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Serotonin and its metabolism in basal deuterostomes: insights from *Strongylocentrotus purpuratus* and *Xenoturbella bocki*

Leah N. Squires[1], Stanislav S. Rubakhin[1], Andinet Amare Wadhams[1], Kristen N. Talbot[1], Hiroaki Nakano[2,*], Leonid L. Moroz[3] and Jonathan V. Sweedler[1,†]

[1]Department of Chemistry and the Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA, [2]Department of Marine Ecology–Kristineberg, University of Gothenburg, Kristineberg 566, 450 34 Fiskebäckskil, Sweden and [3]Department of Neuroscience and the Whitney Laboratory for Marine Bioscience, University of Florida, Gainesville and St Augustine, FL 32080, USA

*Present address: Shimoda Marine Research Center, University of Tsukuba, Shimoda, Shizuoka, 415-0025, Japan
†Author for correspondence (jsweedle@illinois.edu)

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SUMMARY

Serotonin (5-HT), an important molecule in metazoans, is involved in a range of biological processes including neurotransmission and neuromodulation. Both its creation and release are tightly regulated, as is its removal. Multiple neurochemical pathways are responsible for the catabolism of 5-HT and are phyla specific; therefore, by elucidating these catabolic pathways we glean greater understanding of the relationships and origins of various transmitter systems. Here, 5-HT catabolic pathways were studied in *Strongylocentrotus purpuratus* and *Xenoturbella bocki*, two organisms occupying distinct positions in deuterostomes. The 5-HT-related compounds detected in these organisms were compared with those reported in other phyla. In *S. purpuratus*, 5-HT-related metabolites include *N*-acetyl serotonin, γ-glutamyl-serotonin and 5-hydroxyindole acetic acid; the quantity and type were found to vary based on the specific tissues analyzed. In addition to these compounds, varying levels of tryptamine were also seen. Upon addition of a 5-HT precursor and a monoamine oxidase inhibitor, 5-HT itself was detected. In similar experiments using *X. bocki* tissues, the 5-HT-related compounds found included 5-HT sulfate, γ-glutamyl-serotonin and 5-hydroxyindole acetic acid, as well as 5-HT and tryptamine. The sea urchin metabolizes 5-HT in a manner similar to both gastropod mollusks, as evidenced by the detection of γ-glutamyl-serotonin, and vertebrates, as indicated by the presence of 5-hydroxyindole acetic acid and *N*-acetyl serotonin. In contrast, 5-HT metabolism in *X. bocki* appears more similar to common protostome 5-HT catabolic pathways.

Key words: indoleamine, capillary electrophoresis, neurotransmitters, catabolism.

INTRODUCTION

Serotonin (5-hydroxytryptamine or 5-HT), one of the earliest identified signaling molecules, is widely distributed across various systematic groups. In addition, 5-HT and the related auxins may even be involved in cell-to-cell communication in plants and unicellular eukaryotes (Azmitia, 1999; Azmitia, 2007; Garattini and Valzelli, 1965; Pasternak et al., 2005). Shown to function as a classical neurotransmitter, neuromodulator and trophic factor in well-investigated animal phyla, 5-HT controls a wide array of somatic and visceral functions. The degree of scientific interest in the compound is evidenced by more than 100,000 publications on 5-HT since its discovery ~50 years ago.

Worth noting is that the visceral and non-neuronal functions of 5-HT are likely conserved across phyla. Even within mammals, ~95% of 5-HT is found in the enteric nervous system, platelets and skin, with smaller amounts found in the brain (Kim and Camilleri, 2000; Squires et al., 2006). It also plays an important role in early development and embryonic homeostasis (Buznikov et al., 1993; Buznikov et al., 1996; Buznikov et al., 1999; Emanuelsson, 1992; Emanuelsson et al., 1988). In echinoderms, for example, the oocytes of starfish contain 5-HT as well as surface receptors for the compound; later in development, it is thought to function as a modulator for maturation hormones and has been found in the zygotes and blastomeres of starfish as well as in adults. In mollusks, 5-HT is also found in oocytes and participates in various stages of development ranging from fertilization to gastrulation and neurulation (Buznikov et al., 1999). In cuttlefish, *Sepia* oviductal concentrations of 5-HT are directly related to contractions of the oviduct in the release of eggs for fertilization (Zatylny et al., 2000). In vertebrates, 5-HT is present in the oocytes of amphibians, which contain both surface and intracellular receptors for the compound. It is linked to the regulation of a number of developmental stages in amphibians, not only in gastrulation and neurulation but also in neurotransmission after uptake into neural tube cells (Buznikov et al., 1993; Buznikov et al., 1996; Trandaburu and Trandaburu, 2007).

Not surprisingly, less is known about comparative biochemical aspects of 5-HT signaling in the majority of phyla. Although the synthesis of 5-HT is well conserved among species, its fate after release is not. Indeed, one important physiological process by which the levels of any compound in a living organism are affected is catabolism. Catabolic pathways for 5-HT show considerable differences among studied metazoan groups, including mollusks, insects and mammals (Paxon et al., 2005; Squires et al., 2006; Stuart et al., 2003).

The major 5-HT metabolic pathways are illustrated in Fig. 1. Although there are multiple catabolic pathways, it is unclear how their presence or absence is linked to phylogenetic or functional constraints in a given animal lineage. By far the predominant 5-HT degradation pathway in mammals is the monoamine oxidase (MAO) pathway, which results in the production of 5-hydroxyindole acetic

acid (5-HIAA). However, it seems that this pathway may not be dominant in mollusks or arthropods. Secondary pathways in mammals produce *N*-acetyl serotonin (NAS), melatonin, 5-hydroxyindole thiazolidine carboxylic acid (5-HITCA) and, in some cases, 5-HT sulfate (Squires et al., 2006; Squires et al., 2007). Conversely, in mollusks (e.g. *Pleurobranchaea* and *Aplysia*), 5-HT sulfate and γ-glutamyl-serotonin (γ-Glu 5-HT) are major 5-HT metabolic products (Fuller et al., 1998; Hatcher et al., 2008; Stuart et al., 2004; Stuart et al., 2003). γ-Glu 5-HT is also the predominant serotonin metabolic product in the earthworm *Lumbricus terrestris* (Sloley, 1994).

Melatonin has also been detected in gastropod mollusks but apparently it is less abundant than other metabolites (Abran et al., 1994; Waissel et al., 1999). Interestingly, several reports suggest the presence of 5-HIAA in gastropods [e.g. *Aplysia*, *Tritonia*, *Helix* and *Lymnaea* (Fickbohm et al., 2001; Michaelidis et al., 2002; Singh and Agarwal, 1984; Stuart et al., 2003)], but a putative catabolic enzyme ('molluskan MAO') has not yet been found among existing genomic and transcriptomic resources (Moroz et al., 2006).

In insects, two principal metabolites of 5-HT have been detected, with NAS apparently being the major degradation product in a diverse group of insect species (Macfarlane et al., 1990; Paxon et al., 2005; Sloley and Downer, 1984; Sparks and Geng, 1992). As is the case in mollusks, the major mammalian 5-HT metabolite 5-HIAA has also been reported in insects (Barreteau et al., 1991; Kaufman and Sloley, 1996; Rubio et al., 1983; Sparks and Geng, 1992). These examples suggest that there may be different 5-HT metabolic pathways in distinct animal lineages. Of course, the observed presence (or absence) of selected degradation pathways might well reflect limited sampling techniques or measurement capabilities. In fact, 5-HT pathways have been investigated well in only three animal classes (vertebrates, gastropods and insects) and little is known about the neurochemical pathways in many sister groups.

We focus here upon the analysis of 5-HT metabolism in marine invertebrates representing two basal deuterostome lineages, the purple sea urchin, *Strongylocentrotus purpuratus* Stimpson 1857 (the phylum Echinodermata), and *Xenoturbella bocki* Westblad 1949 [the newly established phylum Xenoturbellida (Bourlat et al., 2006)]. By examining the serotonin systems of invertebrate deuterostomes it is possible to gain insight into the evolution of these systems and shed light on several apparently less-used pathways for 5-HT catabolism, such as sulfation in mammals.

Several factors contribute to making *S. purpuratus* an interesting model for the study of 5-HT metabolism. Specifically, its important positioning in the evolutionary tree, unusual nervous system organization, the essential role it plays in the investigation of fundamental developmental mechanisms and the availability of its genomic information (Burke et al., 2006). The distribution and levels of serotonin and serotonin-like compounds in the embryos of sea urchins have been reported previously (Buznikov et al., 2005; Manukhin et al., 1981; Morikawa et al., 2001; Renaud et al., 1983; Shmukler and Tosti, 2001). Surprisingly, however, little research has focused on the 5-HT pathways in adult sea urchins.

A second deuterostome of interest in the study of serotonergic system evolution is *X. bocki*, an organism that has intrigued and puzzled scientists as to its proper phylogenic placement since it was first discovered in 1949 (Westblad, 1949). This marine worm-like organism has many features that can be described as ancestral characteristics for bilaterial animals. For example, it has no through gut, gonads or anus (Telford, 2008) and its nervous system is composed of a nerve net without a distinct brain (Raikova et al., 2000; Westblad, 1949). Originally classified within flatworms, a relationship with bivalve mollusks was subsequently suggested (reviewed in Bourlat et al., 2008; Bourlat et al., 2003). Later it was found that samples were contaminated with molluskan eggs and the organism was placed in a phylum of its own, Xenoturbellida, within the superphylum Deuterostomia (Bourlat et al., 2006). More recently, a genome-wide survey confirmed this distinct phylogenetic position of Xenoturbellida (Bourlat et al., 2009; Dunn et al., 2008; Philippe et al., 2009; Telford, 2008); however, some studies imply that it may be an even more basal lineage within the bilaterian superclade (Hejnol et al., 2009).

Employing a highly specialized capillary electrophoresis (CE) system with laser-induced fluorescence detection (LIF) (Fuller et al., 1998; Zhang et al., 2001), we examined the 5-HT catabolic pathways of adult sea urchins and *X. bocki*, relating these to known molluskan, arthropod and vertebrate pathways. This system has been proven to be well suited for the detection of both known and unknown indoles (Squires et al., 2006; Stuart et al., 2004; Stuart et al., 2003). This study shows that in different locations of the urchin and *X. bocki* nerves, rectal tissues and gonads, catabolism of 5-HT occurs *via* pathways similar to those found in the previously investigated groups.

MATERIALS AND METHODS

Capillary electrophoresis

A laboratory-assembled CE instrument with LIF detection, as previously described (Fuller et al., 1998; Park et al., 1999; Zhang et al., 2001), was used for this study. Briefly, approximately 3 nl of sample was electrokinetically (2.5 V) injected onto a capillary with an inner diameter of 50 μm and an outer diameter of 150 μm. After separation *via* CE, analyte molecules were excited in a sheath flow cuvette by 257 nm radiation from a frequency-doubled argon-ion laser. The native fluorescence of the molecules was then collected at a 90 deg angle, focused onto a spectrograph, and detected using a charge-coupled device to obtain a complete emission profile. Identification of the compounds was based on migration time and comparison of the fluorescence with that of known standards. Quantification was based on a calibration curve using the fluorescence intensities of known standard concentrations.

Sea urchin experimental procedures

Materials

All reagents were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA) at analytical grade or higher with no additional purification unless otherwise stated. Artificial seawater (ASW) was made as follows: NaCl (460 mmol l^{-1}), KCl (10 mmol l^{-1}), $CaCl_2$ (10 mmol l^{-1}), $MgCl_2$ (22 mmol l^{-1}), $MgSO_4 \cdot 7H_2O$ (26 mmol l^{-1}) and Hepes (10 mmol l^{-1}), pH 7.8. Borate running buffer for the CE and extraction solutions was prepared by dissolving 3.0 g of boric acid (H_3BO_3) and 9.2 g of sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) in 1.0 l of ultrapure water (Siemens water filtration system, Siemens Water Technologies, Warrendale, PA, USA).

Tissue extraction

Adult sea urchins (~5 cm body diameter) were collected off the California shore by Charles Hollahan (Santa Barbara Marine Biologicals, Santa Barbara, CA, USA). Sample isolation was guided by the description of sea urchin anatomy as previously described (Sherman and Sherman, 1970). Animals were anesthetized by injection of 20 ml of a $MgCl_2$ solution isotonic to ASW into the main body cavity and rectal, gonadal and nervous tissues surgically

dissected. Tissues were then placed in 1 µl mg^{-1} by wet mass of an extraction solution containing 49.5% borate buffer at pH 8.8, 49.5% MeOH and 1% acetic acid. Tissues remained in the extraction solution at room temperature for 90 min, after which the extraction medium was removed and frozen at –20°C until analysis *via* CE–LIF within 6 h. Trials were repeated four times.

5-HT incubation

Rectal tissues and radial nerves were removed from adult sea urchins and the tissues homogenized in ASW to a final density of 0.097 g ml^{-1} by wet mass. For heat-treated samples, homogenate was placed in a vial heater at 95°C for 10 min; 50 µl of homogenate was then combined with 50 µl of 0.4 mmol l^{-1} serotonin creatine sulfate solution. Incubations took place at room temperature, protected from light for 1 h. Samples were then centrifuged at 10,600 ***g*** at 25°C for 10 min and frozen at –20°C until analysis of supernatant by CE–LIF within 6 h. Trials were repeated four times.

5-HTP incubation

Radial nervous and gonadal tissues were placed separately in an incubation solution (5 µl mg^{-1} by wet mass) of 0.8 mmol l^{-1} 5-hydroxy tryptophan (5-HTP) and 0.8 mmol l^{-1} clorgyline in ASW. Tissues remained in this incubation solution for 1 h at room temperature, protected from light. After 1 h the incubation solution was removed, tissues were rinsed twice with ASW and then placed in 1 µl mg^{-1} of the previously described extraction solution. Tissues remained in extraction media for 90 min, after which the extraction solution was removed and frozen at –20°C until analysis *via* CE–LIF within 6 h. Trials were repeated four times.

5-HT and clorgyline incubation

For this set of experiments, incubations were done in a manner similar to the 5-HTP and clorgyline incubations, except that the incubation solution contained 0.8 mmol l^{-1} of 5-HT and 0.8 mmol l^{-1} of clorgyline in ASW. Trials were repeated three times.

Enzyme search

We searched the sea urchin protein database, downloaded from the NCBI ftp server (ftp://ftp.ncbi.nih.gov/genomes/Strongylocentrotus_purpuratus/protein/), using the names of known enzymes involved in serotonin metabolism in other species.

Xenoturbella bocki experimental procedures

Xenoturbella bocki were collected from muddy sediment in the vicinity of Sven Lovén Centre for Marine Sciences–Kristineberg in the Gullmarsfjord on the west coast of Sweden. The sediment was sieved through a millimetre sieve and individual specimens collected as described previously (Stach et al., 2005; Telford, 2008). Unless otherwise stated, all reagents were obtained from Sigma-Aldrich at analytical grade or higher, without additional purification. The ASW and borate running buffer were prepared as described above.

Tissue extraction and incubation

Animals were separated into three regions, anterior, posterior and middle, with each section being of about equal length. The tissues were then placed in 20–50 µl of an acidified methanol extraction solution containing 49.5% borate buffer, 49.5% methanol and 1% acetic acid for 90 min at room temperature in order to extract the compounds of interest. Next, the tissue was removed from the extraction solution and the solution was frozen until it was analyzed *via* CE–LIF within 48 h of the original dissection.

For the tissue incubation with 5-HTP, animals were separated into four regions (one anterior, two middle and one posterior). These pieces were then cut in half to form control and experimental matched sets. Experimental samples were placed in 10–40 µl of a combination solution of 0.4 mmol l^{-1} dihydroxyphenylalanine (L-DOPA) and 0.4 mmol l^{-1} 5-HTP for 1 h at +4°C while control samples were placed in an equal volume of ASW. Tissues were then removed from the incubation solution and rinsed with filtered seawater from Skagerrak on the Swedish west coast in order to remove as much of the incubation compounds as possible. Next, the tissue was placed in 10–20 µl of the acetified methanol extraction solution containing 49.5% water, 49.5% methanol and 1% acetic acid for 90 min. After extraction, tissues were removed from the media and media was frozen until analysis by CE–LIF, which was completed within 48 h of the original dissection.

RESULTS

5-HT metabolism in sea urchin tissues

The 5-HT catabolic pathways in *S. purpuratus* were investigated to determine their similarities and differences to known pathways in other animals. By sampling the compounds in the tissue at a particular time, one can obtain a snapshot of the 5-HT-related

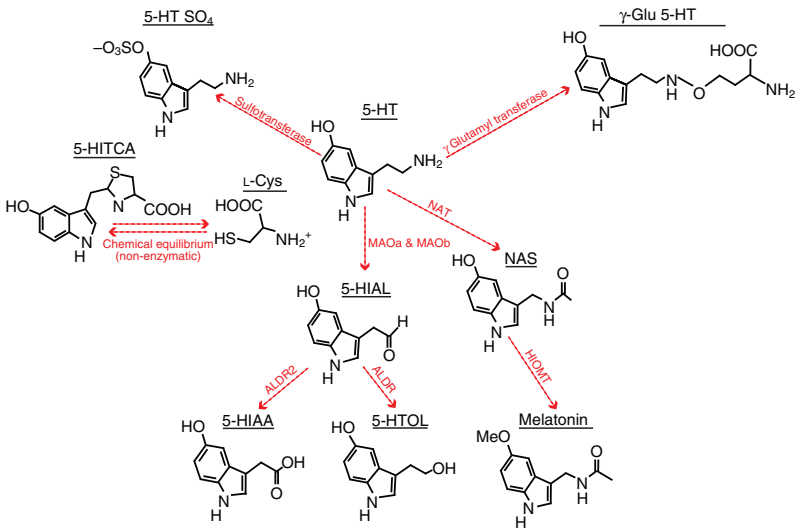

Fig. 1. The major 5-HT catabolic pathways. Serotonin sulfate (5-HT-SO_4); serotonin (5-HT); γ-glutamyl-serotonin (γ-Glu 5-HT); 5-hydroxyindole thiazolidine carboxylic acid (5-HITCA); L-cysteine (L-Cys); 5-hydroxyindole acetaldehyde (5-HIAL); *N*-acetylserotonin (NAS); 5-hydroxyindole acetic acid (5-HIAA); 5-hydroxytryptophol (5-HTOL); 5-methoxy-*N*-acetylserotonin (melatonin); sulfotransferase; γ-glutamyl transferase; chemical equilibrium (non-enzymatic); monoamine oxidase forms a and b (MAOa and MAOb); *N*-acetyltransferase (NAT); aldehyde dehydrogenase (type 2 in the CNS; ALDH2); aldehyde reductase (ALDR); hydroxyindole *O*-methyltransferase (HIOMT).

compounds present at that moment. In this investigation we also used an incubation approach to provide information on enzyme activity and formation of 5-HT catabolites. Specifically, 5-HT incubations with tissue homogenates were used, while keeping enzymes functional, in order to follow the conversion of exogenous 5-HT to various catabolites. This allowed multiple pathways to be unequivocally identified (Squires et al., 2006; Stuart et al., 2004; Stuart et al., 2003).

Identification of 5-HT catabolites

Briefly, the dissected radial nerves and rectal areas were incubated with exogenous 5-HT. Upon incubation the radial nerves formed the serotonin metabolites NAS, γ-Glu 5-HT and 5-HIAA, as shown in Fig. 2A. The average observed concentration of each metabolite was 2.1 μmol l^{-1}, 8.6 μmol l^{-1} and 7.1 μmol l^{-1}, respectively (Fig. 3). As a control, radial nerve samples that were heat treated prior to incubation with 5-HT did not form these or any other serotonin metabolites. A secondary area containing muscles and elements of the nervous system and rectum was also incubated with 5-HT. Upon incubation, the rectal tissue produced NAS and 5-HIAA but did not produce γ-Glu 5-HT (Fig. 2B). The average NAS concentration produced in the rectal tissue was determined to be 0.85 μmol l^{-1}, and the average 5-HIAA concentration was 112 μmol l^{-1}. However, the presence of 5-HT sulfate was not confirmed in any of the tissues analyzed.

Tryptamine was also detected in intact tissue extractions from nerve and gonad samples. However, the levels of tryptamine were variable, ranging from ~10 nmol l^{-1} to 2.4 μmol l^{-1}; in a few cases, it was either not present or was present below 10 nmol l^{-1} (the typical limit of detection for tryptamine for the CE–LIF instrument used).

5-HT synthesis in sea urchin tissues

Endogenous 5-HT was not detected in the tissues studied, most likely because of low levels and rapid catabolism. Therefore, in order to investigate the tissues in which 5-HT metabolism was observed and determine their ability to form 5-HT, incubations were performed with a combination of the 5-HT precursor 5-HTP and the MAO inhibitor clorgyline. When intact radial nerve samples were incubated with these compounds before extraction, 5-HT was formed at an average concentration of 3.1 μmol l^{-1}. Also, γ-Glu 5-HT and an unidentified compound eluting around 22 min were synthesized (Fig. 4A). In contrast, intact gonadal tissues incubated with 5-HTP and clorgyline did not form 5-HT or any of the 5-HT metabolites. Interestingly, the unidentified compound was observed in the nerve ring tissues incubated with 5-HTP and clorgyline, as shown in Fig. 4B.

5-HT metabolism in the presence of clorgyline

An investigation of the metabolites formed when intact nerve tissues were incubated with 5-HT and clorgyline before extraction showed the formation of the two 5-HT metabolites γ-Glu 5-HT and 5-HIAA, but not NAS. The average concentration of γ-Glu 5-HT formed was 29 μmol l^{-1} while that of 5-HIAA was only 1.2 μmol l^{-1}. These incubations did not produce the unidentified peak seen in tissues incubated with 5-HTP and clorgyline.

5-HT metabolism-related enzymes

Application of bioinformatics approaches allowed us to identify sea urchin counterparts of the appropriate vertebrate enzymes in the databases, including a partial sequence of *N*-acetyl transferase (XP_001194555.1) necessary to produce NAS. Also identified were MAO-A (XP_794084.2, XP_001186275.1) and MAO-B (XP_794729.2, XP_001190130.1), one of which is involved in the formation of 5-HIAA. A second enzyme required for the production of 5-HIAA, aldehyde dehydrogenase, was also found (XP_001178451, XP_001202450.1, XP_790286.2, XP_001176560.1, XP_788424.2, XP_780104.2, XP_001185717.1, XP_793156.2, XP_001183277.1, XP_001178148.1). Finally, the enzyme responsible for forming γ-Glu 5-HT, γ-glutamyl transferase, was also uncovered (XP_001190010.1, XP_791194.2).

5-HT metabolites in *X. bocki* tissues

Extraction of intact tissues from *X. bocki* with acidified methanol revealed the presence of a number of 5-HT metabolites – 5-HT sulfate, γ-Glu 5-HT and 5-HIAA – but not NAS, as shown in Fig. 5. Tryptamine and 5-HT also appeared natively in these samples.

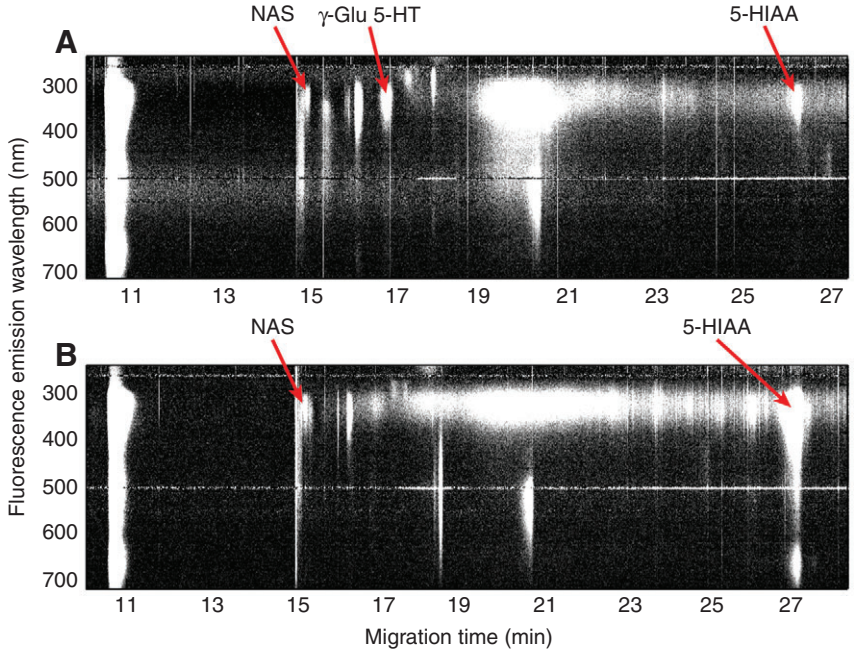

Fig. 2. (A) Wavelength-resolved electropherogram from an incubation of urchin radial nerves with 5-HT shows the appearance of NAS, γ-Glu 5-HT and 5-HIAA. (B) Incubation of rectal tissues with 5-HT shows the formation of NAS and 5-HIAA. Areas of white represent areas of highest fluorescence intensity while areas of black represent areas of lowest fluorescence intensity.

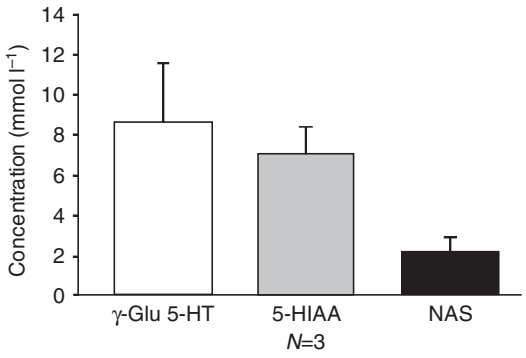

Fig. 3. When nerve tissue homogenates are incubated with 5-HT, γ-Glu 5-HT forms at an average of 8.6 μmol l^{-1}, 5-HIAA at an average of 7.1 μmol l^{-1} and NAS at an average of 2.1 μmol l^{-1}. Error bars show standard deviation between trials.

Although concentrations of these two compounds varied from 0.2 to 2.6 μmol l^{-1} for tryptamine and 0.4 to 6.4 μmol l^{-1} for 5-HT, for individual creatures and regions 5-HT was consistently found at higher levels than tryptamine. Because of limited tissue availability, only incubation experiments were performed on *X. bocki*.

5-HTP and L-DOPA incubations

In samples where 5-HT was detected in the control group, the concentrations ranged from 5.8 to 6.2 μmol l^{-1}. Furthermore, there was no significant increase observed in samples incubated with 5-HTP and L-DOPA, where 5-HT levels ranged from 5.8 to 6.0 μmol l^{-1}. Fig. 6 shows a comparison of the control samples, in which 5-HT appears natively, with the incubated samples. In a few samples where no 5-HT was detected in the control groups, 5-HT was detected upon incubation with 5-HTP at levels similar to those seen in both control and experimental animals in the first group, ranging from 5.9 to 6.4 μmol l^{-1}.

DISCUSSION

Although a significant body of research has focused on the detection of tryptamine and serotonin in sea urchin embryos (Buznikov et al., 2005; Manukhin et al., 1981; Morikawa et al., 2001; Renaud et al., 1983; Shmukler and Tosti, 2001), this marks one of the first studies of 5-HT catabolism in adult sea urchins. Our focus has been on elucidating the 5-HT-related pathways in several deuterostomes. Incubations with a combination of the 5-HT precursor 5-HTP and the MAO inhibitor clorgyline demonstrate that radial nerves are capable of forming 5-HT. This is in contrast to the gonadal tissues, which did not form 5-HT when incubated with 5-HTP and clorgyline. This finding demonstrates that 5-HT synthesis in the sea urchin is tissue specific and that 5-HT is not synthesized at the higher levels found in the mammalian enteric nervous system.

Moreover, our results indicate that the sea urchin metabolizes 5-HT in a manner similar to gastropod mollusks, annelids and vertebrates. In fact, our observation of γ-Glu 5-HT is similar to the results obtained when molluskan tissues are incubated with 5-HT (Sloley, 1994; Stuart et al., 2003). Along with this typically molluskan 5-HT metabolite, the formation of 5-HIAA and NAS was also seen. Although *N*-acetylation is a pathway found in insect 5-HT metabolism (Paxon et al., 2005; Sloley and Downer, 1984), sea urchin 5-HIAA is likely formed *via* MAO, the major mammalian catabolic pathway. Not only do we show that the sea urchin metabolizes 5-HT using a greater variety of pathways than vertebrates but also our results are consistent with the finding that all enzymes necessary to produce γ-Glu 5-HT, 5-HIAA and NAS are present in the sea urchin genome. Interestingly, when intact tissues from the radial nerves and gonads of the sea urchin were placed in extraction solution to observe the levels of indoleamine compounds, tryptamine was found to be a prominent native serotonin-like compound. These results are similar to those reported for sea urchin embryos (Manukhin et al., 1981). Tryptamine was detected in levels ranging from 0.6 to 2.4 μmol l^{-1} in only half of the animals investigated (four trials total). Whether the lack of detection of the compound in some of these animals was a result

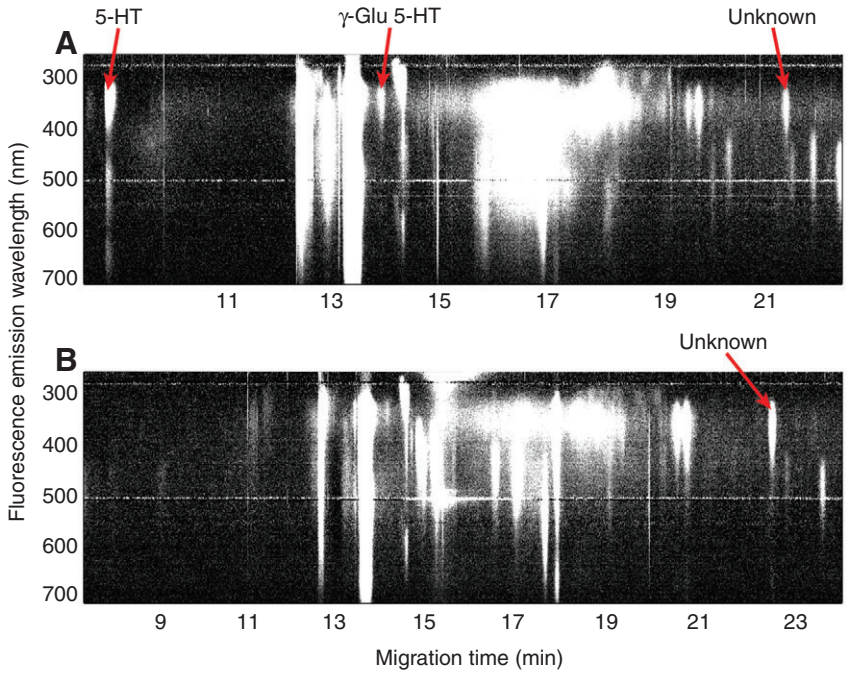

Fig. 4. Wavelength-resolved electropherograms of (A) radial nerves incubated with 5-hydroxytryptophan (5-HTP) and clorgyline showing 5-HT, γ-Glu 5-HT and an unidentified peak. (B) Incubation of gonad tissues with 5-HTP and clorgyline produces only the unidentified peak.

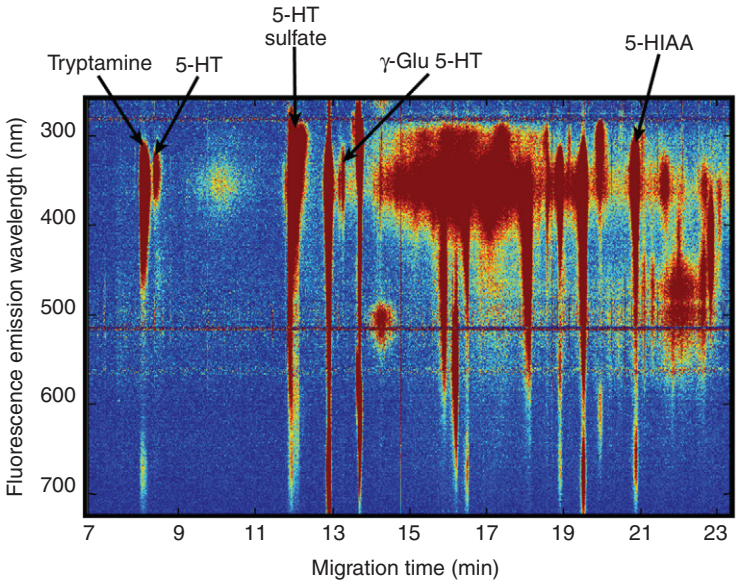

Fig. 5. A wavelength-resolved electropherogram where areas of red represent high fluorescence intensity and areas of blue show low intensity. These data are from the posterior region of *X. bocki*. The following peaks have been identified: tryptamine, 5-HT, 5-HT sulfate, γ-Glu 5-HT and 5-HIAA.

of levels falling below the limits of detection (~10 nmol l^{-1}) for our CE–LIF instrument, or whether the compound was simply not present, its variable formation is intriguing. Such significant fluctuations in observed compound levels can be representative of the physiological state of an organism, such as hunger and satiation [similar to some 5-HT metabolites reported for predatory mollusks (Hatcher et al., 2008)]. Based on other reports that levels of tryptamine vary through the stages of sea urchin embryonic development (Manukhin et al., 1981), it is possible that adult animals have variable tryptamine levels based on their stage in the reproductive cycle, although the reproductive stage of these wild-caught animals was not determined. Interestingly, the measured tryptamine concentrations were higher in a set of animals shipped to us earlier in the year. Clearly, to understand this variation in tryptamine levels would require a study of adult sea urchins at various stages in their reproductive cycle.

Another interesting finding from the sea urchin experiments is the appearance of an unknown compound eluting at ~22 min after incubation of nerve tissues with 5-HTP and clorgyline. Based on fluorescence emission, this compound is an indole; using migration time information, this is a negatively charged compound at pH 8.8 and it is likely slightly smaller than 5-HIAA. Although we were not able to identify this substance using standards that elute around this same time period, such as 5-HITCA (Squires et al., 2006), it remains of interest for further study.

The data from our *X. bocki* experiments showed greater variability for the levels of tryptamine, 5-HT and other 5-HT metabolites detected in these samples. We note that these animals are difficult to obtain and maintain far away from their natural habitats. *Xenoturbella bocki* development, growth, feeding ecology and diet are also largely unknown. Thus, it may be that the observed differences in detected concentrations of 5-HT-related metabolites

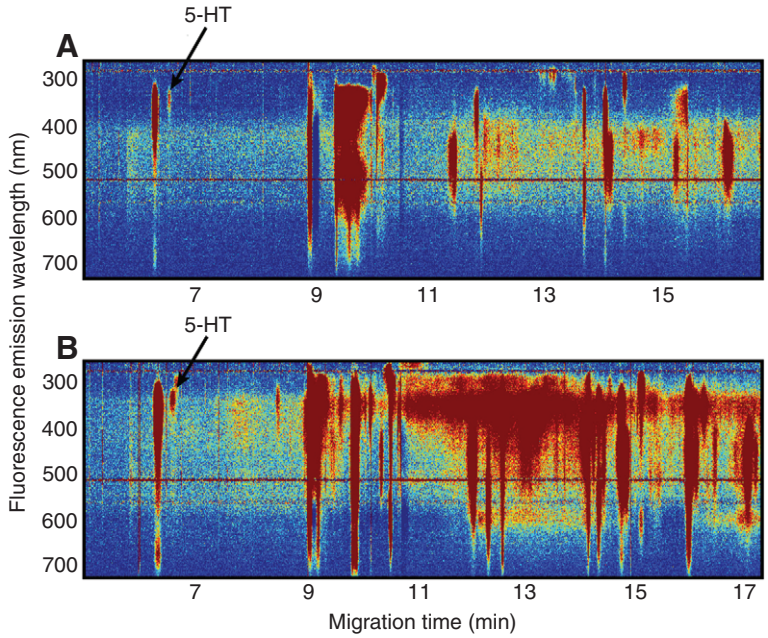

Fig. 6. In *Xenoturbella* samples, 5-HT is observed at similar levels whether the samples were (A) or were not (B) incubated with 5-HTP and L-DOPA.

are due to animal health, nutritional status or physiological conditions.

Because of the phylogenic placement of *X. bocki*, it is of interest to understand its serotonergic system in order to gain possible insight into the origins of the serotonergic systems of present day bilaterians, and deuterostomes in particular. We observed the formation of known 5-HT metabolites in this organism, 5-HT sulfate, γ-Glu 5-HT and 5-HIAA. Probably of greatest interest is that the appearance of 5-HIAA suggests that the MAO pathway of 5-HT catabolism is present in *X. bocki*, along with the γ-glutamyl transferase and phenolsulfotransferase pathways, which are shown in Fig. 1. However, in contrast to our data on sea urchins, we did not detect NAS in this animal. The lack of genomic information prevents the identification of appropriate enzymes.

CONCLUSIONS

Our initial survey of 5-HT metabolism demonstrates a surprising variety of 5-HT-related catabolic pathways in two basal deuterostome lineages. This catabolic diversity suggests that vertebrates may have lost their 5-HT metabolic pathways (e.g. leading to γ-Glu 5-HT). At the same time, our data also suggest that MAO activity can be an ancient 5-HT inactivation strategy in deuterostomes.

Nevertheless, the described pathways are apparently not identical between the two selected deuterostomes studied here. In the sea urchin, 5-HT is metabolized into γ-Glu 5-HT, NAS and 5-HIAA, but not 5-HT sulfate. One unidentified compound was produced upon incubation with 5-HTP, and clorgyline and native tryptamine levels were observed to change, possibly based on the animal's age. Future work will aim to identify this newly observed substance, in addition to conducting controlled age studies of tryptamine levels in the nervous and gonadal tissues of adult sea urchins. In contrast, *X. bocki* 'shared' more identified 5-HT metabolites with mollusks.

Overall, our findings suggest that 5-HT can be a prominent signaling molecule in basal deuterostomes, justifying continued study of the serotonergic systems in these animals. Our results also provide evidence that morphologically 'simpler' animals contain surprising biochemical complexity in well-known transmitter pathways, perhaps reflecting the preservation of earlier enzymatic pathways from ancestral lineages and a large degree of parallel evolution in transmitter systems. Upcoming investigations will characterize these signaling pathways in other members of this diverse superclade and also be expanded to examine additional transmitter pathways.

LIST OF ABBREVIATIONS

ASW	artificial seawater
CE	capillary electrophoresis
L-DOPA	dihydroxyphenylalanine
γ-Glu 5-HT	γ-glutamyl-serotonin
5-HIAA	5-hydroxyindole acetic acid
5-HITCA	5-hydroxyindole thiazolidine carboxylic acid
5-HT	serotonin or 5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
LIF	laser-induced florescence
MAO	monoamine oxidase
NAS	*N*-acetyl serotonin

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