Published in final edited form as:

Neurogastroenterol Motil. 2021 August ; 33(8): e14014. doi:10.1111/nmo.14014.

Multicolor sparse viral labeling and 3D digital tracing of enteric plexus in mouse proximal colon using a novel adeno-associated virus capsid

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Abstract

Background: Intravenous administration of adeno-associated virus (AAV) can be used as a noninvasive approach to trace neuronal morphology and links. AAV-PHP.S is a variant of AAV9 that effectively transduces the peripheral nervous system. The objective was to label randomly and sparsely enteric plexus in the mouse colon using AAV-PHP.S with a tunable two-component multicolor vector system and digitally trace individual neurons and nerve fibers within microcircuits in three dimensions (3D).

Methods: A vector system including a tetracycline inducer with a tet-responsive element driving three separate fluorophores was packaged in the AAV-PHP.S capsid. The vectors were injected retro-orbitally in mice, and the colon was harvested 3 weeks after. Confocal microscopic images of enteric plexus were digitally segmented and traced in 3D using Neurolucida 360, neuTube, or Imaris software.

Key Results: The transduction of multicolor AAV vectors induced random sparse spectral labeling of soma and neurites primarily in the myenteric plexus of the proximal colon, while neurons in the submucosal plexus were occasionally transduced. Digital tracing in 3D showed various types of wiring, including multiple conjunctions of one neuron with other neurons, neurites *en route*, and endings; clusters of neurons in close apposition between each other; axon–

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AUTHOR CONTRIBUTIONS

LW: experiments, image acquisition, digital tracing and writing the manuscript; CC: AAV production and injection in mice, and writing the manuscript; SL: image acquisition, processing and 3D digital segmentation; CCF: digital tracing and writing the manuscript; SRK: AAV production; P-QY: imaging, data interpretation; YFT: writing the manuscript.

CONFLICT OF INTEREST

The authors have no competing interests.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

axon parallel conjunctions; and intraganglionic nerve endings consisting of multiple nerve endings and passing fibers. Most of digitally traced neuronal somas were of small or medium in size.

Conclusions & Inferences: The multicolor AAV-PHP.S-packaged vectors enabled random sparse spectral labeling and revealed complexities of enteric microcircuit in the mouse proximal colon. The techniques can facilitate digital modeling of enteric micro-circuitry.

Keywords

3D imaging; adeno-associated virus; colonic enteric neuron; digital tracing; mouse

1 | INTRODUCTION

Adeno-associated viruses (AAVs) are commonly used as vectors for gene therapy,^{1,2} functional research,^{3,4} and also as a tool for neuroanatomical tracing.^{5–8} AAV8 and AAV9 have been validated for efficient delivery to colonic enteric neurons by iv injections in newborn and/or young mice,^{9,10} and guinea pigs and primates.¹¹ PHP.S is a new engineered variant of AAV9 that has been reported to transduce peripheral neurons with increased efficiency.^{12,13} Recently, a tunable two-component multicolor four-vector system (AAV-PHP.S:hSyn-tTA:TRE-XFP) was established to trace individual neurons and connectivity via random and combinatorial expression of spectrally distinct fluorescent proteins (XFP).¹² The first component of the vector system is an inducer (AAV-PHP.S:hSyn-tTA), which was engineered to contain the synapsin-I promoter driving the expression of the tetracycline (tet) off transactivator (tTA). The second component consists of three vectors that use tet-responsive elements (TRE) to drive the expression of fluorescent proteins, for example, each carrying one of the red, green, and cyan colors. Doses of the inducer vector could be adjusted to induce random sparse labeling in neurons where vectors carrying XFP are expressed with random color mix contributing to the spectral variability.^{12,14}

In the present study, we injected the multicolor two-component tunable AAV-PHP.S vector system, AAV-PHP.S:hSyn-tTA:TRE-XFP^{12,14} to label systemically and randomly the enteric plexus in the mouse proximal colon, and digitally traced individual neurons and fibers in 3D confocal images. We focused initially on the proximal colon because of its large enteric ganglia¹⁵ and specific functions.¹⁶ We tested the inducer at several doses to optimize sparse labeling for digital tracing of individual neurons and nerve fibers and used newly developed tracing software to reveal in 3D specific neuronal morphologies and microcircuits wiring inside the myenteric ganglia.

2 | EXPERIMENTAL PROCEDURES

2.1 | Animals

Experiments were performed in mice C57BL/6J (Jackson Laboratories), male and female, 6–10 weeks old. Animal care and experimental procedures followed institutional ethics committee guidelines of the Veterans Affairs Greater Los Angeles Healthcare System (animal protocol approval #07013-17) and conformed to the requirements of federal regulations for animal research conduct.

2.2 | Viral constructs

The constructs of pAAV-hSyn-tTA (#99120, Addgene), pAAV-TRE-mNeonGreen (#99131, Addgene), pAAV-TRE-mRuby2 (#99114, Addgene), and pAAV-TRE-mTurquoise2 (#99113, Addgene) were developed in Dr. V. Gradinaru's laboratory (California Institute of Technology, Pasadena, CA).¹⁷ Namely, viruses were packaged with the AAV-PHP.S capsid (pUCmini-iCAP-PHP.S, #103006, Addgene) in HEK-293T cells and purified in-house as detailed previously.¹⁷

2.3 | Retro-orbital injection of AAV

Mice were anesthetized with 2.5% isoflurane in oxygen and injected with the four-vector mix diluted in phosphate-buffer saline into one side of the retro-orbital venous sinus (60 μ l/mouse) using a BD Veo Insulin Syringe with BD Ultra-Fine 6 mm $\times$ 31G needle (324911, Becton, Dickinson and Company). The inducer vector, AAV-PHP.S:hSyn-tTA, was tested in male (M) and female (F) mice at 2.5×10^9 (6 M), 5×10^9 (2 F), 1×10^{10} (7 M, 5 F), 5×10^{10} (5 M, 6 F), or 1×10^{11} (4 M, 4 F) genome copies (GC)/mouse with the same total dose (1×10^{12} GC/mouse) of three fluorescent protein vectors mix (AAV-PHP.S:TRE-mRuby2, AAV-PHP.S:TRE-mNeonGreen, and AAV-PHP.S:TRE-mTurquoise2).

2.4 | Tissue sampling

Three weeks after AAV injection, mice were euthanized by an overdose of isoflurane. The survival time was selected based on previous studies using the same AAV.PHP.S serotype showing that the transduction reaches a steady level after 3–4 weeks.^{12,17} The colon was removed, cleared, and embedded. To compare the labeling of the proximal colon with that in other segments of the gastrointestinal (GI) tract and peripheral ganglia, the different parts of GI tract, the dorsal root ganglion at L6, and celiac and pelvic ganglia were also collected (Methods S1).

2.5 | Image acquisition and processing

Microscopic images were acquired in Zeiss confocal microscopes (LSM 710 and 880). The 3D reconstruction, segmentation, and digital neuronal tracing were performed using Neurolucida 360 (MBF Bioscience), a customized version of neuTube¹⁸ and/or Imaris 9.2 and 9.5 for neuroscientists (Bitplane Inc.). The soma sizes and shapes of myenteric neurons in the proximal colon were measured in Neurolucida Explorer (MBF Bioscience). Details of the methods are described in the Appendix S1, and the photomicrography information is specified in Table S1 in the Appendix S1.

3 | RESULTS

Injection of the inducer vector at the doses of 1×10^{10} , 5×10^{10} , and 1×10^{11} GC/mouse yielded sparse, random labeling of individual neurons and fibers in the proximal colon. The population of labeled neurons induced by a same dose varied which could reflect differences in the efficiency of viral vector batches used in duplicate experiments. The multicolor AAV vectors transduced soma and neurites primarily in the myenteric plexus and in fibers innervating the circular muscle layer (Figure 1A–C; Figure S8C and Video S1), while in the submucosal plexus, nerve fibers and a few weakly labeled somas were

observed (Figure 1D; Video S1). The AAV-transduced myenteric neurons were distributed unevenly in the proximal colon as shown by ganglia containing transduced neurons from many to a few or none labeled cells (Figure 1B,C). Neurons in the transverse and distal colon were not transduced at all doses tested; however, a few nerve fibers were labeled (Figure S1). The gastric antrum and small intestine had less transduction than the proximal colon, and neurons and nerve fibers in the lower esophagus and gastric corpus were rarely labeled, similar to the distal colon (Figure S1). The dorsal root ganglia at L6 and the celiac and pelvic ganglia contained numerous AAV-transduced neurons, demonstrating the effectiveness of the AAV-PHP.S capsid at transducing neurons in peripheral ganglia (Figure S2).

We designated small, medium, and large nerve cell somas by the maximal 2D area and major (longest) diameter, and the round and elongated somas by the ratio of major/minor diameters (Table 1). Majority of nerve cell somas randomly and sparsely transduced by the multicolor AAV system were in medium size (54.8% with major diameter between 20–30 μm and 56.3% with area between 200–400 μm^2) and oval shape (ratio of major/minor diameter at 1.3–2.5). Small somas were 33.6% with major diameter <20 μm and 37.6% with area <200 μm^2 , while large somas with major diameter >30 μm were 11.6% and area >400 μm^2 were only 6.1% (Figure S3). The maximal areas of all the somas were 79–690 μm^2 and major diameters 12–49 μm (Table 1).

AAV-transduced myenteric neurons in the mouse proximal colon were predominantly Dogiel type I-like neurons, although the neuronal morphology did not follow the Dogiel classification. The Dogiel type I-like neurons appeared with somas of various shapes and sizes, short, thick and irregular dendrites and one axon (Figures 1B,C, 2A and 4). Other types were much less prominent and occasionally observed, namely uniaxonal neurons with smooth soma of various shapes and sizes, small neurons with short processes, and multiaxonal neurons (Figures 2 and 3, Figure S4). Many neurons of oval or small round shape did not display processes (Figure 1B,C). We also observed several medium- or large-size neurons with a thick varicose axon containing brighter fluorescence (Figure 1C and Figure S9C). Based on the randomly AAV-labeled neurons in 95 images acquired in confocal microscopes from the proximal colon of 14 mice (7 M, 7 F), we found 1548 AAV-labeled neurons, 272 were Dogiel type I-like, 119 uniaxonal with smooth somas, 36 small somas with short processes and 13 were multiaxonal.

The digital traces in 3D demonstrated various types of neurons and wiring. Namely, somas grouped in a cluster (Figures 2C and 4), close appositions of one neuron with other neurons and nerve fibers and endings (Figure 3A, Figures S6C and S8); small neurons with short projections to passing fibers including ganglion-crossing fibers (Figures 2E and 4B); and uniaxonal neurons with large bright varicosities projecting out of a ganglion (Figure 1C; Figure S9C). Some multiaxonal neurons were observed adjacent to different neural structures inside a ganglion and projections through interganglionic strands (Figures 2B,D and 3A and Figure S4).

Regarding nerve fibers and endings, we observed (a) axons running in close parallel juxtaposition to each other or with nerve fibers (Figure 4, Figure S5–S8); (b) nerve fibers

crossing ganglion with apparent contacts with several neurites *en route* (Figure 4 and Figure S4D); (c) intraganglionic nerve endings, complex type (IGNEx) bearing large beaded enlargements and varicosities in axons, forming a nest with several nerve endings, as well as neuronal processes and passing fibers from interganglionic strands that made presumed junctions (Figure 3, Video S2), but only 2 of them were found in 95 images; and (d) varicose endings were more common than IGNEx (Figure 1B, Figure S9A,B).

Digital traces using Neurolucida 360 and neuTube showed similar neuronal structures and networks (Figure S10).

4 | DISCUSSION

Traditional methods for morphological studies of enteric neurons are silver impregnation,¹⁹ immunohistochemistry,²⁰ intracellular dye filling,^{21–23} and retrograde tracing.²⁴ Although silver impregnation and intracellular dye filling can label enteric neurons with detailed morphology, the monochromaticity of the method makes it difficult to trace connectivity. The morphological profiles depicted by immunohistochemistry labeling of proteins, peptides, and enzymes depend on their locations in neurons. Retrograde tracing only labels neurons projecting to a given target. The advantage of sparse multicolor AAV systems is the transduction of neural elements with various hues in a small population.¹² This enables 3D digital tracing of individual neurons and nerve fibers to unveil their local networks in a microcircuit. However, it is to note that the morphological details were not as precise as intracellular dye filling. A previous study performing 3D reconstruction of individual neurons labeled in guinea pig ileum by intracellular dye demonstrated different types of neurons, but not their connections.²⁵

In the present study, we used primarily major and minor diameters, and ratio, known as parameters for the classification of neuronal somas by sizes and shapes. We showed that 94% of the myenteric neurons transduced by AAV-PHP.S-packaged multicolor AAV vectors in the mouse proximal colon were small and medium in size (100–400 μm^2 ; Figure S3), similar as reported in the rat colon.²⁶ We also found that 95% of the AAV-labeled neurons displayed round and oval shapes, consistent with a previous report that demonstrated by immunostaining and histochemistry, many small round or oval myenteric neurons in the guinea pig proximal colon.²⁷ It should be pointed out that the AAV-labeled neurons do not represent either the whole or any functional subpopulations, but those randomly and sparsely transduced.

The classification of enteric neurons was initially described by A. S. Dogiel based on the histological differentiation, and the possibility of distinguishing between neuronal processes as dendrites and axons.¹⁹ Since 1970s, enteric neurons have been thoroughly studied and classified using immunohistochemistry, retrograde tracing, intracellular dye staining, and electrophysiology,^{20,21,28,29} and the morphology revealed by other methods does not show all Dogiel types.¹⁹ Dogiel type II was the most prominently traced and characterized in several species by intraneuronal dye filling into myenteric neurons.^{22,30,31} Messenger et al.³¹ classified myenteric neurons in the guinea pig proximal colon in 8 categories distinguished morphologically by intraneuronal dye injection after functional

characterization by electrophysiology in AH and S neurons. In the present study, the labeling obtained by multicolor two-component tunable AAV system is random, similar to silver impregnation.¹⁹ Interestingly, the Dogiel type I neurons transduced by the multicolor AAVs were the type most prominently observed, consistent with the previous report in guinea pig using immunofluorescence.²⁷ However, processes of many neurons were not as well labeled as obtained by silver impregnation or intracellular dye injection, although the morphology of some neurons was delineated. Therefore, a comprehensive classification of myenteric neurons could not be achieved to compare with that described in the proximal colon of guinea pigs.³¹ Importantly, the present approach allowed us to highlight the complexity of the myenteric ganglia of mouse proximal colon. In particular, we showed a single neuron making apparent connections with different neural elements, various neuronal structures joining in the IGNE_x, clusters of neurons closely apposed to each other and small neurons with multi-processes wired with long crossing nerves which could be interneurons.¹⁹ These complex micro-circuitries may facilitate communications of neural activity among motor and interneurons, efferent and afferent terminals, and intra- and interganglionic nerve fibers. The spread of projections of multiaxonal neurons support a primary role in responding to physiological stimuli that evoke activation of the enteric network.^{22,32,33}

The study also suggests that AAV vectors may be able to transduce both extrinsic and intrinsic nerve fibers. The long straight circumferential fibers observed in the circular muscle layer may possibly be extrinsic afferent endings, as vagal efferent nerves only terminate in myenteric ganglia, not in muscle layer.³⁴ This is supported by the present observation that AAV-PHP.S densely labeled neurons in the sensory and autonomic ganglia, consistent with previous reports.^{12,13} There is also evidence from anterograde tracing experiments that labeled sympathetic nerve fibers run circumferentially with abundant branches of one terminal innervating several ganglia in the myenteric plexus.³⁵ Here, in the multicolor AAV-PHP.S labeled myenteric plexus, the 3D image and digital tracing demonstrated that nerve fibers were interweaving or passing a basket of nerve endings. The morphology is not typical of afferent endings from the vagus or spinal nerves,^{36–38} as it has neither laminar endings nor conventional varicose endings,³⁷ but a nest of large beaded nerves. A recent study using dual or triple immunofluorescent labeling in the mouse distal colon demonstrated varicose baskets of intrinsic nerve terminals.³⁹ Therefore, we designated the observed endings as intraganglionic nerve endings, complex type (IGNE_x). The unique morphology and multiple components of IGNE_x could function as a micro-unit that triggers activation of various components in the network.

It should be taken into account that these sparse multicolor viral and digital tracing techniques are recently developed and have limitations that will need to be addressed. (a) The random sparse labeling is not suitable for quantitative analysis as we observed an uneven pattern of distribution. (b) Importantly, it was unexpected and yet, unexplained, why neurons were transduced more prominently in the proximal colon while not in the distal colon and the gastric corpus. (c) The segmental selective enteric transduction compared to the abundant transduction in other peripheral ganglia indicates the need for additional research involving the development of new serotype AAV. (d) The digital tracing of nerve fibers often needs manual corrections, which is a time-consuming process as previously pointed out.⁴⁰ The improvement of image resolution using powerful microscopes to

visualize deep layers will enhance the efficiency of detecting fluorescent signals and reduce manual corrections. (e) Software tools for neuronal tracing require additional automated features to trace the detailed morphology of neuronal soma, nerve fibers, and varicosities with precision. For instance, Imaris program has new features improving the tracing of soma and fibers (Figure S4).

In summary, we used a multicolor two-component tunable AAV system and 3D digital tracing to depict individual neural wiring patterns in the enteric microcircuits of mouse proximal colon. This revealed both neuron and fiber units wired in a complex organization. Although these combined approaches still have limitations, the labeling and tracing techniques are undergoing continuous improvement, and may facilitate digital modeling of enteric micro-circuitry. The digital segmentation approaches have potential to be used for tracing neuronal structures labeled by different fluorescent markers or tracers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Dr. V. Gradinaru (Division of Biology, California Institute of Technology, Pasadena, CA) for the supply of AAV, and Ms. Aiden Sullivan and Susan Tappan of MBF Biosciences for support with Neurolucida 360. The authors are grateful to Ms. Honghui Liang and Ming Johnson for their excellent technical assistance.

Funding information

NIH SPARC award OT2OD24899, Department of Veterans Affairs Rehabilitation Research and Development grant 1RX001685, Veterans Administration Senior Research Career Scientist Award, P30 NIHDDK-41301 UCLA/Digestive Diseases Research Core Center Grant.

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Key points

- AAV-PHP.S, a peripheral AAV serotype, was used to deliver intravenously a multicolor vector system that randomly transduced enteric neurons in mouse proximal colon and resulted in sparse multispectral labeling.
- 3D imaging, digital segmentation, and tracing of individual neuronal somas and nerve fibers enabled the identification of novel morphological features.
- Further development of these tools can help with reconstruction and modeling of microcircuits in the enteric nervous system.

FIGURE 1.

Confocal microscopic images of multicolor AAV-PHP.S randomly and sparsely transduced neurons and nerve fibers in mouse proximal colon. Mice were injected retro-orbitally with the inducer vector, AAV-PHP.S-hSyn-tTA (1×10^{10} , 5×10^{10} , or 1×10^{11} GC/mouse) mixed with the fluorophores-carrying vectors, PHP.S-TRE-XFP (total dose of 1×10^{12} GC/mouse). Images were acquired in Zeiss confocal microscope 880 or 710 and visualized in Imaris 9.2. A, Random and sparse AAV transduction. The sample was prepared by removing the mucosa layer to reduce diffusion fluorescent signals that interfere with the focus. The image was acquired in 10X objective and stitched by 9 titles (from a male mouse, inducer dose 1×10^{11} GC/mouse). B, C, Representative images at higher magnification showing the random sparse multicolor AAV transduction in neurons and nerve fibers in the myenteric plexus and circular muscle layer (from a male mouse, inducer dose 1×10^{11} GC/mouse).

*The ganglia containing few/no labeled neurons. A cyan-colored neuron in panel C has a

long thick varicose axon (arrow). B, Varicose fiber branched forming pericellular endings (a yellow fiber from the top). Straight vertical nerve fibers in the images A-C are the nerve endings in the circular muscles layer (also shown in Figure S6C). One bundle is indicated by a yellow arrow in B. D, Occasionally AAV-PHP.S-transduced submucosal plexus (from a female mouse, inducer dose 1×10^{10} GC/mouse)

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FIGURE 2.

Confocal microscopic (A) and digital tracing (B-E) images in 3D of multicolor AAV randomly and sparsely transduced neurons and nerve fibers in mouse proximal colon. The AAV-PHP.S inducer dose was 1×10^{10} GC/mouse, and the sample in A was collected from a female mouse. B, Digital traces of various types of neuronal somas and fibers in a myenteric ganglion in the microphotograph in panel A (same scale): small neurons with short processes in nearby wiring (#4 and #14); medium neurons with smooth soma, 1–2 processes (#5, #9, and #10); and medium (#1, #5) or large (#11) multiaxonal neurons adjacent to other neurons and fibers, projecting out the ganglion (white arrows) or into the interganglionic strands (red arrows). C, Junctions of 3 neurons to each other, and to long passing fibers (yellow horizontal and pink vertical); portion of axons closely apposed (white arrow). D, Wiring of one neuron (#1) to other neuronal somas (#2 and 3) and to passing fibers. E, A small neuron adjacent to 3 nerve fibers

FIGURE 3.

A, Selected 3D digital traces of the wiring of neuron #5 in Figure 2A with other neurons, nerve fibers, IGNEEx (complex type of intraganglionic nerve endings) and a fiber in the circular muscle (magenta arrow). B, Confocal microscopic image cropped in 3D from Figure 2A using Imaris 9.2. C, Digital traces of neurites forming the IGNEEx. The blue fiber has branched endings, more likely an afferent nerve; the purple and cyan fibers are also interweaved into the fiber basket; the orange, rosy, and pink fibers from interganglionic strand and one process of a neuron (orange arrows) cross the IGNEEx making 1–2 conjunctions

FIGURE 4.

A, Confocal microphotograph of random and sparse AAV-transduced multicolored neurons and fibers in a mouse proximal colon. B, Digital traces of panel A (same scale). The sample was obtained from a male mouse injected with the inducer vector at 5×10^{10} GC/mouse. The digital tracing was performed in Neurolucida 360. Various types of labeled neurons located in one large ganglion. Long nerve fibers from different directions cross the ganglion, and two fibers (rose and green) were traced running together in segments (white arrows) *en route* with axons of neurons (white and red). Two axons closely apposed each other (red arrow), so did two somas (red and yellow). One neuron (cyan) had complex wiring with neurons nearby via the axon–soma, axon–dendritic, and axon–axon close appositions. Vertically projecting fibers in the left side of panel A are the fibers innervating the circular muscles

Soma size and shape of the myenteric neurons in the mouse proximal colon, randomly sparsely transduced by intravenous administration of multicolor AAV-PHP.S vector system, grouped by major diameter, area, or shape

TABLE 1

	Soma (n)	Percentage	Area (μm^2)	Diameter major (μm)	Diameter minor (μm)	Major/minor diameter ratio
Major diameter						
Small	160	33.6	167.38 $\pm$ 2.66	17.78 $\pm$ 0.14	12.6 $\pm$ 0.16	1.45 $\pm$ 0.02
Medium	261	54.8	253.56 $\pm$ 3.48	23.88 $\pm$ 0.15	14.6 $\pm$ 0.17	1.70 $\pm$ 0.02
Large	55	11.6	392.79 $\pm$ 13.56	34.56 $\pm$ 0.58	15.60 $\pm$ 0.45	2.32 $\pm$ 0.08
Area						
Small	179	37.6	163.3 $\pm$ 1.98	18.74 $\pm$ 0.22	11.82 $\pm$ 0.13	1.63 $\pm$ 0.03
Medium	268	56.3	267.57 $\pm$ 3.02	24.66 $\pm$ 0.25	15.00 $\pm$ 0.14	1.69 $\pm$ 0.03
Large	29	6.1	469.91 $\pm$ 13.83	35.04 $\pm$ 0.95	18.42 $\pm$ 0.46	1.95 $\pm$ 0.09
Shape						
Round	96	20.2	226.83 $\pm$ 7.43	19.31 $\pm$ 0.35	15.79 $\pm$ 0.79	1.22 $\pm$ 0.01
Oval	354	74.4	242.31 $\pm$ 4.87	23.39 $\pm$ 0.27	13.75 $\pm$ 0.02	1.72 $\pm$ 0.02
Elongated	26	5.5	269.50 $\pm$ 18.01	32.45 $\pm$ 1.36	10.96 $\pm$ 0.40	2.98 $\pm$ 0.09

Note: Neuronal somas ($n = 476$) were digitally traced in NeuroLucida 360 in 3D confocal images and measured in NeuroLucida Explorer. The soma size and shape were defined as follows. Major diameter (range 12–49 μm): small <20, medium 20–30, and large >30 μm . Area size (range 79–690 μm^2): small <200, medium 200–400, and large >400 μm^2 . Shape defined by major/minor diameter ratio (range 1.1–4.4): round <1.3, oval 1.3–2.5, and elongated >2.5.