# Electrochemical Quantification of Serotonin in the Live Embryonic Zebrafish Intestine

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# Electrochemical quantification of serotonin in the live embryonic zebrafish intestine

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## Abstract

We monitored real-time *in vivo* levels of serotonin release in the digestive system of intact zebrafish embryos during early development (5 dpf) using differential pulse voltammetry with implanted carbon fiber microelectrodes modified with carbon nanotubes dispersed in nafion. A detection limit of 1 nM, a linear range between 5 to 200 nM and a sensitivity of 83.65 nA·μM$^{-1}$ were recorded. The microelectrodes were implanted at various locations in the intestine of zebrafish embryos. Serotonin levels of up to 29.9(±1.13) nM were measured *in vivo* in normal physiological conditions. Measurements were performed in intact live embryos without additional perturbation beyond electrode insertion. The sensor was able to quantify pharmacological alterations in serotonin release and provide the longitudinal distribution of this neurotransmitter along the intestine with high spatial resolution. In the presence of fluvoxamine, a selective serotonin reuptake inhibitor (SSRI), concentrations of 54.1(±1.05) nM were recorded while in the presence of p-chloro-phenylalanine (PCPA), a tryptophan hydroxylase inhibitor, the serotonin levels decreased to 7.2(±0.45) nM. The variation of serotonin levels was correlated with immunohistochemical analysis. We have demonstrated the first use of electrochemical microsensors for in *vivo* monitoring of intestinal serotonin levels in intact zebrafish embryos.

## Introduction

Serotonin (5-hydroxytryptamine or 5-HT) is a neurotransmitter involved in a large number of physiological processes including motility and secretion in the digestive system. Enteric neurons and enterochromaffin cells (EC) within the intestinal epithelial layer store approximately 95% of the 5-HT within the vertebrate body.[1–3] EC cells are positioned within the epithelium to transduce mechanical and nutrient stimulation from the lumen to processes of intestinal sensory neurons within the mucosa.[4–8] Transduction of these signals through EC cells is necessary because sensory neurons do not directly innervate intestinal epithelial cells due to their high rate of turn-over. Stimulation of EC cells results in secretion of 5-HT into the mucosa, initiating action potentials mediated by 5-HT receptors on the processes of intrinsic primary afferent neurons (IPANs) as well as primary extrinsic afferent neurons.[9–12] IPANs synapse with interneurons and motor neurons within the submucosal and myenteric plexuses to create peristaltic and secremotor responses[12–16] while primary afferent extrinsic neurons communicate with the central nervous system, creating visceral sensation including nausea and discomfort.[17–19] 5-HT is charged at physiological pH and does not cross cellular membranes.

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Supporting Information Available: This material is available free of charge via the internet at http://pubs.acs.org.

5-HT is catabolized by monoamine oxidases and glucuronyl transferases after transport across enterocyte membranes by the serotonin transporter.[20, 21]

Altered serotonin levels within the digestive system have been implicated in disease states. Celiac disease has both an increase in enterochromaffin cells[22, 23] and defects in mucosal 5-HT removal.[24] Irritable Bowel Syndrome results in alteration of mucosal 5-HT levels and decreased serotonin transporter expression affecting transit time and secretion within the intestine.[24–26] Therefore measurements of 5-HT are important in understanding mechanisms of gastrointestinal functions and monitoring pathophysiological conditions within the intestine. In this study, we describe the first use of an electrochemical microsensor for measuring 5-HT *in vivo* in the intestine of a zebrafish embryo.

Zebrafish is a widely accepted model organism in developmental biology used to understand many areas of early pattern formation, organ development, metabolism, and toxicity response. Zebrafish share a similar anatomy and development of the digestive system to other vertebrates including humans.[27]

5-HT levels in the brain of adult zebrafish28 and in the gastrointestinal tissue of guinea-pigs[29] have been assayed by extraction followed by high precision liquid chromatography (HPLC) analysis. The quantity of tissue and 5-HT levels are low in the embryonic zebrafish intestine, making this technique less feasible in this organ. Moreover, these methods are slow, require extensive sample treatment, do not provide real-time quantitative information, and do not have spatial resolution. In previous studies, the presence of 5-HT in the zebrafish intestine has been determined immunohistochemically[30, 31] but there have been no quantitative assessments.

Electrochemical microsensors are used traditionally for the detection of neurotransmitters in the brain and can provide real-time measurements in vivo.[32, 33] 5-HT is an electrochemically active compound and can be measured directly using carbon fiber microelectrodes. Several electrochemical methods have been described in the literature for detection of 5-HT including fast scan cyclic voltammetry,[32] amperometry, and differential pulse voltammetry.[34, 35] In previous studies, 5-HT has been determined electrochemically in the mucosa of 3–4 month old guinea-pig and rabbit ileum using boron doped diamond coated Pt wire microelectrodes,36 and with carbon fiber microelectrodes to monitor 5-HT release from EC cells37،38 In these studies, segments of intestine have been surgically removed and measurements with the microelectrode were performed on dissected segments of ileum.[36] Since this is an invasive procedure, measurements may not reflect normal physiological concentrations due to stress from surgery and dissection. In other studies fast scan cyclic voltammetry with carbon fiber microelectrodes have been used to quantify changes in extracellular monoamines (5-HT and dopamine) in isolated larval fly ventral nerve cord[37] and nafion modified microelectrodes have been used for endogenous recording of 5-HT in the mammalian brain.32 To our knowledge there have been no electrochemical studies examining neurotransmitter release in intact embryos.

The goal of this study is to demonstrate the feasibility of electrochemical measurement of neurotransmitter release in zebrafish embryos using implantable carbon-fiber microelectrodes. Sensors consist of a carbon fiber microelectrode with a diameter of 30 μm modified with carbon nanotubes dispersed in nafion, that have been reported previously to improve the detection of catecholamine neutotransmitters[39] and prevent surface fouling.32، 34، 40، [41] We determine real-time in vivo concentrations of 5-HT within the digestive system of 5 day post-fertilization (dpf) zebrafish embryos with high temporal and spatial resolution. The sensor identifies differences in concentrations of 5-HT along the anterior-posterior axis of the intestine as well as changes of 5-HT due to pharmacological manipulation. Pharmacological manipulation can

also be correlated with immunohistochemical analysis. These studies suggest that electrochemical microsensors can be a powerful tool to study neurodevelopmental mechanisms. Electrochemical sensors can provide a greater understanding of the roles of 5-HT signaling in digestive system function both in zebrafish and other vertebrates using a simple and rapid procedure.

## Materials and Methods

### Reagents

Fluvoxamine, nafion, (5% solution in a mixture of lower aliphatic alcohols and water) Proteinase K, rabbit anti-Serotonin, uric acid, 5-hydroxyindolacetic acid (5-HIAA) and PCPA (4-Chloro-DL-phenylalanine methyl ester hydrochloride) were purchased from Sigma-Aldrich. Serotonin was purchased from Acros organics. Melatonin was purchased from AKC Scientific Inc. Tyramine was purchased from Spectrum Chemical. Carbon wire and Dumont #5 forceps were purchased from WPI (World Precision instruments Inc, Sarasota, FL). Reserpine (Cat # 2742) and Pirlinodole mesylate, a highly specific inhibitor of monoamine oxidase type A (MAOI) (Cat # 0724) were purchased from Tocris Bioscience. Sodium phosphate monobasic was from Fisher Scientific. Sodium phosphate (dibasic, anhydrous) was from J. T. Baker (Phillipsburg, NJ). Secondary antibody (Alexa Fluor 488 anti-rabbit Ig (1:500)) was from Molecular Probes-Invitrogen and primary antibody (rabbit anti-Serotonin (1:500 dilutions) was from Sigma. Carbon nanotubes (CNTs) were obtained from Nanodynamics (Buffalo, NY).

### Instrumentation

Differential pulse voltammetry experiments were performed with a CH Instrument electrochemical analyzer potentiostat (CH Instruments Inc., Austin, TX). All optimization experiments were carried out using a conventional electrochemical cell equipped with a Ag/AgCl/3M NaCl (BAS MF-2052, RE-5B) as reference electrode, a platinum wire (BAS MW-1032) as counter electrode and a modified carbon fiber microelectrode as working electrode. All the potentials were referred to the Ag/AgCl reference electrode. Vectashield was from Vector Laboratories. Micromanipulator was purchased from WPI (World Precision instruments Inc, Sarasota, FL).

### Preparation of Carbon Fiber Microelectrodes

Carbon fiber microelectrodes (~30 μm diameter) were fabricated from carbon fibers from WPI. Fibers were glued to a copper wire using a conductive silver paste and aspirated into a pulled glass capillary tube. Both ends of the capillary tube were sealed using an epoxy resin and cured at a temperature of 120°C for 5 minutes. The fiber extending from glass was cut using a scalpel so that it extended up to ~0.5 mm from the glass seal. Before modification the electrodes were electrochemically treated by repeated overoxidation in 0.1 M PB pH 7.0 in the potential range −0.4 V to 1.4 V at 500 V/s until a current of 1 μA was achieved. Modification with oxidized carbon nanotubes (treated in 2 M nitric acid for 24 hrs41 to remove impurities) and nafion was performed using a dip-coating procedure of a suspension of carbon nanotubes in nafion, 39 as described by Swamy & Venton.34 In brief, 1 mg of the oxidized nanotubes was suspended in 1 mL of 2.5 % Nafion (diluted with isopropanol) and sonicated for 30 min. The carbon-fiber microelectrodes were dipped for 1 sec in the nanotube suspension and then immediately rinsed by dipping it in isopropanol for 30 s to remove loose nanotubes. The electrodes were allowed to air dry for at least 10 min before testing. For comparison, the response to 5-HT was evaluated for the bare and carbon nanotubes coated microelectrodes. All values are reported as the mean ± standard deviation for at least three replicate measurements; in vivo measurements are reported as the mean ± standard deviation for measurements with at least three embryos. A background differential pulse voltammogram (DPV) obtained in vitro in absence of 5-HT was

subtracted from each DPV obtained in presence of 5-HT. The microelectrodes were electrochemically treated in between in vivo measurements by potential cycling in the range −0.4 to 1.4 V at 25 V/s for 1400 cycles in 0.1 M PB pH 7.0.

## Fish Stocks

Fish maintenance and matings were performed as previously described.[42,43] AB wild type fish were used for all procedures.[42] Pigmented five dpf larvae were used for electrochemical sensing and pharmacological manipulation.

## Immunohistochemistry

Embryos were fixed in 4% formaldehyde for 2 h to overnight. Fixed embryos were then permabilized with Proteinase K in phosphate buffer solution (PBS) for 20 minutes at room temperature and incubated at 4° overnight. After fixation, embryos were washed for 45 minutes in PBS and the primary antibody (rabbit anti-serotonin antibody; 1:500 dillution Sigma) was added overnight. Embryos were washed in PBS for 45 minutes and incubated with Alexa Fluor conjugated secondary anti-rabbit antibody (1:500 dilution), Molecular Probes -Invitrogen, Carlsbad, CA for two hours. Embryos were washed in PBS for 45 minutes and whole mount embryos were analyzed on a Nikon Eclipse TE2000 inverted compound fluorescence microscope. Images were recorded using a Hamamatsu Orca camera in IP lab software as described previously.[31]

## Embryo Visualization for Immunohistochmistry

The digestive system is visible in whole mount but portions of the embryos needs to be dissected for efficient viewing. For fluorescence immunohistochemical analysis of the distribution of the 5-HT containing neurons in the intestine, skin was removed, followed by any remaining yolk, and the digestive system was dissected from the embryo by grasping a region in the pharynx with forceps and pulling away from body. The digestive system (pharynx, esophagus, and intestine) was then mounted separately in Vectashield. Dumont #5 forceps were used for dissection on a Leica MZ12.5 stereomicroscope.

## In vivo Electrochemistry Measurements in Intact Embryos

Agarose was fixed in one of a six well plate and placed under a stereomicroscope. 5 dpf zebrafish embryos were immobilized on the agarose bed and carbon nanotube–coated microelectrodes were inserted into various positions along the posterior intestine using a micromanipulator. Electrochemical measurements in vivo were carried out in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM $MgSO_4$) as the electrolyte. Measurements were performed using differential pulse voltammetry at a scan increment of 4.0 mV. The pulse amplitude, width and period were 50 mV, 50 ms and 200 ms respectively. All differential pulse voltammograms are reported with the background subtracted (the background electrode response in the measuring buffer). Electrochemical measurements *in vivo* were initiated after electrode insertion. Experiments were performed in live embryos to measure normal physiological 5-HT concentrations as well as altered levels after serotonin specific pharmaceutical manipulations to either inhibit or over-express 5-HT production in the intestine.

## Pharmacological Manipulation

Each of the compounds fluvoxamine, 4-Chloro-DL-phenylalanine methyl ester hydrochloride (PCPA), Pirlinodole mesylate (highly selective monoamine oxidase type A inhibitor- MAOI) and reserpine were diluted in embryo medium (E3) and embryos were exposed for two hours before electrochemical detection was performed. Working concentrations were: fluvoxamine 10 μM PCPA 100 μM, reserpine 100 μM and 100 μM pirlinodole mesylate. Fluvoxamine, PCPA, and pirlinodole mesylate are water soluble however a stock of reserpine (82 mM) was

dissolved in Dimethyl sulfoxide (DMSO) and then diluted into E3. Controls with equivalent concentrations of DMSO in E3 were performed in parallel with reserpine exposure.

## Results and Discussion

### Electrochemical Characterization of the Carbon Fiber Microelectrodes

Before making *in vivo* measurements of zebrafish embryos, carbon fiber microelectrodes were first characterized using differential pulse voltammetry in standard solutions of 5-HT ranging from 10 nM to 2 μM. Response to serotonin was examined before and after modification with a stable dispersion of carbon nanotubes and nafion. Nafion is used as a linker to solubilize the carbon nanotubes and to efficiently attach the nanotubes onto the carbon fiber surface.41 This surface treatment has been shown previously to dramatically prevent surface fouling and increase detection capability for catecholamines39–41 and 5-HT.[34] Indeed, the sensitivity of the sensor after modification increased from 75.16 (±2.13) nA/μM to 83.65 (±0.95) nA/μM. The background current also increased for the modified electrode due to a larger surface area. As expected, the most dramatic effect of adding carbon nanotubes and nafion was observed in terms of surface fouling and reusability. Due to reduced surface fouling, the carbon nanotube–coated microelectrode could be reused for three measurements with brief electrochemical conditioning in between runs. Figure 1 shows typical differential pulse voltammograms for increasing concentrations of 5-HT ranging from 10 to 80 nM and the corresponding calibration curve for this concentration range obtained with a carbon nanotube – nafion modified microelectrode. A well-defined voltammetric peak is present at 360 mV vs. Ag/AgCl reference electrode, corresponding to the oxidation of 5-HT was observed for both modified and unmodified electrode, with a higher signal of ~ 10% for the electrode with carbon nanotube-nafion. This peak is proportional with the concentration of 5-HT and the recorded current response at 360 mV was used to plot the calibration curve of the microsensor. For comparison, the background current was featureless. In other works, oxidation peaks at 290 mV *vs.* Ag/AgCl were reported for bare 30 μm diameter carbon fiber microelectrodes and at 500 mV vs. Ag/AgCl for boron doped diamond deposited on 40 μm Pt wire.[44] In its optimized form, in the presence of carbon nanotubes and nafion, the detection limit (determined according to the $3S_b/m$ criteria,[45] where m is the slope of the linear calibration plot, and $S_b$ is the relative standard deviation of the amperometric signal of the blank for n = 5) was 1 nM and the linear range spanned from 5–200 nM for background-subtracted signals. This detection limit is significantly lower than that reported in other works for 5-HT using differential pulse voltammetry with bare carbon fiber (1.05 μM) and boron doped diamond (3.08 μM) microelectrodes.[44] This is also lower than the detection limit obtained using high-liquid performance chromatography with electrochemical detection (241 nM).29 The low detection limit and high sensitivity of our sensors allowed us to use this technology for determining 5-HT levels in the digestive system of intact embryos, where the concentrations are low. All embryonic experiments were performed with the carbon nanotube - nafion modified microelectrodes.

In addition to 5-HT, other electrochemically active compounds could be responsible for the recorded peak. Both uric acid and 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-HT, are electrochemically active and may contribute to the 5-HT peak. Addition of uric acid up to concentrations of 500 μM demonstrated no response. The unresponsiveness of these electrodes to uric acid can be attributed to electrostatic repulsions between the negatively charged uric acid and the negatively charged nafion.[46] In contrast, 40 nM 5-HIAA produced a well-defined voltammetric peak at 360 mV vs Ag/AgCl reference electrode (Supplemental Figure S1). This suggests that a portion of the 5-HT peak at 360 mV in vivo could be due to the presence of 5-HIAA.

## Electrochemical Sensing of 5-HT in the Intact Zebrafish Intestine of Live Embryos

Serotonin-containing enteric neurons develop late in embryogenesis first in the anterior and later in the posterior.[31] While immunohistochemistry can be used to visualize the distribution and relative levels of 5-HT within the intestine, quantification of the concentration of 5-HT during embryogenesis is not possible using this technique. To measure the concentration of 5-HT, microsensors were inserted in the intestine, posterior to the swim bladder of a 5 dpf embryo, approximately halfway between the posterior swim bladder and the end of the intestine, as shown in Figure 2A. By 5 dpf, serotonin neurons are distributed throughout the intestine with EC cells present in the intestinal epithelium posterior to the position of the swim bladder (Figure 2B). Figure 2C shows the corresponding differential pulse voltammogram recorded *in vivo* with the microelectrode. The voltamogram shows a well-defined peak at ~360 mV vs Ag/AgCl corresponding to 5-HT, at the same potential value as the one obtained in standard solutions (Figure 1A). Using the calibration curve in pure 5-HT solutions, the recorded current at 360 mV corresponds to concentrations of 29.9(±1.13) nM in normal physiological conditions. In addition, a second peak at ~570 mV vs Ag/AgCl was observed which was not present in pure 5-HT solutions.

To confirm the identity of the second peak, melatonin, a paracrine molecule produced by intestinal EC cells and synthesized from 5-HT by 5-hydroxyindole-ortho-methyltransferase47, was tested as well as tyramine, which was previously suggested to potentially be present in the gastrointestinal track and oxidized at higher potentials38, [47] Using a mixture of 5-HT and melatonin, a calibration curve was obtained in the concentration range (20 nM to 100 nM) with melatonin producing a peak at a potential of ~620 mV (Supplemental Figure S2). The oxidation potential of tyramine was found at 620 mV, which overlapped the melatonin peak (Supplemental Figure S3). Similar electrochemical behavior for the compounds, 5-HT, melatonin and tyramine using carbon fiber electrodes was reported by Bertrand, 2004[38]. Release of melatonin was reported previously and measured with voltammetric methods in the EC cells of guinea pig[47] and rabbit ileum where it was found together with 5-HT. However, the *in vivo* peak in zebrafish intestine appears at ~570 mV vs Ag/AgCl and therefore cannot be directly attributed to neither melatonin nor tyramine.

Microsensors can be used to measure 5-HT levels at different locations within the intestine. Immunohistochemistry reveals nearly equivalent levels of 5-HT in the mid and posterior intestine with lower levels in the anterior (Figure 3) at 5 dpf. Higher 5-HT concentrations in mid and posterior segments were confirmed by differential pulse voltammetry. Voltammetric responses in the intestine anterior to the swim bladder demonstrates 5-HT concentrations of 5.4 (±0.65) nM estimated with the calibration curve at 360 mV vs Ag/AgCl in standard solutions. Low 5-HT levels correlate with immunohistochemistry in this region, showing low numbers of 5-HT producing enteric neurons and no EC cells (Figure 3B). The highest 5-HT levels were found at the middle posterior of the intestine (29.9 (±1.13) nM) followed by the lower posterior end (25.9 (±0.5) nM).

## Pharmacological Manipulations Result in Altered Levels of 5-HT

Although the recorded peak corresponds 5-HT and the metabolite 5-hydroxyindoleacetic acid (5-HIAA), pharmacological manipulation of 5-HT further demonstrates the identity of the compound and that it is regulated in a manner similar to other vertebrates. Levels of 5-HT were reduced in the intestine using PCPA, an inhibitor of the tryptophan hydroxylase, a rate-limiting enzyme in the synthesis of 5-HT, and reserpine, which inhibits the vesicular monoamine transporter from packaging 5-HT into vesicles. Levels of 5-HT were increased using the selective serotonin reuptake inhibitor (SSRI) fluvoxamine. Changes in 5-HT levels were confirmed by both immunohistochemical and electrochemical methods. The microsensor was implanted in the 5 dpf embryonic intestine in the mid segment, between the posterior swim

bladder and the end of the intestine as shown in Figure 2. This region contains the highest 5-HT levels in unaltered physiological conditions, estimated at 29.9 (±1.13) nM by voltammetry (Figure 3C).

5 dpf embryos were exposed to PCPA and reserprine for two hours and compared to controls (Figure 4). As expected, application of the tryptophan hydroxylase inhibitor (PCPA) results in a visible decrease of 5-HT by fluorescence immunohistochemistry (compare Figure 4A and 4B). The concentration of 5-HT from the voltammetric data is estimated at 7.2 (± 0.45) nM, which is significantly lower that that of the control. The second peak at 570 mV remains essentially the same in the presence and absence of PCPA. Addition of reserpine, results in nearly undetectable levels of 5-HT by immunohistochemistry (compare Figure 4A and 4B). The near complete depletion of serotonin is similar to the non-specific release of 5-HT from rabbit intestine[48] and rat brain.[49] Thus, 5-HT is synthesized and turned over within the embryonic intestine similar to other vertebrates. When reserpine was used, no voltammetric peak was observed (Figure 4C). In contrast, addition of fluvoxamine results in an increase in 5-HT levels compared to unexposed controls (compare Figure 5A to 5B) suggesting that, similar to other vertebrates, SERT is the main transporter removing 5-HT from the extracellular space. Levels of 5-HT of up to 54.1 (±1.05) nM were determined in this case. The fact that the electrochemical measurements were able to follow the variations of the 5-HT levels as a result of serotonin specific pharmacological manipulations, as predicted from immunohistochemistry further confirms that the compound measured *in vivo* is 5-HT. In addition, appropriate responses due to pharmacological manipulation, suggests that the zebrafish serotonin signaling system is operating in a similar manner to other vertebrates.

Transport of 5-HT into cells containing monoamine oxidase (MAO) results in metabolism into 5-hydroxyindoleacetic acid (5-HIAA). MAO converts 5-HT to 5-hydroxyindole-3-acteticaldehyde (5-HIAD) which is further oxidized by aldehyde dehydrogenase to 5-HIAA.[50, 51] 5-HIAA is an electrochemically active and contributes to the 5-HT peak. To determine the contribution of the 5-HIAA to the 5-HT peak, pirlindole mesylate, a highly selective reversible inhibitor of monoamine oxidase type A (MAOI), was utilized in order to inhibit the intracellular breakdown of 5-HT. Addition of this MAOI should drastically reduce the level of 5-HIAA and the resultant peak should be comprised primarily of 5-HT. However, with MAO inhibition, recorded levels of 5-HT will be dependent on penetration of the sensor into the cells as opposed to treatment with fluvoxamine which results in additional 5-HT outside the cells. Comparison between embryos exposed to pirlinodole mesylate for 2 hours and control embryos demonstrate an increase in 5-HT levels to 41.7 (±6.0) nM (Figure 6B). This level is higher than the control (29.9 (±1.13) nM) but about half as much as with the SSRI fluvoxamine (54.1 (±1.05) nM). A portion of the difference in the 5-HT peak is likely to be due to the near absence of 5-HIAA with the MAOI but in order for the sensor to detect increased levels, the cell must be penetrated or lysed upon insertion of the electrode. Additionally, with SSRI treatment, higher 5-HT levels will be more accessible to the sensor due to availability in the extracellular matrix. As a result, some of the difference in 5-HT levels between the SSRI and MAOI treatment will be do to 5-HIAA levels but it is difficult to determine how much due to the differential accessibility of 5-HT to the sensor.

SSRI treatment should exclude 5-HT from the cell while the MAOI should increase 5-HT concentration within the cell. Comparison of the immunohistochemistry however, appears to produce opposite results. Addition of fluvoxamine appears to increase 5-HT levels (compare Figure 5A to 5B) while pirlindole mesylate lowers levels (compare Figure 6A to 6B). One explanation may be in the response of the cell to altered levels of 5-HT. Increased 5-HT after MAOI addition may reduce further synthesis while lowering cellular levels of 5-HT may induce additional synthesis resulting in a more concentrated nuclear staining.

In summary, quantitative information about concentrations of 5-HT is provided by differential pulse voltammetry in an *in vivo* setting, which correlates with observed immunohistochemical changes. In addition, DPV is rapid (one analysis can be completed within several seconds in vivo) and does not require addition of reagents or incubation steps. Of particular advantage is that measurements are performed in intact live embryos without additional perturbation beyond electrode insertion. To our knowledge this is the first demonstration of direct electrochemical measurements of neurotransmitter release with an implanted microelectrode in live embryos at organ level.

## Conclusions

We showed that carbon fiber microelectrodes functionalized with carbon nanotubes dispersed in nafion, in conjunction with differential pulse voltammetry can be successfully used to determine *in vivo* concentrations of 5-HT in the intestine of zebrafish embryos, with high temporal and spatial resolution. The sensor can measure nanomolar levels of 5-HT with a detection limit of 1 nM, a linear range of 5 – 200 nM and a sensitivity of 83.65 (±0.95) nA/μM. The electrochemical signal recorded in vivo with the implanted microelectrode corresponded to a value of 5-HT of 29.9 (±1.13) nM in normal physiological conditions. The electrochemical signals were further confirmed by serotonin specific pharmacological manipulations using tryptophan hydroxylase and SERT inhibitors and the results were confirmed with fluorescence immunohistochemistry. We have also determined the longitudinal distribution along the intestine at the anterior, mid, and posterior segments of the 5 dpf embryonic intestine.

In summary, we used this technology to demonstrate the first measurements of 5-HT release in live embryos within the digestive system. We are currently applying this technique to study mechanisms of development and control of gastrointestinal functions for understanding a variety of biological processes, obtain toxicological information, and study diseases. We plan to extend such measurements to other neurotransmitters and other organs including cardiac and the central nervous systems.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

1. Costa M, Furness JB, Cuello AC, Verhofstad AA, Steinbusch HW, Elde RP. Neuroscience 1982;7:351–363. [PubMed: 6210850]
2. Gershon MD. Curr Opin Pharmacol 2003;3:600–607. [PubMed: 14644011]
3. Gershon MD, Tamir H. Neuroscience 1981;6:2277–2286. [PubMed: 6276814]
4. Braun T, Voland P, Kunz L, Prinz C, Gratzl M. Gastroenterology 2007;132:1890–1901. [PubMed: 17484882]
5. Fukumoto S, Tatewaki M, Yamada T, Fujimiya M, Mantyh C, Voss M, Eubanks S, Harris M, Pappas TN, Takahashi T. Am J Physiol Regul Integr Comp Physiol 2003;284:R1269–1276. [PubMed: 12676748]
6. Kellum JM, Albuquerque FC, Stoner MC, Harris RP. Am J Physiol 1999;277:G515–520. [PubMed: 10484375]

7. Kim M, Christofi FL, Xue J, Robinson JM, Cooke HJ. Neurogastroenterol Motil 2007;19:309–317. [PubMed: 17391247]
8. Kim M, Javed NH, Yu JG, Christofi F, Cooke HJ. J Clin Invest 2001;108:1051–1059. [PubMed: 11581306]
9. Furness, JB. Blackwell Pub; Malden, Mass: 2006.
10. Grider JR, Foxx-Orenstein AE, Jin JG. Gastroenterology 1998;115:370–380. [PubMed: 9679042]
11. Jin JG, Foxx-Orenstein AE, Grider JR. J Pharmacol Exp Ther 1999;288:93–97. [PubMed: 9862758]
12. Pan H, Gershon MD. J Neurosci 2000;20:3295–3309. [PubMed: 10777793]
13. Cooke HJ, Sidhu M, Wang YZ. Neurogastroenterol Motil 1997;9:181–186. [PubMed: 9347474]
14. Kirchgessner AL, Liu MT, Gershon MD. J Comp Neurol 1996;371:270–286. [PubMed: 8835732]
15. Kirchgessner AL, Tamir H, Gershon MD. J Neurosci 1992;12:235–248. [PubMed: 1729436]
16. Sidhu M, Cooke HJ. Am J Physiol 1995;269:G346–351. [PubMed: 7573444]
17. Blackshaw LA, Grundy D. J Auton Nerv Syst 1993;45:41–50. [PubMed: 8227963]
18. Grundy D, Blackshaw LA, Hillsley K. Dig Dis Sci 1994;39:44S–47S. [PubMed: 7995214]
19. Sugiuar T, Bielefeldt K, Gebhart GF. J Neurosci 2004;24:9521–9530. [PubMed: 15509739]
20. Chen JX, Pan H, Rothman TP, Wade PR, Gershon MD. Am J Physiol 1998;275:G433–448. [PubMed: 9724254]
21. Wade PR, Chen J, Jaffe B, Kassem IS, Blakely RD, Gershon MD. J Neurosci 1996;16:2352–2364. [PubMed: 8601815]
22. Moyana TN, Shukoor S. Mod Pathol 1991;4:419–423. [PubMed: 1681534]
23. Wheeler EE, Challacombe DN. Arch Dis Child 1984;59:523–527. [PubMed: 6378103]
24. Coleman NS, Foley S, Dunlop SP, Wheatcroft J, Blackshaw E, Perkins AC, Singh G, Marsden CA, Holmes GK, Spiller RC. Clin Gastroenterol Hepatol 2006;4:874–881. [PubMed: 16797248]
25. Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, Moses PL. Gastroenterology 2004;126:1657–1664. [PubMed: 15188158]
26. Miwa J, Echizen H, Matsueda K, Umeda N. Digestion 2001;63:188–194. [PubMed: 11351146]
27. Wallace KN, Pack M. Dev Biol 2003;255:12–29. [PubMed: 12618131]
28. Chatterjee D, Gerlai R. Behav Brain Res 2009;200:208–213. [PubMed: 19378384]
29. Chau RM, Patel BA. Biomed Chromatogr 2009;23:175–181. [PubMed: 18814194]
30. Shigematsu N, Yamamoto K, Higuchi S, Fukuda T. Brain Research 2006;1075:110–116. [PubMed: 16460713]
31. Olden T, Akhtar T, Beckman SA, Wallace KN. Genesis 2008;46:484–498. [PubMed: 18781646]
32. Hashemi P, Dankoski EC, Petrovic J, Keithley RB, Wightman RM. Anal Chem. 2009
33. Venton BJ, Wightman RM. Analytical Chemistry 2003;75:414a–421a.
34. Swamy BEK, Venton BJ. Analyst 2007;132:876–884. [PubMed: 17710262]
35. Sazonova, N.; Njagi, JI.; Marchese, ZS.; Ball, MS.; Andreescu, S.; Schuckers, S. IEEE. 2009. Accepted
36. Bian XC, Patel B, Dai XL, Galligan JJ, Swain G. Gastroenterology 2007;132:2438–2447. [PubMed: 17570217]
37. Bertrand PP. The Journal of Physiology 2006;577:689–704. [PubMed: 16959854]
38. Bertrand PP. Neurogastroenterology and Motility 2004;16:511–514. [PubMed: 15500507]
39. Wang J, Musameh M, Lin YH. Journal of the American Chemical Society 2003;125:2408–2409. [PubMed: 12603125]
40. Wang J, Deo RP, Musameh M. Electroanalysis 2003;15:1830–1834.
41. Hocevar SB, Wang J, Deo RP, Musameh M, Ogorevc B. Electroanalysis 2005;17:417–422.
42. Westerfield, M. The zebrafish book : a guide for the laboratory use of zebrafish (Brachydanio rerio). M. Westerfield; Eugene, OR: 1993.
43. Wallace KN, Akhter S, Smith EM, Lorent K, Pack M. Mechanisms of Development 2005;122:157–173. [PubMed: 15652704]
44. Patel BA. Analyst 2008;133:516–524. [PubMed: 18365122]

45. Currell, C. Analytical Instrumentation Performmance Characteristics and Quality. 1. John Wiley and Sons Ltd; Dartford: 2000.

46. Brazell MP, Kasser RJ, Renner KJ, Feng J, Moghaddam B, Adams RN. Journal of Neuroscience Methods 1987;22:167–172. [PubMed: 2893860]

47. Patel BA, Bian XH, Quaiserova-Mocko V, Galligan JJ, Swain GM. Analyst 2007;132:41–47. [PubMed: 17180178]

48. Pletscher A, Shore PA, Brodie BB. Science 1955;122:374–375. [PubMed: 13246642]

49. Reimann W, Schneider F. Eur J Pharmacol 1998;349:199–203. [PubMed: 9671098]

50. Dryhurst G. Chemical Reviews 1990;90:795–811.

51. Wang Y, Berndt TJ, Gross JM, Peterson MA, So MJ, Knox FG. Am J Physiol Regul Integr Comp Physiol 2001;280:R248–254. [PubMed: 11124158]

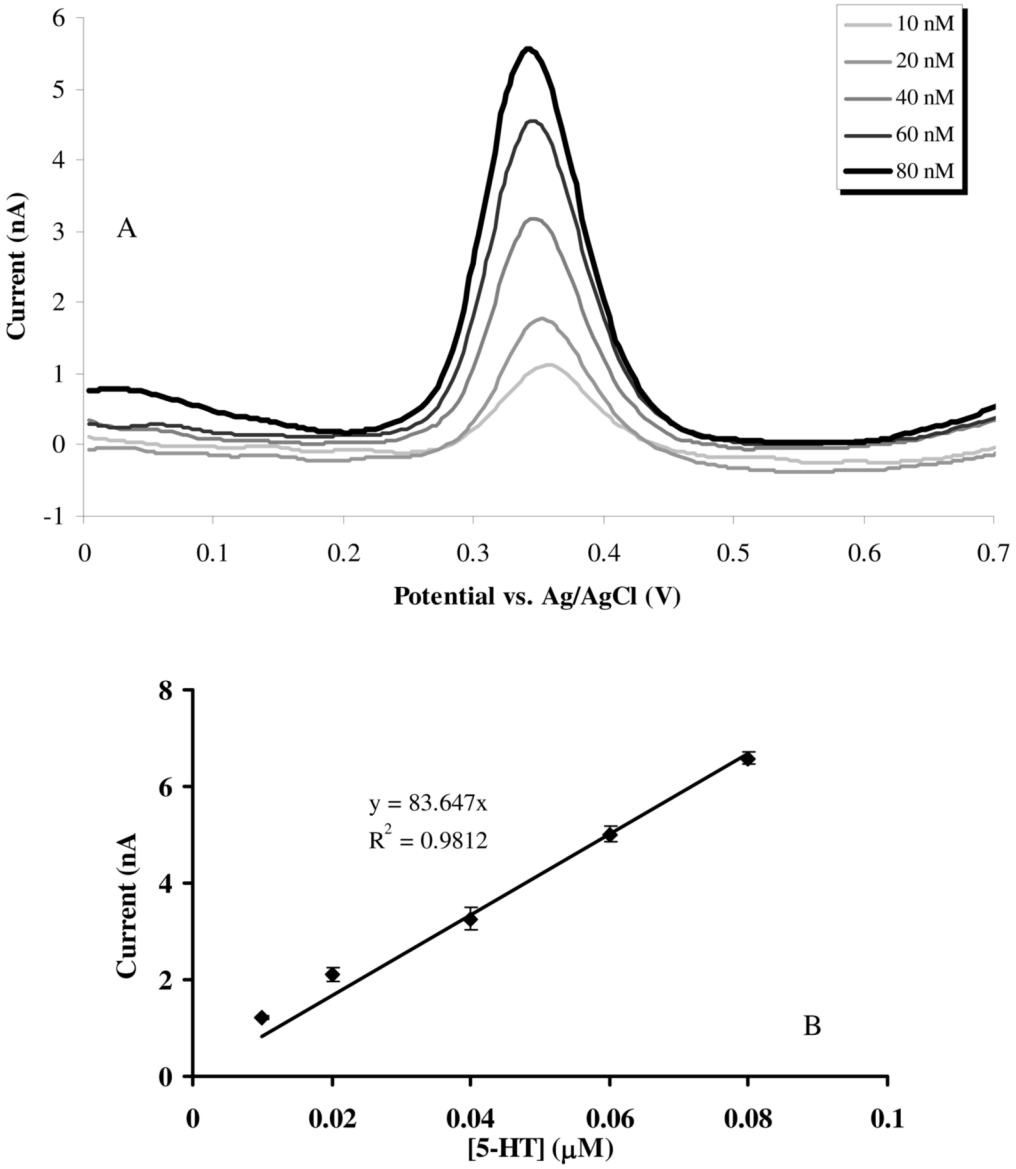


**Figure 1.**

**(A)** Differential pulse voltammograms of the various concentrations of 5-HT at a carbon nanotube –modified microelectrode after background subtraction. **B:** The corresponding calibration curves for 5-HT (the error bars represent standard deviation for n=3 replicate measurements). The supporting electrolyte was 0.1 M phosphate buffer at pH 7.4.

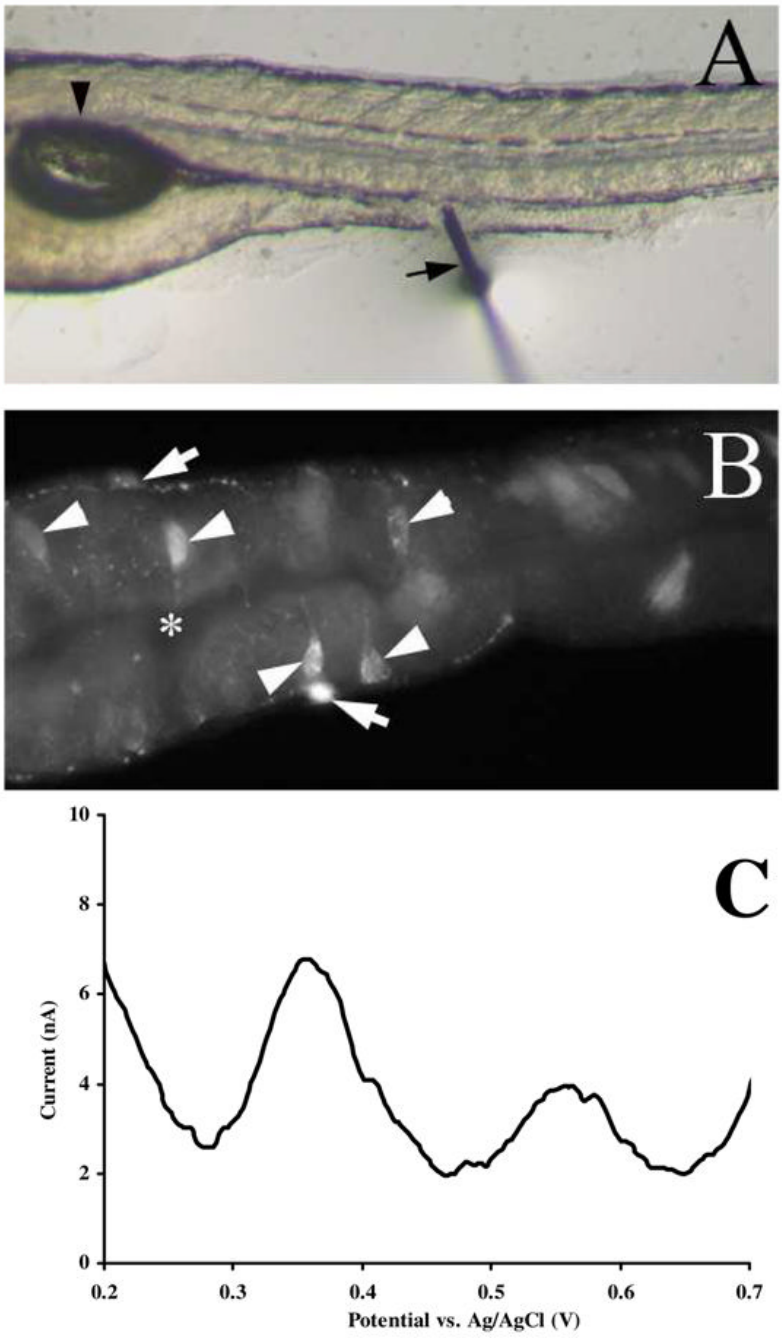


**Figure 2.**

**A:** Insertion of the electrochemical microsensor into the intact embryonic intestine between the posterior swim bladder (*) and the end of the intestine (#) at 5 dpf. Arrowhead indicates swim bladder and arrow shows point of insertion of the microelectrodes. **B:** 5-HT immunohistochemistry reveals a subset of enteric neurons (arrows) as well as enterochromaffin cells within the epithelium (arrowheads). Enteric neurons are on the periphery of the intestine between the thin circular and longitudinal smooth muscle layers. Enterochromaffin cells are situated more toward the basal surface of the epithelium that sense contents of the lumen (asterisk) using thin processes. **C:** Typical differential pulse voltammogram obtained *in vivo* with the implanted microelectrode as shown in A. The supporting electrolyte was E3 medium.

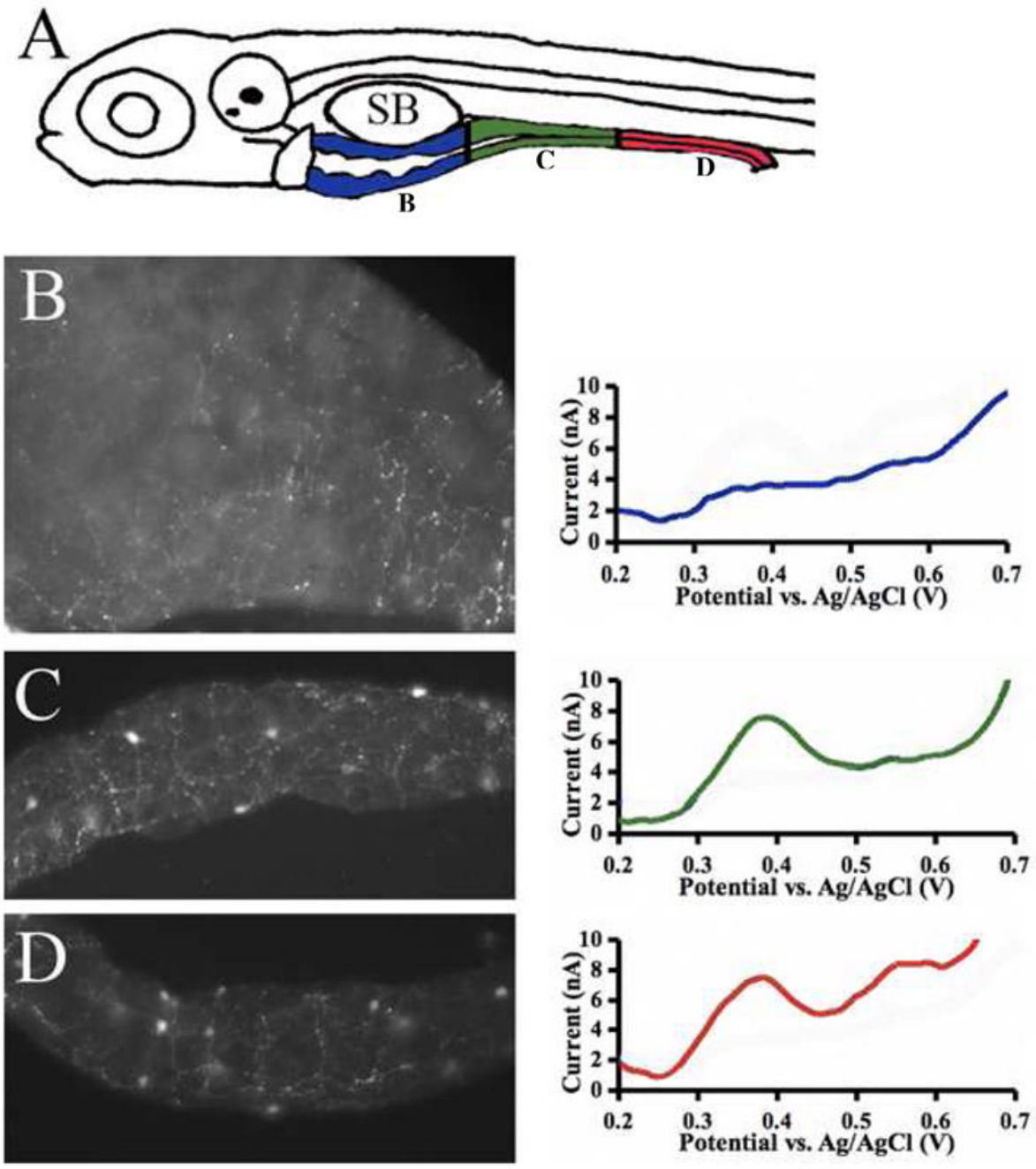


**Figure 3.**

**A:** Schematic representation depicting anterior (blue-B), mid (green–C) and posterior (red–D) segments of the 5 dpf embryonic intestine. The colors match the line color of the voltammetric profiles measured with the microsensor *in vivo* at the respective locations within the intestine. **B, C, D:** Anti-5-HT immunohistochemistry (fluorescence images on the left panel) and differential pulse voltammograms (diagrams on the right panel) measured at the anterior (B), mid (C) and posterior (D) parts of the intestine. The supporting electrolyte was E3 medium. All images have anterior to the left and posterior to the right. All images are identical in scale.

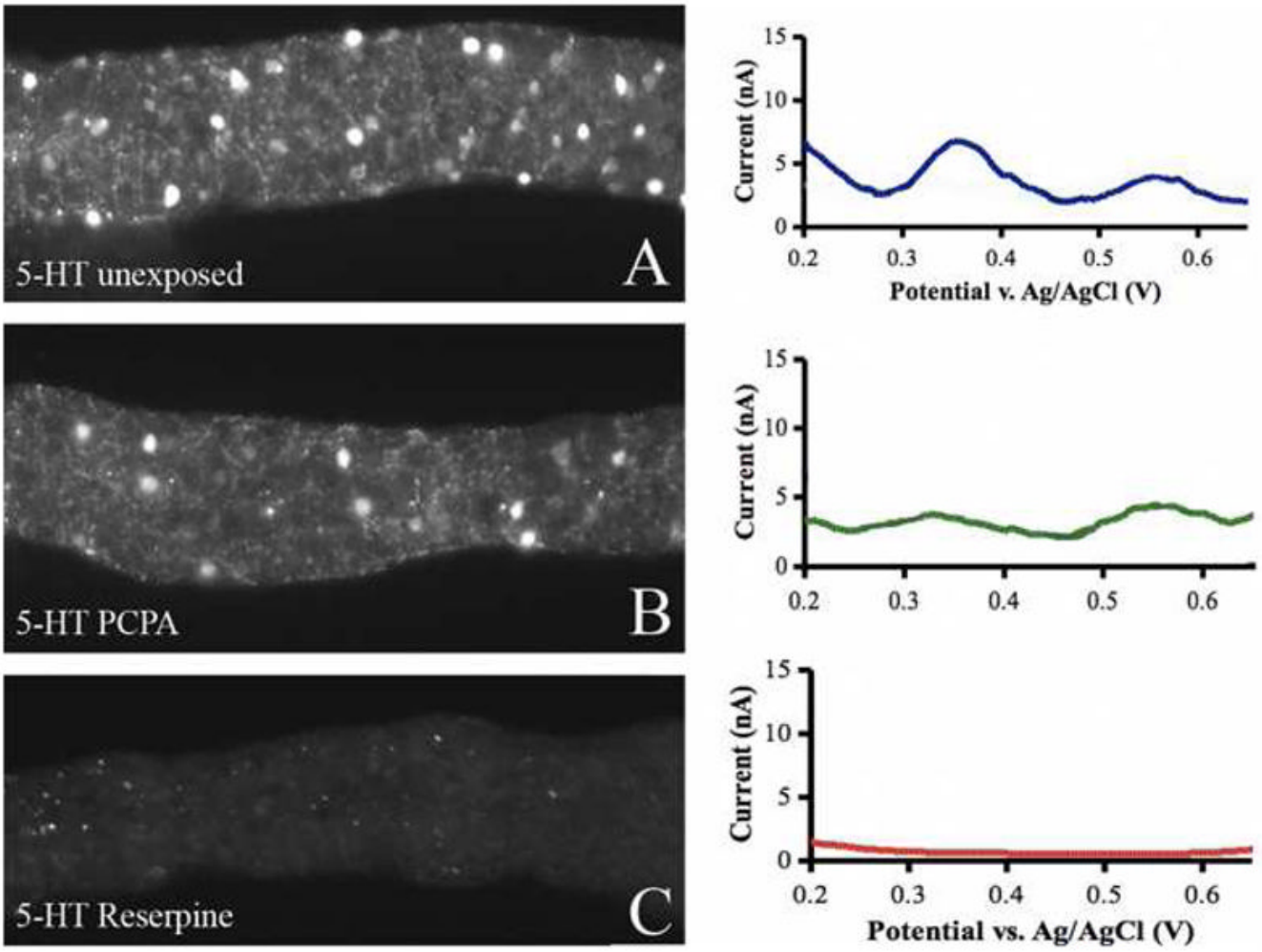


**Figure 4.**

Reduction in 5-HT levels after pharmacological manipulation. Anti-5-HT immunohistochemistry (fluorescence images on the left panel) and the corresponding differential pulse voltammograms of microelectrodes (graphs on the right panel) measured for unexposed embryos (A), for embryos exposed to the tryptophan hydroxylase inhibitor PCPA (B), and a monoamine transporter, reserprine (C). Application of PCPA to whole 5 dpf embryos results in visible decreases in 5-HT levels within the intestine (B). In contrast, reserpine drastically reduces 5-HT levels throughout the intestine(C). All images have anterior to the left and posterior to the right. All images are identical in scale.

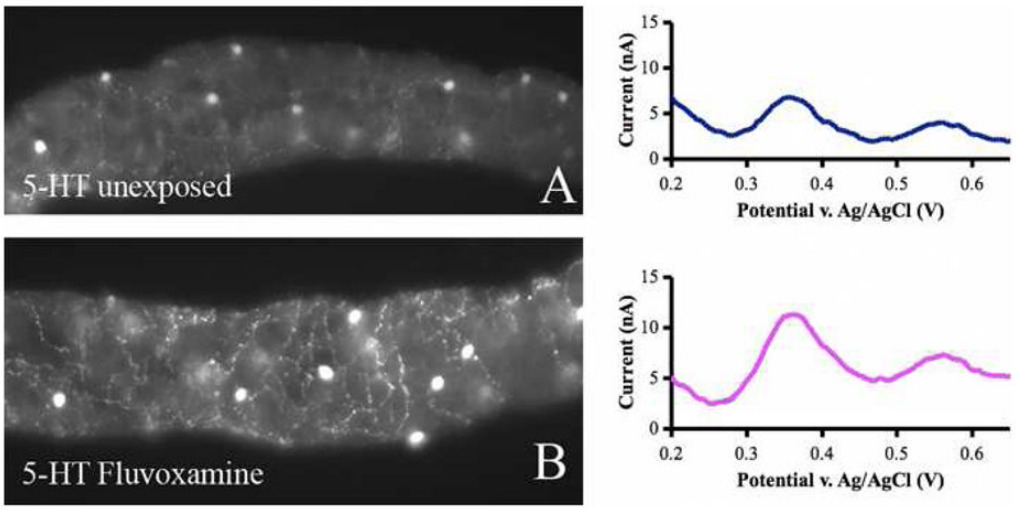


**Figure 5.**

5-HT increase after SERT inhibition. Anti-5-HT immunohistochemistry demonstrates higher levels of serotonin within the intestine coupled with a higher reading from differential pulse voltammograms of microelectrodes after treatment of whole embryos with fluvoxamine (B) when compared to unexposed controls (A). All images have anterior to the left and posterior to the right. All images are identical in scale.

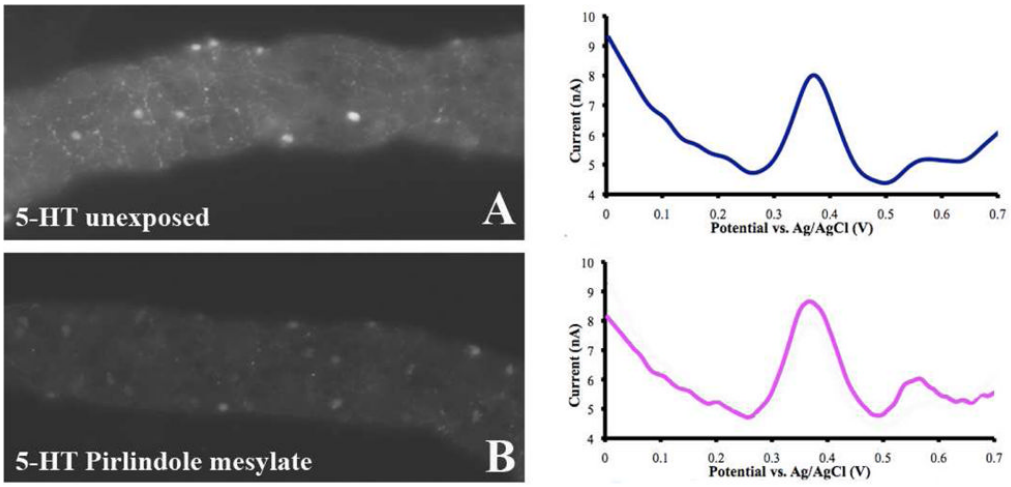


**Figure 6.**

5-HT increase after monoamine oxidase inhibition (MAOI). Anti-5HT immunohistochemistry reveals similar to lower serotonin levels within the intestine of MAOI treated 5 dpf embryos (B) when compared to unexposed controls (A). Differential pulse voltammograms of microelectrodes record an overall increase in 5-HT levels (compare right graph B to right graph A). All images have anterior to the left and posterior to the right. All images are identical in scale.