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Research Report

# 5-Hydroxytryptamine$_{2C}$ receptors on pudendal motoneurons innervating the external anal sphincter

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**Abstract**

The aim of this study was to determine the localization of 5-hydroxytryptamine$_{2C}$ (5-HT$_{2C}$) receptors on the motoneurons innervating the external anal sphincter (EAS) of male rats. Motoneurons were retrogradely labeled after percutaneous intramuscular injection of Fluorogold (FG) into the EAS. Using fluorescent immunohistochemistry, FG-positive EAS motoneurons that were immunoreactive for the 5-HT$_{2C}$ receptor (5-HT$_{2C}$-IR) were targeted for specific examination with widefield microscopy or confocal laser scanning microscopy with spectral separation. Widefield microscopy revealed distributions of FG-positive EAS motoneurons in the L5–S1 gray matter corresponding to the dorsomedial cell group. 5-HT$_{2C}$-IR positive cells were distributed in the intermediolateral cell column and the ventral horn. Ventral horn 5-HT$_{2C}$-IR labeling included the dorsomedial cell group as well as the dorsolateral, ventromedial and ventrolateral areas. Confocal analysis of FG-positive EAS motoneurons and 5-HT$_{2C}$-IR positive motoneuron profiles adjacent to EAS motoneurons that were not labeled with FG but presumably innervate the bulbospongiosus muscle confirmed that EAS motoneurons were immunopositive for the 5-HT$_{2C}$ receptor. These data suggest that previously identified descending serotonergic immunopositive fibers observed terminating on EAS motoneurons might mediate their input through the activation of 5-HT$_{2C}$ receptors.


*Theme:* Endocrine and autonomic regulation
*Topic:* Gastrointestinal and urogenital regulation

*Keywords:* Continence; Defecation; Gastrointestinal function; Serotonin

## 1. Introduction

The ventrolateral medulla is a major source of descending serotonergic pathways [7,55,63]. Included in this region are neurons within the gigantocellular and lateral paragigantocellular nucleus (Gi–lPGi, inclusive). Descending serotonergic control of pudendal reflexes has received considerable attention in the control of sexual function [1,33,34,43,45]. In light of the widespread distribution of the terminal fields of descending projections from the Gi–lPGi complex [21], it is not surprising that the ventrolateral medulla has been implicated in the serotonergic regulation of sexual reflexes [58].

In the male rat, the motoneurons innervating the bulbospongiosus and external anal sphincter (EAS) muscles have been shown to fully co-mingle within a dorsomedially located region of the L5–S1 spinal cord [20,21,44,54]. These motoneurons respectively innervate the striated muscles necessary for the somatic motor control of erection (e.g., the bulbospongiosus muscles [18,50]) and defecation (e.g., the EAS [12]). These motoneurons receive terminal appositions from the Gi–lPGi [21] as well as the nucleus raphe obscurus (nRO) originating within the caudal medulla [20].

The motoneurons of both the bulbospongiosus and ischiocavernosus have been demonstrated as being immunoreactive for the 5-HT$_{2C}$ receptor [3]. Similar results for pudendal motoneurons have been reported using autoradiographic techniques [60]. The verification of 5-HT receptors

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on EAS motoneurons is less clear, despite the apposition of fibers immunoreactive for serotonin (5-HT-IR) in intact and long-term spinal-cord-injured rats [26]. The report by Bancila and colleagues did note 5-$HT_{2C}$ immunoreactivity in unidentified cells located within the dorsomedial nucleus [3]. These cells were not labeled with the tracer that was applied to the bulbospongiosus muscle. Whether this was due to immunoreactive labeling of components of the bulbospongiosus that did not receive tracer or to immunoreactive labeling of EAS motoneurons could not be determined. The present studies were designed to verify the presence of 5-$HT_{2C}$ receptors on a specific sub-population of pudendal motoneurons involved in the control of eliminative reflexes.

## 2. Materials and methods

### 2.1. Animals

Long–Evans male rats ($n$ = 11; Simonsen Laboratories, Gilroy, CA), weighing 200–500 g, were maintained in a temperature-controlled room on a 12:12-h light–dark cycle. Animals were housed two to a cage in hanging plastic tubs and had access to food and water ad libitum. All procedures were performed according to National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center. The vivarium is an AALAC-approved facility under the direction of a licensed laboratory animal veterinarian.

### 2.2. Experimental design

Experimental conditions consisted of adjacent serial sectioned tissue sets from experimental animals that received percutaneous injections of the retrograde tracer Fluorogold (Fluorochrome LLC, Denver, CO) into the EAS 1 week prior to sacrifice. One set of tissue received both primary and secondary antibody immunohistochemical treatment. Adjacent sets were used for control slides in which the primary antibody was omitted.

### 2.3. Surgical preparations

All animals were deeply anesthetized with isoflurane (3–5%/L $O_2$; Baxter, Deerfield, IL). Percutaneous injection of 2% Fluorogold dissolved in sterile water was made in 5 μL increments at 4 uniformly spaced locations in the EAS (20 μL total injection). Following recovery from isoflurane anesthesia, animals were returned to their home cages. Animals were maintained for 7 days following the surgical procedure.

### 2.4. Histology

Following terminal nembutal anesthesia (75 mg/kg, IP), animals were transcardially perfused with a bolus of heparinized lidocaine in ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The spinal cord was removed in one 15-mm block, encompassing L4 through S2. Tissue was post-fixed overnight at 4 °C in paraformaldehyde with 20% sucrose and processed the next day. Spinal cord blocks were cut frontally at 40 μm on a freezing stage microtome (Microm HM400, Richard-Allan Scientific, Kalamazoo MI) and the sections mounted on Chrom-Alum subbed slides. For all tissue preparations, two complete sets of slides containing alternating sections were made. Tissue sections were dried overnight to ensure that they attached thoroughly to the gelatin coating on the slides.

### 2.5. Serotonin type$_{2C}$ receptor immunofluorescence

Following rinsing (3×) with phosphate-buffered saline (PBS, pH 7.4), mounted sections were incubated 1 h in blocking solution (0.25% Triton + 5% normal goat serum). Sections were incubated overnight in primary antibody (5-$HT_{2C}$ rabbit antibody—1:500, Immunostar, Inc, Hudson, WI) at 4 °C. The primary antibody was raised against a synthetic peptide corresponding to sequence 439–460 in the rat 5-$HT_{2C}$ receptor. This sequence corresponds to the 5-$HT_{2C}$ c-terminus in the rat. After rinsing (3×) with PBS, the sections were incubated in secondary antibody-conjugated to Alexafluor 488 (goat anti-rabbit, 2.5 h incubation, 1:200, Molecular Probes) at room temperature. Slides were rinsed a final time (3×) with PBS and coverslipped with Vectashield Hard Set (Vector, Inc.) mounting media. Negative controls consisted of omitting the primary (5-$HT_{2C}$) antibody. The lack of a commercially available synthetic peptide for the 5-$HT_{2C}$ receptor precluded running a preabsorption control with a corresponding antigen. The preponderance of 5-$HT_{2C}$ positive cells in the spinal cord, combined with the small number of EAS motoneurons, also precluded performing controls utilizing a Western blotting technique of spinal cord homogenates since specificity of the tissue to exclusively contain EAS-derived 5-$HT_{2C}$ receptors cannot be guaranteed. Both techniques were indicated as having been performed to test the specificity of the antibody by the commercial supplier (Immunostar, Inc.).

### 2.6. Widefield fluorescence and laser scanning microscopy

Low power observations of labelled sections were made using an Everest Microscopy Workstation (Slidebook Ver. 4.0.8.0, 3i Systems, Denver, CO.) attached to a Zeiss Axioplan microscope with a motorized $x–y–z$ stage. Pseudo-color low power images were captured with a Coolsnap HQ CCD camera (Roper Scientific, Munich, Germany) at 1392 × 1040 resolution. Sections were screened for Fluorogold and Alexa488 labeling, and low power images were generated with a 10× Fluar objective using the automatic montage-capturing feature of the Everest software. Sections with positive double labeling were identified for later confocal analysis.