaw apparatus (Murakami et al. 2004).

Dorsal-ventral patterning

Each pharyngeal arch acquires distinct identities, critical for skeletal shaping, also along the DV axis. It has been proposed that the DV specification of branchial arches is mediated by another nested expression of homeobox genes, the Dlx1–Dlx6 genes (Dlx code). The term "Dlx code" was coined in accordance with the Hox code that specifies AP identity, and the craniofacial patterning of the gnathostomes was generally thought to depend on a sort of Cartesian pattern of Hox/Dlx codes that provides specific combinations of homeobox gene transcripts in each neural crest-derived pharyngeal arch. Although the lamprey also has six Dlx genes, it remains controversial as to the presence or absence of the Dlx code in their embryos (cf. Kuratani 2012). However, more recent research shows that the DV patterning is more complex than a static nested code. In fact, during migration the NCCs encounter signals in the facial microenviroment that influence DV pattern. In particular the ectoderm and endoderm of the pharyngeal pouches are important sources of signaling molecules that influence gene expression. Recent evidence suggests that multiple signaling pathways such as fibroblast growth factor (FGF), endothelin-1 (Edn1), bone morphogenetic protein (BMP) and jagged-notch are needed to pattern skeletal NCCs precursors along the DV axis. These signaling molecules form an integrated network that establishes discrete DV gene expression domains, and act through several families of transcription factors, including Dlx, Msx and Hand, to establish dynamic zones of skeletal differentiation (Clouthier et al. 2010; Medeiros & Crump 2012; Takechi et al. 2013; Jandzik et al. 2014). One of the most-studied signaling pathways in pharyngeal DV patterning is endothelin, a small peptide mainly secreted by the ventral ectoderm of the pouches, that acts on NCCs that express endothelin type A receptors (Ednras) (Clouthier et al. 2010). In the context of the jaws development endothelin signaling appears to be of particular importance. In fact, in zebrafish the abrogation of Ednra receptors results in the loss of the jaw joint (Nair et al. 2007).

Xenopus laevis *as a model system*

In the complex scenario of craniofacial morphogenesis, novel insights into the molecular mechanisms underlying craniofacial development were provided using a genetic manipulation approach in *Xenopus* embryos, which have proven to be a very useful and tractable developmental model system (Ori et al. 2013, 2015) (Figure 2).

The gene loss-of-function strategy, based on the injection of antisense morpholino oligonucleotides (MO), is still a valid way to disrupt a gene function in *Xenopus* embryos. MOs are nucleic acids characterised by morpholine rings, which replace the ribose or deoxyribose moieties. Antisense MOs are targeted to
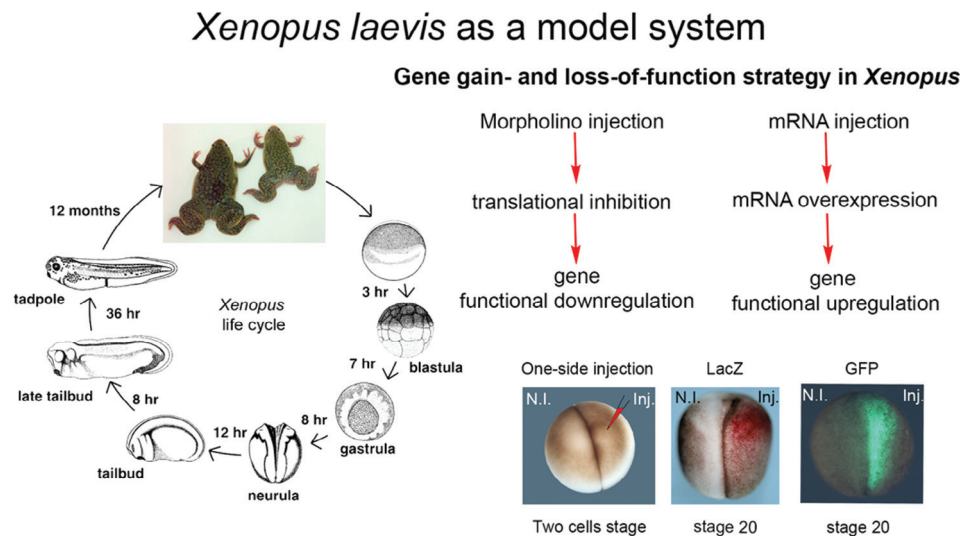

Figure 2. *Xenopus laevis* as a model system to study gene function. On the left, the life cycle of *Xenopus laevis*. On the right, the gene gain- and loss-of-function strategy that allows functional gene gain and loss of function studies in *Xenopus* embryos. The embryo cleavage pattern allows injecting the mRNA in just one side of the embryo, and the injected side can be immediately compared with the control side. Antisense morpholinos are targeted to sequences close to the translation start site to specifically block the translation of a gene of interest. The injected side is visualised by the co-injection of a reporter mRNA such as LacZ or GFP. Examples of chromogenic reaction for beta galactosidase (red) and GFP (green fluorescence) are shown in neural stage embryos. N.I. = non-injected side; Inj. = injected side. Modified from Ori et al. (2013). Reproduced with permission.

sequences close to the translation start site of a gene of interest, specifically blocking its translation by steric hindrance. *Xenopus* embryo cleavage pattern allows injecting MOs in just one side of the embryo, and the injected side can be visualised by co-injection of a reporter gene mRNA. For this unique feature, the injected side can be immediately compared with the control side in the same embryo. Functional up-regulation of a given gene can also be obtained by side-specific mRNA microinjection (Figure 2).

This review reports loss- and gain-of-function genetic manipulations performed in *Xenopus* embryos and how these experiments unveiled some unsuspected actors in the development of craniofacial structures. For the first time the involvement of a serotonin receptor signaling pathway in the development of the mandibular arch, and particularly in the jaw joint formation, was demonstrated.

Serotonin and serotonin receptors in development

Serotonin (5-HT) is a biogenic monoamine that appeared very early in evolution, being present not only in vertebrates and invertebrates but also in plants (cf. Sullivan & Levin 2016). First discovered as the secretory product of enterocromaffin cells ("enteramine", Erspamer & Asero 1952), it has been mostly studied for its role as neurotransmitter in the nervous system as it is involved in the etiology of many human psychiatric disorders, including depression, anxiety, obsessive-compulsive disorder (OCD) and autism (cf. Araragi & Lesch 2013; Benza & Chugani 2015). Serotonin effects are mediated by multiple different receptors. At least 14 receptor subtypes have been identified in mammals and are grouped into seven families (5-HT1-5-HT7) (Hannon & Hoyer 2008).

Since the pioneer studies of Buznikov and his collaborators (reviewed in Buznikov 2007), several laboratories have pursued the idea that 5-HT could function as a humoral morphogen before being co-opted as neurotransmitter in the central nervous system, but only in recent years has such a role been demonstrated by in vivo studies. It is now well accepted that serotonin is involved in the establishment of left–right asymmetry (Fukumoto et al. 2005; Carneiro et al. 2011; Beyer et al. 2012; Vandenberg & Levin 2013), in different cellular processes involved in health and disease (Kubera et al. 2009; Hummerich & Schloss 2010; Blackiston et al. 2011, 2015) and in nervous system development (Migliarini et al. 2013). Accordingly, specific serotonin receptors have been implicated in developmental processes; in particular the 5-HT2B receptor signaling was found to be involved in mouse heart development. In fact, its targeted inactivation leads to embryonic and neonatal death caused by heart defects (Nebigil et al. 2000). Further experiments carried out in the model *Xenopus laevis* have revealed other important and unsuspected roles of this receptor in development such as craniofacial and eye morphogenesis (Reisoli et al. 2010; Ori et al. 2013). Multiple sources of serotonin during *Xenopus* development have been described including maternal serotonin storage in the eggs. Later in embryo development, serotonin is actively produced in the brain and in the gut by serotonergic cells. In *Xenopus* embryos other cells could accumulate serotonin, such as specialised skin cells or amacrine cells in the retina (Ori et al. 2013).

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

A first hint for a possible role of the 5-HT2B signaling in cranio-facial morphogenesis derived from gene gain of function experiments: 5-HT2B overexpression resulted, in fact, in a morphological change of the craniofacial skeleton due to the formation of an ectopic cartilaginous element and by a reduction in the quadrate and subocular cartilages (Figure 3A). Such skeletal alterations were associated with altered muscular connectivity: the orbitohyoideus muscle maintained a correct insertion on the cerathyal but it was anchored to the ectopic cartilage instead of to the reduced quadrate (Figure 3C). Significant information on the origin of the ectopic cartilage derived from the analysis of the gene expression pattern of bap, an important molecular marker of the mandibular arch. bap is the *Xenopus* ortholog of bagpipe in *Drosophila* and of Bapx1 (Nkx3.2) in vertebrates, a gene codifying for a transcription factor expressed in the precursor cells of the jaw joint region (Newman et al. 1997). In 5-HT2B-overexpressing embryos, bap mRNA was ectopically expressed and resembled a mirror-image duplication of the wild-type bap mRNA expression site (Figure 3B). On the whole these findings suggest that the ectopic cartilage derives, at least in part, from the first branchial arch NCCs. Enhanced 5-HT2B signaling is able to influence visceral arch morphogenesis, but does it have a physiological role in craniofacial development? The answer to this question came from a morpholino experimental approach to specifically knock down 5-HT2B function in *Xenopus* embryos. 5-HT2B morphants were characterised by a specific skeletal defect consisting in a hypomorphic quadrate fused with the Meckel's cartilage into a single element, leading to the loss of the jaw joint (Figure 4A, A'). In line with this finding, 5-HT2B *Xenopus* morphants showed a strong reduction of bap mRNA
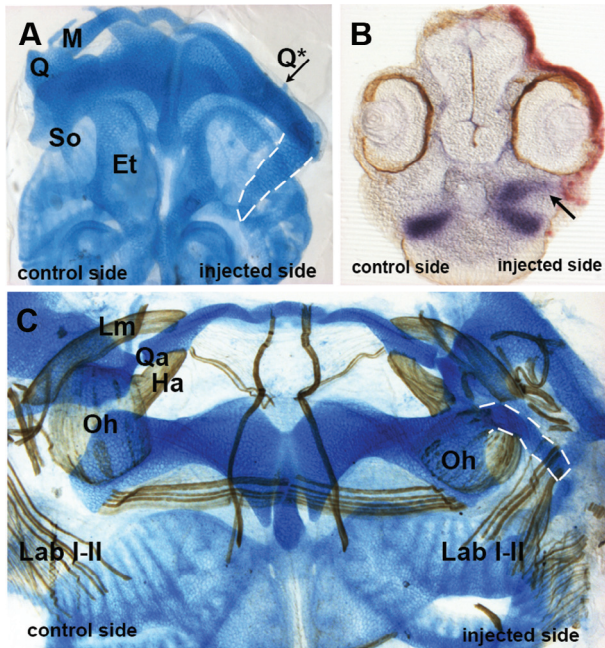
Figure 3. Skeletal and muscular alteration and ectopic expression of branchial arch markers in 5-HT2B-overexpressing tadpoles. A, flat-mount skeleton of a stage 49 injected embryo. Dashed lines showed the ectopic cartilage, black arrow and Q* indicate the reduction of the quadrate (Q). B, vibratome section of a stage 37 *Xenopus* embryo after in situ hybridisation to reveal the bap gene expression pattern. The injected side is on the right. C, the flat-mount of an injected embryo (stage 49) double stained for cartilage (blue) and muscles (brown). Dashed lines indicate the ectopic cartilage. The orbitohyoideus muscle (Oh) is anchored to the ectopic cartilage instead of to the reduced quadrate cartilage. Note the disorganisation of the levatores arcuum branchialum muscles I-II (Lab I-II) on the injected side. Ha = hyangularis; Qa = quadratoangularis; Lm = levator mandibulae; orbitohyoideus (Oh). Modified from Reisoli et al. (2010). Reproduced with permission.
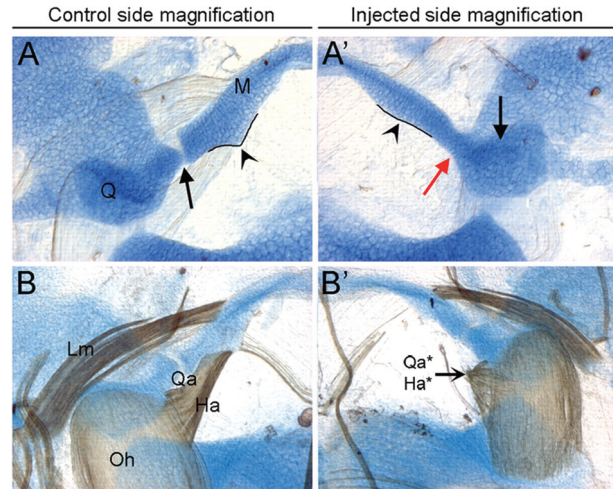

Figure 4. Skeletal and muscular connectivity alterations in 5-HT2B morphants. A, A', flat-mount preparation of an Alcian Blue-stained 5-HT2BMO-injected *Xenopus* embryo. Magnification of the jaw joint region of the control (A) and injected (A') side of the embryo. Note the lack of the jaw joint (red 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, B', flat-mount preparation of a 5-HT2B-MO-injected embryo double stained for cartilage (blue) and muscle (brown). Magnification of the jaw joint region of the uninjected (B) and injected (B') sides of the embryo. Note the abnormal development of the hyangularis (Ha*) and quadratoangularis (Qa*) muscles (arrow in B'). Normal hyangularis (Ha) and quadratoangularis (Qa) muscles (B). Modified from Reisoli et al. (2010). Reproduced with permission.

expression in the first visceral arch. Meckel's cartilage was also altered in shape due to the lack of the muscular process necessary for the attachment of the hyoangularis and quadratoangularis muscles, and its absence resulted in the abnormal development of such muscles, which failed to reach their target cartilage (Figure 4B, B'; Reisoli et al. 2010). The consequence of both the skeletal and the muscular abnormalities was a critical functional impairment of the mouth opening, leading to the death of the tadpole. It is interesting to note that such skeletal alterations of the first arch give rise to an unjointed cartilaginous rod that resembles that one present in the first vertebrates (agnathans). These functional experiments clearly demonstrated a positive regulation of the bap expression by 5-HT2B signaling. The other signaling pathway able to positively control bapx1 expression is endothelin (Edn1), which

induces signaling from its cognate receptor type A (Ednra) (Ivey et al. 2003). The abrogation of Ednra results in down regulation of Bapx1 expression and in the loss of the jaw joint in zebrafish morphants (Nair et al. 2007), a phenotype similar to that observed in 5-HT2B *Xenopus* morphants. Endothelin signaling appears to be conserved among vertebrates and it has been shown that the phospholipase C beta 3 gene (plcb3) is the downstream effector of the Edn1 signal transduction pathway in zebrafish (Walker et al. 2007). A functional analysis of the *Xenopus* ortholog of the Plcb3 gene has shown that it represents the 5-HT2B effector as well, and that this transduction pathway is crucial for regulating bap expression and for the correct development of the jaw joint (Reisoli et al. 2010). These findings suggest that endothelin and 5-HT2B signaling may cooperate in modulating bap expression through the plcb3 pathway during the development of the mandibular arch jaw joint.

So far, evidence has been reported that serotonin and 5-HT2B receptor signaling is required in

craniofacial morphogenesis in both mice and zebra-fish (Moiseiwitsch 2000; Bashammakh et al. 2014) suggesting a conservation of this signaling pathway across vertebrate evolution. Interestingly, selective serotonin reuptake inhibitors (SSRi), the most commonly prescribed drugs for the treatment of depression, when used in pregnancy have been linked to craniofacial and cardiac malformations in both mice and humans (Alwan et al. 2007; Cray et al. 2014; Hanley & Oberlander 2014).

Conclusions

The acquisition of a jaw was a turning point in vertebrate evolution because it allowed primitive vertebrates to capture and process large preys. How the unjointed gill bars of jawless ancestors gave rise to the jointed gnathostome pharyngeal skeleton and jaws is still an open question. However, in the last few years evolutionary developmental studies have tried to explain this novelty at the molecular and cellular level. Recent evidence suggests a scenario for jaw evolution as a progression of changes in the developmental patterning of the rostral-most pharyngeal arch (mandibular arch).

Functional studies performed in a suitable embryogenesis model such as *Xenopus* have revealed for the first time that the serotonin 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. 5-HT2B receptor shares a transduction cascade, via plcb3, with the endothelin pathway, and possibly these two signaling pathways cooperate to define and sustain bap gene expression domains in order to shape the mandibular arch skeletal elements. This serotonin signaling pathway appears to be conserved across vertebrate evolution. Accordingly, serotonin can be added to the complex interactive network of extrinsic factors that regulates mandibular arch morphogenesis, thus contributing to one of the major vertebrate successes in evolution. Serotonin is an ancient molecule that has been used and readapted several times to different purposes. Incidentally, evidence suggests the contribution of serotonin and its receptors to both the innate and adaptive responses, another vertebrate innovation (Baganz & Blakely 2013). These data further confirm that during evolution serotonin has played many roles as a morphogen-like signal in different developmental programmes.

Acknowledgements

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