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(71) Applicant: TRANSFERT PLUS, SOCIÉTÉ EN COM-
MANDITE [CA/CA]; 355 Rue Peel, Carrefour INGO, Bu-
reau 503, Montreal, Québec H3C 2G9 (CA).

(72) Inventors: SORET, Rodolphe; 4421 Rue Rivard, Mon-
tréal, Québec H2J 2N1 (CA). PILON, Nicolas; 1458 Rue
Gilford, Montréal, Québec H2J 1R9 (CA).

(74) Agent: LAVERY, DE BILLY, LLP; 1, Place Ville Marie,
Bureau 4000, Montréal, Québec H3B 4M4 (CA).

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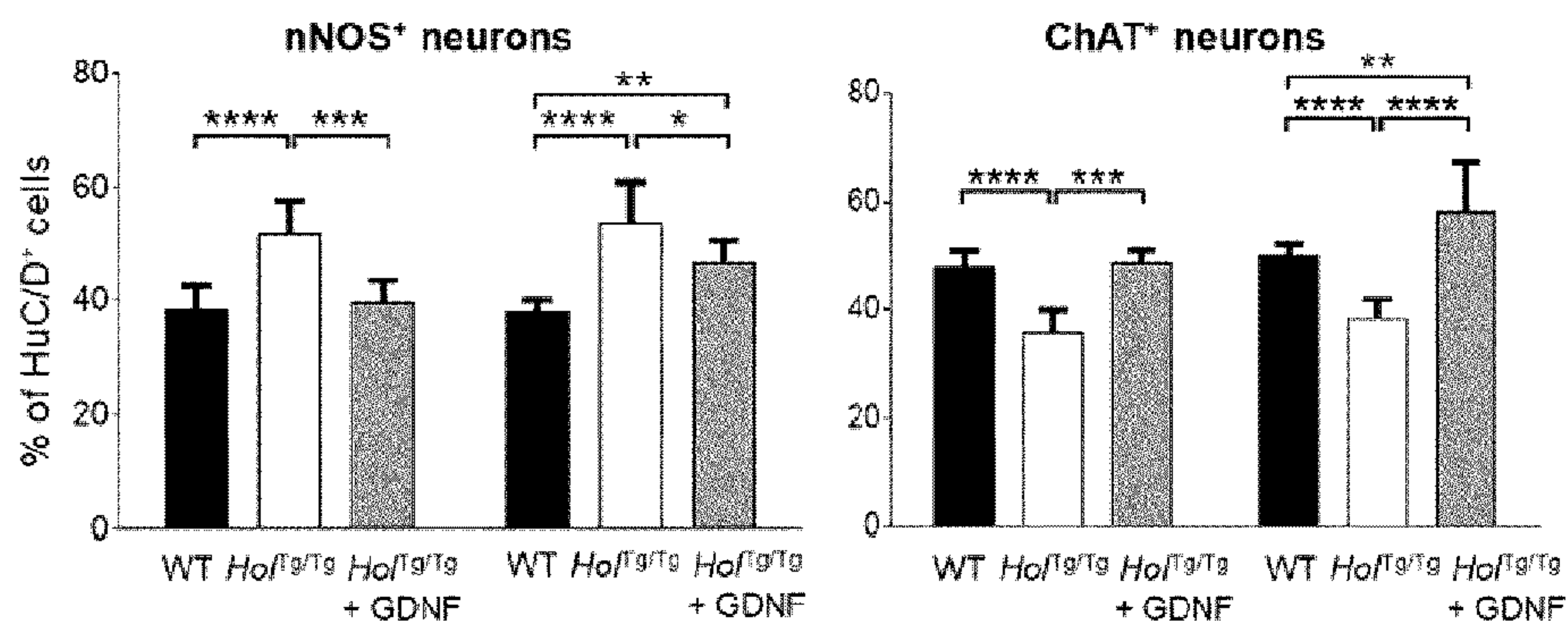


FIG. 7B

(57) Abstract: The present application relates to a method for inducing neurogenesis in an aganglionic or hypoganglionic segment of the distal colon of a human subject suffering from an enteric neuropathy such as Hirschsprung disease (HSCR) or intestinal hypoganglionosis through the administration of an effective dose of recombinant Glial cell line-Derived Neurotrophic Factor (GDNF) polypeptide into the distal colon of the subject. The method also permits to correct the imbalance of nitrergic and cholinergic neuron subtypes located upstream of the aganglionic or hypoganglionic segment and restore distal colon function (e.g., motility and epithelial barrier) in the subject.



TITLE OF INVENTION

USE OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF) FOR THE TREATMENT OF ENTERIC NEUROPATHIES

CROSS REFERENCE TO RELATED APPLICATIONS

5 The present application claims the benefit of U.S. provisional application serial No. 62/950,781, filed on December 19, 2019, which is incorporated herein by reference.

TECHNICAL FIELD

The present invention generally relates to the treatment of enteric neuropathies such as Hirschsprung disease (HSCR) and intestinal hypoganglionosis.

10 BACKGROUND ART

The enteric nervous system (ENS) extends along the entire gastrointestinal tract to control bowel motility, blood flow and epithelial activity in response to sensory stimuli (1). Interconnected enteric ganglia containing neurons and glia develop from neural crest-derived progenitors that migrate through the intestine during prenatal development. Incomplete
15 colonization of distal colon by ENS progenitors causes Hirschsprung disease (HSCR), a condition affecting 1 in 5000 newborns (2,3). In HSCR, distal colon without neural ganglia (i.e., aganglionic colon) remains tonically contracted and does not propagate contractions, causing functional intestinal obstruction. HSCR symptoms include refractory constipation with retention of stool and air, abdominal distension, growth failure, occasional vomiting, bowel inflammation (enterocolitis)
20 and a risk of bacterial translocation into blood causing sepsis and premature death (2).

HSCR is clinically subdivided into short-segment (S-HSCR) and long-segment forms (L-HSCR) (4). S-HSCR, which occurs in >80% of cases, means the ENS is absent from rectum and sigmoid colon. L-HSCR means longer regions of distal bowel are aganglionic. HSCR etiology remains incompletely understood, but many genes influence HSCR risk (2). Furthermore, genetic
25 risk variants may combine with non-genetic factors to prevent full bowel colonization by ENS progenitors (5). This non-Mendelian inheritance occurs because many proteins must work together for normal ENS development.

Since 1948, most children with HSCR have had their life saved by surgical removal of distal aganglionic bowel (16, 17). However, this procedure is far from ideal. Post-surgical
30 complications are common and can also be long-lasting, impacting survival (e.g. enterocolitis) and/or quality of life (e.g., fecal incontinence or obstructive symptoms) (18-20). One promising approach would be "regenerative medicine" to rebuild the ENS and reduce the need for surgery. This idea prompted many groups to develop cell transplantation-based HSCR therapies (21). However, despite many encouraging results, some difficulties remain (22). The optimal source of

stem cells, ideal amplification and/or differentiation strategies prior to transplantation, methods of cell delivery, and cell function and fate after transplantation are not yet well defined. Additionally, non-autonomous cell transplantation may require immunosuppression.

5 Hypoganglionosis, also known as intestinal hypoganglionosis, is a disorder causing a reduced number of nerves in the intestinal wall. Intestinal hypoganglionosis can mimic HSCR; patients with both conditions may present with chronic constipation, intestinal obstruction, and enterocolitis (inflammation of the intestines). Patients with hypoganglionosis may also suffer from severe complications including fecaloma (hardening of the feces inside the colon), bleeding or perforation of the intestine, and breathing problems resulting from a distended colon. The exact
10 cause of hypoganglionosis is often not known. In some cases, it is due to factors present at birth (congenital), while other times it is believed to be an acquired condition. The management of isolated hypoganglionosis generally involves surgery to remove the affected bowel segment.

There is thus clearly a need for alternative treatments for enteric neuropathies such as HSCR and intestinal hypoganglionosis, notably treatments aimed at inducing neurogenesis in the
15 distal colon and restoring distal colon motility in HSCR and intestinal hypoganglionosis patients.

The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The present disclosure relates to the use of GDNF for the treatment of one or more
20 pathological features of enteric neuropathies such as Hirschsprung disease.

In aspects and embodiment, the present disclosure relates to the following items 1 to 90:

1. A method for inducing enteric neurogenesis in an aganglionic or hypoganglionic segment of the distal colon of a human subject suffering from an enteric neuropathy, the method comprising administering a pharmaceutical composition comprising an effective dose of a recombinant Glial
25 cell line-Derived Neurotrophic Factor (GDNF) polypeptide and a pharmaceutically acceptable carrier into the distal colon of the subject.
2. The method of item 1, wherein the GDNF polypeptide comprises an amino acid sequence having at least 70% identity with amino acids 78-211 of SEQ ID NO:1.
3. The method of item 2, wherein the GDNF polypeptide comprises an amino acid sequence
30 having at least 90% identity with amino acids 78-211 of SEQ ID NO:1.
4. The method of item 3, wherein the GDNF polypeptide comprises an amino acid sequence having at least 95% identity with amino acids 78-211 of SEQ ID NO:1.
5. The method of item 4, wherein the GDNF polypeptide comprises amino acids 78-211 of SEQ ID NO:1.

6. The method of any one of items 1 to 5, wherein the effective dose of recombinant GDNF polypeptide administered to the human subject corresponds to a dose of about 5 µg to about 20 µg in a mouse pup.
7. The method of any one of items 1 to 6, wherein the pharmaceutically acceptable carrier
5 comprises a saline solution or a gelling agent.
8. The method of any one of items 1 to 7, wherein the pharmaceutical composition is administered rectally through enema.
9. The method of any one of items 1 to 7, wherein the pharmaceutical composition is administered by injection into the distal colon wall.
10. 10. The method of any one of items 1 to 9, wherein the pharmaceutical composition is administered once-a-day up to four times a day.
11. The method of any one of items 1 to 10, wherein the pharmaceutical composition is administered for at least 2 consecutive days.
12. The method of any one of items 1 to 11, wherein the method is performed prior to surgical
15 removal of the aganglionic or hypoganglionic segment in the subject.
13. The method of any one of items 1 to 11, wherein the method is performed after surgical removal of the aganglionic or hypoganglionic segment in the subject.
14. The method of item 12 or 13, wherein the surgical removal of the aganglionic or hypoganglionic segment is through pull-through surgery.
- 20 15. The method of any one of items 1 to 14, wherein the enteric neuropathy is intestinal hypoganglionosis.
16. The method of any one of items 1 to 14, wherein the enteric neuropathy is Hirschsprung disease (HSCR).
17. The method of item 16, wherein the subject suffers from short-segment HSCR.
- 25 18. The method of item 16 or 17, wherein the HSCR is sporadic HSCR.
19. The method of any one of items 16 to 18, wherein the HSCR is associated with a reduced expression or activity of the RET receptor.
20. The method of item 19, wherein the HSCR is associated with a mutation in the RET gene.
21. The method of any one of items 1 to 20, wherein the enteric neurogenesis comprises
30 production of enteric neurons and/or enteric glial cells.
22. The method of item 21, wherein the neurogenesis comprises production of enteric neurons and enteric glial cells.
23. The method of item 21 or 22, wherein the production of enteric neurons and/or enteric glial cells comprises proliferation of enteric neurons and/or enteric glia progenitors.
- 35 24. The method of any one of items 1 to 23, wherein the method corrects the imbalance of nitrergic and cholinergic neuron subtypes located upstream of the aganglionic or hypoganglionic segment.

25. The method of any one of items 1 to 24, wherein the method restores distal colon motility in the subject.

26. The method of any one of items 1 to 25, wherein the method restores the proportions of lymphoid and/or myeloid immune cells in the distal colon of the subject.

5 27. The method of any one of items 1 to 26, wherein the human subject is less than 5 years-old, or less than 1-year-old.

28. The method of item 27, wherein the human subject is less than 6-month-old.

29. The method of any one of items 1 to 28, wherein the pharmaceutical composition is administered into the rectum and/or the sigmoid colon.

10 30. The method of item 29, wherein the pharmaceutical composition is administered or is for administration into the rectosigmoid region.

31. A pharmaceutical composition comprising a recombinant Glial cell line-Derived Neurotrophic Factor (GDNF) polypeptide and a pharmaceutically acceptable carrier for inducing enteric neurogenesis in an aganglionic or hypoganglionic segment of the distal colon of a human
15 subject suffering from an enteric neuropathy, wherein the composition is for administration into the distal colon of the subject.

32. The pharmaceutical composition for use according to item 31, wherein the GDNF polypeptide comprises an amino acid sequence having at least 70% identity with amino acids 78-211 of SEQ ID NO:1.

20 33. The pharmaceutical composition for use according to item 32, wherein the GDNF polypeptide comprises an amino acid sequence having at least 90% identity with amino acids 78-211 of SEQ ID NO:1.

34. The pharmaceutical composition for use according to item 33, wherein the GDNF polypeptide comprises an amino acid sequence having at least 95% identity with amino acids 78-
25 211 of SEQ ID NO:1.

35. The pharmaceutical composition for use according to item 34, wherein the GDNF polypeptide comprises amino acids 78-211 of SEQ ID NO:1.

36. The pharmaceutical composition for use according to any one of items 31 to 35, wherein the dose of recombinant GDNF polypeptide used corresponds to a dose of about 5 µg to about
30 20 µg in a mouse pup.

37. The pharmaceutical composition for use according to any one of items 31 to 36, wherein the pharmaceutically acceptable carrier is a saline solution or a gelling agent.

38. The pharmaceutical composition for use according to any one of items 31 to 37, wherein the pharmaceutical composition is for rectal administration through enema.

35 39. The pharmaceutical composition for use according to any one of items 31 to 38, wherein the pharmaceutical composition is for administration by injection into the distal colon wall.

40. The pharmaceutical composition for use according to any one of items 31 to 39, wherein the pharmaceutical composition is for administration once-a-day up to four times a day.

41. The pharmaceutical composition for use according to any one of items 31 to 40, wherein the pharmaceutical composition is for administration for at least 2 consecutive days.

5 42. The pharmaceutical composition for use according to any one of items 31 to 41, wherein the pharmaceutical composition is for use prior to surgical removal of the aganglionic or hypoganglionic segment in the subject.

43. The pharmaceutical composition for use according to any one of items 31 to 41, wherein the pharmaceutical composition is for use after surgical removal of the aganglionic or
10 hypoganglionic segment in the subject.

44. The pharmaceutical composition for use according to item 42 or 43, wherein the surgical removal of the aganglionic or hypoganglionic segment is through pull-through surgery.

45. The pharmaceutical composition for use according to any one of items 31 to 44, wherein the enteric neuropathy is intestinal hypoganglionosis.

15 46. The pharmaceutical composition for use according to any one of items 31 to 44, wherein the enteric neuropathy is Hirschsprung disease (HSCR).

47. The pharmaceutical composition for use according to item 46, wherein the subject suffers from short-segment HSCR.

48. The pharmaceutical composition for use according to item 46 or 47, wherein the HSCR is
20 sporadic HSCR.

49. The pharmaceutical composition for use according to any one of items 46 to 48, wherein the HSCR is associated with a reduced expression or activity of the RET receptor.

50. The pharmaceutical composition for use according to item 49, wherein the HSCR is associated with a mutation in the RET gene.

25 51. The pharmaceutical composition for use according to any one of items 31 to 50, wherein the enteric neurogenesis comprises production of enteric neurons and/or enteric glial cells.

52. The pharmaceutical composition for use according to item 51, wherein the enteric neurogenesis comprises production of enteric neurons and enteric glial cells.

53. The pharmaceutical composition for use according to item 51 or 52, wherein the
30 production of enteric neurons and/or glial cells comprises proliferation of enteric neuron and/or enteric glia progenitors.

54. The pharmaceutical composition for use according to any one of items 31 to 53, wherein the pharmaceutical composition corrects the imbalance of nitrergic and cholinergic neuron subtypes located upstream of the aganglionic or hypoganglionic segment.

35 55. The pharmaceutical composition for use according to any one of items 31 to 54, wherein the pharmaceutical composition restores distal colon motility in the subject.

56. The pharmaceutical composition for use according to any one of items 31 to 55, wherein the pharmaceutical composition restores the proportions of lymphoid and/or myeloid immune cells in the distal colon of the subject.

57. The pharmaceutical composition for use according to any one of items 31 to 56, wherein
5 the human subject is less than 5 years-old, or less than 1-year-old.

58. The pharmaceutical composition for use according to item 57, wherein the human subject is less than 6-month-old.

59. The pharmaceutical composition for use according to any one of items 31 to 58, wherein the pharmaceutical composition is for administration into the rectum and/or the sigmoid colon.

10 60. The pharmaceutical composition for use according to item 59, wherein the pharmaceutical composition is for administration into the rectosigmoid region.

61. Use of a pharmaceutical composition comprising a recombinant Glial cell line-Derived Neurotrophic Factor (GDNF) polypeptide and a pharmaceutically acceptable carrier for the manufacture of a medicament for inducing enteric neurogenesis in an aganglionic or
15 hypoganglionic segment of the distal colon of a human subject suffering from an enteric neuropathy, wherein the medicament is for administration into the distal colon of the subject.

62. The use according to item 61, wherein the GDNF polypeptide comprises an amino acid sequence having at least 70% identity with amino acids 78-211 of SEQ ID NO:1.

63. The use according to item 62, wherein the GDNF polypeptide comprises an amino acid
20 sequence having at least 90% identity with amino acids 78-211 of SEQ ID NO:1.

64. The use according to item 63, wherein the GDNF polypeptide comprises an amino acid sequence having at least 95% identity with amino acids 78-211 of SEQ ID NO:1.

65. The use according to item 64, wherein the GDNF polypeptide comprises amino acids 78-211 of SEQ ID NO:1.

25 66. The use according to any one of items 61 to 65, wherein the dose of recombinant GDNF polypeptide used corresponds to a dose of about 5 µg to about 20 µg in a mouse pup.

67. The use according to any one of items 61 to 66, wherein the pharmaceutically acceptable carrier is a saline solution or a gelling agent.

68. The use according to any one of items 61 to 67, wherein the pharmaceutical composition
30 is for rectal administration through enema.

69. The use according to any one of items 61 to 67, wherein the pharmaceutical composition is for administration by injection into the distal colon wall.

70. The use according to any one of items 61 to 69, wherein the pharmaceutical composition is for administration once-a-day up to four times a day.

35 71. The use according to any one of items 61 to 70, wherein the pharmaceutical composition is for administration for at least 2 consecutive days.

72. The use according to any one of items 61 to 71, wherein the pharmaceutical composition is used prior to surgical removal of the aganglionic or hypoganglionic segment in the subject.

73. The use according to any one of items 61 to 72, wherein the pharmaceutical composition is used after surgical removal of the aganglionic or hypoganglionic segment in the subject.

5 74. The use according to item 72 or 73, wherein the surgical removal of the aganglionic or hypoganglionic segment is through pull-through surgery.

75. The use according to any one of items 61 to 74, wherein the enteric neuropathy is intestinal hypoganglionosis.

10 76. The use according to any one of items 61 to 75, wherein the enteric neuropathy is Hirschsprung disease (HSCR).

77. The use according to item 76, wherein the subject suffers from short-segment HSCR.

78. The use according to item 76 or 77, wherein the HSCR is sporadic HSCR.

79. The use according to any one of items 76 to 78, wherein the HSCR is associated with a reduced expression or activity of the RET receptor.

15 80. The use according to item 79, wherein the HSCR is associated with a mutation in the RET gene.

81. The use according to any one of items 61 to 80, wherein the enteric neurogenesis comprises production of enteric neurons and/or enteric glial cells.

20 82. The use according to item 81, wherein the enteric neurogenesis comprises production of enteric neurons and enteric glial cells.

83. The use according to item 81 or 82, wherein the production of enteric neurons and/or glial cells comprises proliferation of enteric neuron and/or enteric glia progenitors.

25 84. The use according to any one of items 61 to 83, wherein the method corrects the imbalance of nitrergic and cholinergic neuron subtypes located upstream of the aganglionic or hypoganglionic segment.

85. The use according to any one of items 61 to 84, wherein the medicament restores distal colon motility in the subject.

86. The use according to any one of items 61 to 85, wherein the medicament restores the proportions of lymphoid and/or myeloid immune cells in the distal colon of the subject.

30 87. The use according to any one of items 61 to 86, wherein the human subject is less than 5 years-old, or less than 1-year-old.

88. The use according to item 87, wherein the human subject is less than 6-month-old.

89. The use according to any one of items 61 to 88, wherein the medicament is for administration into the rectum and/or the sigmoid colon.

35 90. The use according to item 89, wherein the medicament is for administration into the rectosigmoid region.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF DRAWINGS

5 In the appended drawings:

FIGs. 1A-H show the set-up of GDNF therapy parameters in *Hol^{Tg/Tg}* mice. (**FIG. 1A-B**) Distribution of 10µl methylene blue enemas in the colon of P4 (**FIG. 1A**) and P8 (**FIG. 1B**) *Hol^{Tg/Tg}* pups. (**FIG. 1C**) Impact of GDNF concentration on survival of *Hol^{Tg/Tg}* pups that received 10µl enemas once daily between P4-P8. Indicated amounts correspond to the total quantity of GDNF that was administered each day. (**FIG. 1D**) Impact of treatment time window (P4-P8 vs. P8-P12), duration (1d, 5d or 10d; starting at P4) and frequency (once or twice a day, for 5 days) on the survival of *Hol^{Tg/Tg}* pups treated with GDNF enemas (quantity of GDNF administered per single enema was kept constant at 10µg in 10µl). (**FIG. 1E**) Survival rate of *Hol^{Tg/Tg}* pups that were administered 10µl enemas containing the indicated neurotrophic molecule (Noggin, Endothelin-3, or the serotonin receptor agonist RS67506; all at 1µg/µl final concentration) once daily between P4-P8. (**FIG. 1F**) Impact of food consistency (regular chow vs gel diet) on survival of *Hol^{Tg/Tg}* pups that received GDNF enemas (10µg in 10µl) on a daily basis between P4-P8. (**FIG. 1G**) Impact of coadministration of ascorbic acid (Vit.C; 100µM final concentration), serotonin (5-HT; 1µg/µl final concentration) and Endothelin-3 (ET3; 1µg/µl final concentration) on survival of *Hol^{Tg/Tg}* pups that received GDNF enemas (10µg in 10µl) once daily between P4-P8. (**FIG. 1H**) Neuron density in the colon (expressed in % of surface area) and associated health status of P20 *Hol^{Tg/Tg}* mice that received GDNF enemas (10µg in 10µl) on a daily basis between P4-P8.

FIGs. 2A-E show that GDNF enemas rescue aganglionic megacolon in HSCR mouse models. (**FIGs. 2A-C**) Daily administration of GDNF enemas to *Hol^{Tg/Tg}* (**FIG. 2A**), *Ednrb^{S-/S-}* (**FIG. 2B**) and *TashT^{Tg/Tg}* (**FIG. 2C**) mice between P4-P8 positively impacts both megacolon symptoms (i.e. distal blockage and accumulation of fecal material in the colon, retarded growth, lethargy, and hunched posture) and survival rates (Mantel-Cox statistical test, comparing GDNF-treated and non-treated groups in the case of *Hol^{Tg/Tg}* and *Ednrb^{S-/S-}*, or GDNF-treated and PBS-treated groups in the case of *TashT^{Tg/Tg}*). (**FIG. 2D**) Whole-mount immunofluorescence staining of colonic muscle strips from P20 mice shows that the 5-day GDNF treatment induces myenteric ganglia containing HuC/D⁺ neurons and Sox10⁺ glia in the otherwise aganglionic region of *Hol^{Tg/Tg}* mice. Dashed outline marks area occupied by extrinsic nerve fibers. Below each representative image is a schematic representation of the average neuronal density for each colon sub-region (represented by cylinders) along the length of the colon. Neuronal density is expressed as the percentage of area occupied by HuC/D⁺ cells in a single confocal slice at the level of the myenteric plexus within the bowel wall (n=6 mice per group; 3 fields of view per sub-region). For each distal

colon subregion, the neuronal density is also given as a numerical value. **(FIG. 2E)** Immunofluorescence analysis of distal colonic muscularis from P20 mice that received intraperitoneal injections of EdU during the 5-day GDNF treatment demonstrates that a subset of induced myenteric neurons (arrowheads) and glia (arrows) were generated from a dividing precursor during the treatment period. Dashed outline marks area occupied by a single ganglion. **(FIG. 2F)** Quantification of EdU incorporation in myenteric neurons and glia in the distal colon. Results are expressed as the number of EdU⁺ cells per mm² (n=3 WT and 3 GDNF-treated *Hol^{Tg/Tg}* mice; 2-7 fields of view per animal; ****P*<0.001; *****P*<0.0001; one-way ANOVA with post-hoc Sidak's test). All displayed images represent a z-stack projection at the level of the myenteric plexus. Scale bars, 100 μm (**FIG. 2D**) and 50 μm (**FIG. 2E**).

FIG. 3 shows an overview of myenteric plexus and submucosal plexuses in the distal colon of WT, untreated *Hol^{Tg/Tg}* or GDNF-treated *Hol^{Tg/Tg}* mice at P20. Immunofluorescence analysis of TuJ1⁺ neuronal structures in myenteric (upper panels) and submucosal (lower panels) plexus. Arrows point to GDNF-induced ganglia that contain neuronal bodies. Insets are zoomed-in views of GDNF-induced ganglia in dashed boxes. GDNF-treated mice received 10μg GDNF in 10μL enemas once daily from P4-P8. All images show a z-stack projection representative of observations made from 3 mice. Scale bar, 100μm (large panels) and 50μm (insets).

FIGs. 4A-C show an analysis of myenteric ganglion size and neuronal density in the colon of P20 *Hol^{Tg/Tg}* and *TashT^{Tg/Tg}* mice that were treated or not with GDNF between P4-P8. **(FIG. 4A)** Analysis of myenteric ganglion size in *Hol^{Tg/Tg}* mice. **(FIG. 4B)** Analysis of neuronal density in *TashT^{Tg/Tg}* mice. The average neuronal density is indicated for each colon sub-region (represented by cylinders) along the length of the colon. Neuronal density is expressed as the percentage of area occupied by HuC/D⁺ cells in a single focal plane at the level of the myenteric plexus within the bowel wall (n=6 mice per group; 3 fields of view per sub-region). For each distal colon subregion, the neuronal density is also given as a numerical value. **(FIG. 5C)** Analysis of myenteric ganglion size in *TashT^{Tg/Tg}* mice.

FIGs. 5A-C show an analysis of EdU incorporation in myenteric and submucosal ganglia of the colon from P20 WT and GDNF-treated *Hol^{Tg/Tg}* mice that were administered EdU between P4-P8. **(FIG. 5A)** Example of EdU incorporation in a z-stack projection of submucosal neurons (arrowheads) and glia (arrows) in the distal colon. Dashed outline marks area occupied by a single ganglion. Scale bar, 50 μm. **(FIG. 5B)** Quantitative analysis of EdU incorporation in submucosal neurons (HuC/D⁺) and glia (SOX10⁺) in mid and distal colon. Results are expressed as the number of EdU⁺ cells per mm² (n=3 WT and 3 GDNF-treated *Hol^{Tg/Tg}* mice; 2-7 fields of view per animal; **P*<0.05; one-way ANOVA with post-hoc Sidak's test). **(FIG. 5C)** Quantitative analysis of EdU incorporation in myenteric (left panel) and submucosal (right panel) neurons (HuC/D⁺) and glia (SOX10⁺) in distal colon. Results are expressed in percentage of EdU⁺ cells per ganglion (n=3 WT and 3 GDNF-treated *Hol^{Tg/Tg}* mice; 2-7 fields of view per animal; ****P*<0.001;

**** $P < 0.0001$; one-way ANOVA with post-hoc Sidak's test). GDNF-treated mice received 10 μ g GDNF in 10 μ L enemas once daily from P4-P8.

FIGs. 6A-K show the phenotypic and functional characterization of the GDNF-induced ENS in P20 *Hol^{Tg/Tg}* mice. **(FIGs. 6A-B)** Immunofluorescence-based quantitative analysis of GDNF-induced myenteric ganglia from the distal colon of *Hol^{Tg/Tg}* mice shows WT-like neuron (HuC/D⁺) to glia (Sox10⁺) ratio (**FIG. 6A**), and proportions of nitrergic (nNOS⁺ HuC/D⁺) and cholinergic (ChAT⁺ HuC/D⁺) neurons (**FIG. 6B**) (n=6 mice per group; 3 fields of view per animal). **(FIG. 6C)** Analysis of GDNF-induced myenteric ganglia not only shows the presence of nitrergic and cholinergic neurons but also other neuron subtypes expressing either tyrosine hydroxylase (TH), substance P (SubP), calretinin (CalR), or vasoactive intestinal peptide (VIP). All images are single focal planes representative of observations made from 3 mice per subtype marker, with arrows pointing to examples of indicated neuron subtypes. Scale bar, 20 μ m. **(FIG. 6D)** *In vivo* bead latency test indicates partial recovery of colonic motility in some GDNF-treated *Hol^{Tg/Tg}* mice (n=8-9 mice per group, * $P < 0.05$; one-way ANOVA with post-hoc Sidak's test). Note that time to expel the glass bead after rectal insertion was capped at 30 min. in order to simplify the analysis without impacting statistical significance. **(FIG. 6E)** *Ex vivo* analysis of electric field-stimulated and drug-modulated patterns of longitudinal smooth muscle contraction-relaxation in an organ bath equipped with a force transducer. Contractile strength (expressed in g/s) is calculated from the difference from baseline of the area under the curve (AUC) values obtained after electric field stimulation. Mean Δ AUC values for the *Hol^{Tg/Tg}* + GDNF group are divided into two sub-groups as a function of contractility response. About half of the colonic muscle strips from GDNF-treated *Hol^{Tg/Tg}* mice (4 out of 7) showed a WT-like pattern of response to sequential inhibition of nitrergic and cholinergic signaling (using L-NAME and atropine, respectively), whereas the other half (3 out of 7) remained poorly responsive similar to muscle strips from untreated *Hol^{Tg/Tg}* mice (n=6 WT and *Hol^{Tg/Tg}*, n=7 *Hol^{Tg/Tg}* + GDNF; ** $P < 0.01$; **** $P < 0.0001$; two-way ANOVA with post-hoc Tukey's test). **(FIG. 6F)** *Ex vivo* measurement of distal colon mucosal permeability to FITC-labeled 4kDa dextran (FD4) in Ussing chambers indicate increased permeability in *Hol^{Tg/Tg}* colons that is reversed in two-thirds (4 out of 6) of GDNF-treated *Hol^{Tg/Tg}* mice (n=6 mice per group; * $P < 0.05$; ** $P < 0.01$; one-way ANOVA with post-hoc Sidak's test). **(FIG. 6G-I)** H&E staining of transverse sections of the distal colon shows that the GDNF treatment rescues both the increased thickness of smooth muscles (see brackets in **FIG. 6G** and quantification in **FIG. 6H**) and the increased number of neutrophils in the submucosa (see asterisks in **FIG. 6G** and quantification in **FIG. 6I**) (n=6 mice per group; Scale bar, 150 μ m; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; one-way ANOVA with post-hoc Sidak's test). **(FIGs. 6J-K)** Microbiome profiling shows that GDNF treatment at least partially rescues the microbiota imbalance normally observed in untreated *Hol^{Tg/Tg}* mice (n=5 mice per group). The bar histograms (**FIGs. 6J**) display the average relative abundance of 16S rRNA gene sequences at the genera level (* $P < 0.05$; one-way ANOVA with post-hoc Tukey's test). Beta-

diversity comparisons (**FIG. 6K**) with 95% confidence interval ellipses are based on non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity of the relative abundance of operational taxonomic units among samples ($P < 0.001$; PERMANOVA).

FIGs. 7A-B show the proportion of nitrergic and cholinergic myenteric neurons in the proximal and mid colon of WT, untreated *Hol^{Tg/Tg}* or GDNF-treated *Hol^{Tg/Tg}* mice at P20. (**FIG. 7A**) Qualitative analysis of the proportion of nitrergic (left panel) and cholinergic (right panel) neurons. Scale bar, 50 μ m. (**FIG. 7B**) Quantitative analysis of the proportion of nitrergic (left panel) and cholinergic (right panel) neurons ($n=3$ WT and 3 GDNF-treated *Hol^{Tg/Tg}* mice; 3 fields of view per animal; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$; one-way ANOVA with post-hoc Sidak's test). GDNF-treated mice received 10 μ g GDNF in 10 μ L enemas once daily from P4-P8.

FIGs. 8A-B show supporting information for *in vivo* and *ex vivo* analyses of motility in the distal colon of WT, untreated *Hol^{Tg/Tg}* or GDNF-treated *Hol^{Tg/Tg}* mice at P20. (**FIG. 8A**) Correlation between neuron density in distal colon and time for bead expulsion in GDNF-treated *Hol^{Tg/Tg}* mice at P20 (in support of **FIG. 6D**). (**FIG. 8B**) Examples of electric field-stimulated and drug-modulated patterns of longitudinal smooth muscle contraction-relaxation in an organ bath equipped with a force transducer (in support of **FIG. 6E**). In responsive tissues, electric field stimulation (EFS) triggers contractions of colonic muscles that can be slightly increased by L-NAME-mediated inhibition of nitrergic signaling, and robustly counteracted by atropine-mediated inhibition of cholinergic signaling. GDNF-treated mice received 10 μ g GDNF in 10 μ L enemas once daily from P4-P8.

FIGs. 9A-B show an analysis of smooth muscle thickness in the distal colon of WT, untreated *Hol^{Tg/Tg}* or GDNF-treated *Hol^{Tg/Tg}* mice at P20. (**FIG. 9A**) Representative H&E-stained cross-sections of different colon segments, with smooth muscle thickness indicated by red brackets. Scale bar, 150 μ m. (**FIG. 9B**) Average muscle thickness for each colon segment ($n=6$ mice per group; $**P < 0.01$; $***P < 0.001$; one-way ANOVA with post-hoc Tukey's test). GDNF-treated mice received 10 μ g GDNF in 10 μ L enemas once daily from P4-P8.

FIGs. 10A-I show that extrinsic Schwann cell precursors (SCPs) are a source of GDNF-induced neurons and glia in the otherwise aganglionic colon. (**FIG. 10A**) Western blot analysis of GDNF distribution in different sub-regions of the GI tract from WT, *Hol^{Tg/Tg}* and GDNF-treated *Hol^{Tg/Tg}* mice at P8. Endogenous GDNF (eGDNF) is normally restricted to the ileum in all mice whereas recombinant GDNF (rGDNF) is exclusively detected in the distal colon of GDNF-treated *Hol^{Tg/Tg}* mice. In GDNF-treated *Hol^{Tg/Tg}*, eGDNF is expressed in all sub-regions of the colon. The displayed anti-GDNF blots are representative of observations made from 3 mice per group. (**FIGs. 10B-C**) Time-course analysis of the distribution of a 6xHis-tagged version of GDNF (_{His}GDNF) used for enema treatments of *Hol^{Tg/Tg}* mice between P4-P8. Anti-His and anti-RET double staining of the distal colon at 2-day intervals shows that both _{His}GDNF and RET accumulate in the submucosa during the treatment (**FIG. 10B**). Both are also detected in induced myenteric neurons

close to extrinsic nerve fibers at P8 (**FIG. 10C**). All images show a z-stack projection representative of observations made from 3 mice. Scale bar, 20 μm . (**FIG. 10D**) Representative images from 10-hour long time-lapse recordings of aganglionic colon tissues from *HoI^{Tg/Tg};G4-RFP* mice showing that SCP-like cells are dividing (arrows) and migrating (arrowheads) on extrinsic nerve fibers. Explants were prepared from P4 distal colons and pre-cultured for 72h with GDNF before live imaging on a confocal microscope in the continued presence of GDNF. Images are projections of 50 μm -thick z-stacks representative of observations made from 3 explants. Scale bar, 100 μm . (**FIGs. 10E-F**) Anti-SOX10 and anti-Ki67 double labeling demonstrates that exposure to GDNF for 96h markedly increases the rate of SCP proliferation in explants of distal colon prepared from P4 *HoI^{Tg/Tg}* mice. Images in **FIG. 10E** are single focal planes representative of observations made from 3 explants. Scale bar, 50 μm . Each value in **FIG. 10F** corresponds to the average percentage of Ki67⁺ SCPs (i.e., (Ki67⁺SOX10⁺ / SOX10⁺) x 100) calculated from a minimum of 3 fields of view per explant (***P*<0.01; two-tailed Student's *t*-test). (**FIGs. 10G-H**) Immunofluorescence analysis of myenteric ganglia in the distal colon of P20 *HoI^{Tg/Tg};Dhh-Cre^{Tg/+};R26^{YFP/+}* mice that were administered GDNF enemas and EdU via intraperitoneal injections between P4-P8. Four categories of induced neuron are detected: 1) SCP-derived (*Dhh*+ lineage) and EdU-positive (filled grey arrowhead); 2) SCP-derived and EdU-negative (empty grey arrowhead); 3) unknown origin and EdU-positive (filled white arrowhead); 4) unknown origin and EdU-negative (empty white arrowhead). Images in **FIG. 10G** are single focal planes representative of observations made from 3 mice. Scale bar, 50 μm . The relative proportions of the four categories of induced neurons per ganglion plotted in **FIG. 10H** corresponds to the averages calculated from a minimum of 3 fields of view per mouse. Dashed outlines mark area occupied by either an extrinsic nerve fiber (**FIG. 10E**), or an extrinsic nerve fiber and an adjacent single ganglion (**FIGs. 10C and G**). **FIG. 10I**: Schwann cells in the aganglionic distal colon of *HoI^{Tg/Tg}* mice express neural cell adhesion molecule (NCAM) but not RET. Immunofluorescence analysis of NCAM and RET expression in extrinsic nerve fibers (delineated by *dashed lines*) from the distal colon of untreated *HoI^{Tg/Tg}* mice at P20. NCAM but not RET is expressed in SOX10⁺ Schwann cells and putative enteric glia/ENS progenitors (*arrows*). DAPI, 4',6-diamidino-2-phenylindole. The displayed images are single focal planes representative of observations made from 3 mice. Scale bar, 50 μm .

FIG. 11 shows an analysis of GDNF distribution in multiple tissues of GDNF-treated *HoI^{Tg/Tg}* mice at P20. Western bolt analysis of $\alpha\text{Tubulin}$ -normalized levels of endogenous GDNF (eGDNF) and recombinant GDNF (rGDNF) in different tissues of P20 *HoI^{Tg/Tg}* mice that received 10 μg GDNF in 10 μL enemas once daily from P4-P8. The displayed blots are representative of observations made from 3 mice.

FIG. 12 shows a time-course analysis of HisGDNF distribution and RET expression in colonic smooth muscles of P4-P8 *HoI^{Tg/Tg}* mice treated with HisGDNF . Immunofluorescence

analysis of HisGDNF distribution and RET expression in distal colon muscularis of HisGDNF -treated $\text{Hol}^{\text{Tg/Tg}}$ mice. White arrowheads point to RET⁺ neurons that also stain positive for HisGDNF . All images show a single focal plane representative of observations made from 3 mice. Scale bar, 20 μm .

5 **FIGs. 13A-D** show an analysis of SCP-derived neurogenesis in myenteric and submucosal ganglia of $\text{Dhh-Cre}^{\text{Tg/+}};\text{R26}^{\text{YFP/+}}$ and $\text{Hol}^{\text{Tg/Tg}};\text{Dhh-Cre}^{\text{Tg/+}};\text{R26}^{\text{YFP/+}}$ mice at P20. (**FIG. 13A**) Analysis of myenteric neurons (HuC/D⁺) and YFP expression in the proximal colon of $\text{Dhh-Cre}^{\text{Tg/+}};\text{R26}^{\text{YFP/+}}$ (Ctl) and $\text{Hol}^{\text{Tg/Tg}};\text{Dhh-Cre}^{\text{Tg/+}};\text{R26}^{\text{YFP/+}}$ ($\text{Hol}^{\text{Tg/Tg}}$) mice. Yellow arrowheads point to SCP-derived neurons. (**FIG. 13B**) Quantitative analyses of myenteric neurons (HuC/D⁺) and YFP
10 expression in the proximal and mid-colon of $\text{Dhh-Cre}^{\text{Tg/+}};\text{R26}^{\text{YFP/+}}$ (Ctl) and $\text{Hol}^{\text{Tg/Tg}};\text{Dhh-Cre}^{\text{Tg/+}};\text{R26}^{\text{YFP/+}}$ ($\text{Hol}^{\text{Tg/Tg}}$) mice (n=3 Ctl and 3 $\text{Hol}^{\text{Tg/Tg}}$ mice; 3 fields of view per animal; * $P<0.05$; one-way ANOVA with post-hoc Sidak's test). (**FIG. 13C**) Analysis of submucosal neurons (HuC/D⁺) and YFP expression in the distal colon of $\text{Hol}^{\text{Tg/Tg}};\text{Dhh-Cre}^{\text{Tg/+}};\text{R26}^{\text{YFP/+}}$ ($\text{Hol}^{\text{Tg/Tg}}$) mice that were treated with GDNF between P4-P8. Neurons of either SCP (grey arrowhead) or
15 unknown (white arrowhead) origin are detected. (**FIG. 13D**) Analysis of RET-expressing myenteric neurons (HuC/D⁺) and YFP expression in the distal colon of $\text{Hol}^{\text{Tg/Tg}};\text{Dhh-Cre}^{\text{Tg/+}};\text{R26}^{\text{YFP/+}}$ ($\text{Hol}^{\text{Tg/Tg}}$) mice that were treated with GDNF between P4-P8. RET is expressed in a subset of neurons, regardless of SCP (RET⁺, filled grey arrowhead; RET⁻, empty grey arrowhead) or non-SCP (white arrowhead) origin. All displayed images are z-stack projections
20 representative of observations made from 3 mice. Scale bar, 50 μm . Dashed outline marks area occupied by a single ganglion.

FIGs. 14A-H show the *ex vivo* preclinical testing of GDNF therapy on explants of aganglionic colon from $\text{Hol}^{\text{Tg/Tg}}$ mice and human HSCR patients. (**FIGs. 14A-C**) Immunofluorescence-based analysis of explants prepared from the distal colon of P4 $\text{Hol}^{\text{Tg/Tg}}$
25 mice, and cultured for 96h in presence of GDNF and EdU (+GDNF) or EdU alone (ctl). New HuC/D⁺ neurons can be induced by GDNF under these *ex vivo* culture conditions but the total number of neurons per explant is variable (**FIG. 14A**), these neurons only formed very small ganglia, if any (**FIG. 14B**), and they were less likely to show EdU incorporation than SCPs (see arrowhead in **FIG. 14B** and quantification in **FIG. 14C**) (n=7 explants per condition; * $P<0.05$; ** $P<0.01$; *** $P<0.001$; two-tailed Mann-Whitney U test). (**FIGs. 14D-G**) Immunofluorescence-based analysis of explants prepared from samples of aganglionic colon resected from human HSCR patients, and cultured for 96h in presence of GDNF and EdU (+GDNF) or EdU alone (ctl). For all human explants cultured under these conditions GDNF treatment leads to robust incorporation of EdU in SOX10⁺ SCPs but not in HuC/D⁺ neurons (**FIGs. 14D-E**). A significant
35 number of HuC/D⁺ neurons can be induced by GDNF in a subset of explants (**FIG. 14F**), which all originated from patients less than 3 months of age at the time of surgery (**FIG. 14G**) (n=12 explants per condition; * $P<0.05$; two-tailed Mann-Whitney U test). (**FIG. 14H**) Extended culture in

presence of GDNF for a total of 7 days allowed the detection of neurons in human explants from patients ≥ 3 months of age at the time of surgery, including some that incorporated EdU (arrowhead). All displayed images represent a z-stack projection at the level of the myenteric plexus. Scale bars, 50 μm (FIGs. 14B and H) and 100 μm (FIGs. 14D and F). Dashed outline marks area occupied by extrinsic nerve fibers.

FIGs. 15A-B show an analysis of neurogenesis and SCP proliferation in distal colon explants prepared from P4 *Hol^{Tg/Tg}* mice and cultured in presence or absence of GDNF for 96h. Representative images of HuC/D⁺ neurons (FIG. 15A), and EdU⁺ SOX10⁺ proliferating SCPs (arrows in FIG. 15B) in explants of distal colon from *Hol^{Tg/Tg}* mice cultured in presence of GDNF and EdU (+GDNF) or EdU alone (ctl). The displayed images are single focal planes representative of observations made from 7 mice. Scale bar, 50 μm . Dashed outline marks area occupied by extrinsic nerve fibers.

FIG. 16 shows a marker analysis of GDNF-induced neurons in sigmoid colon explants prepared from HSCR patients and cultured in presence of GDNF for 96h. Immunofluorescence analysis showing that human GDNF-induced neurons are closely associated with extrinsic nerves and express β III-Tubulin (TuJ1), RET, PGP9.5 and PHOX2B (in support of FIG. 14F). Arrowheads point to round/ovoid nuclei of PGP9.5⁺ neurons. The displayed images are single focal planes representative of observations made from 3 human samples. Scale bar, 100 μm (upper panels), 50 μm (middle panels) and 25 μm (lower panels).

FIG. 17A shows the amino acid sequence of human GDNF isoform 1 (UniProtKB accession No. P39905, SEQ ID NO:1), with the sequence corresponding to the signal peptide underlined (residues 1-19), the sequence corresponding to the propeptide italicized (residues 20-75) and the sequence corresponding to the mature polypeptide in bold (residues 78-211).

FIGs. 17B-C show the nucleotide sequence of the cDNA encoding human GDNF isoform 1 (RefSeq accession No. NM_000514.4, SEQ ID NO:2), with the sequence encoding the signal peptide underlined (nucleotides 562-618), the sequence encoding the propeptide italicized (nucleotides 619-786) and the sequence encoding the mature polypeptide in bold (nucleotides 793-1194).

DISCLOSURE OF INVENTION

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the technology (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context.

The terms "comprising", "having", "including", and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to") unless otherwise noted.

All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context.

The use of any and all examples, or exemplary language ("e.g.", "such as") provided herein, is intended merely to better illustrate embodiments of the claimed technology and does not pose a limitation on the scope unless otherwise claimed.

No language in the specification should be construed as indicating any non-claimed element as essential to the practice of embodiments of the claimed technology.

Herein, the term "about" has its ordinary meaning. The term "about" is used to indicate that a value includes an inherent variation of error for the device or the method being employed to determine the value, or encompass values close to the recited values, for example within 10% of the recited values (or range of values).

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All subsets of values within the ranges are also incorporated into the specification as if they were individually recited herein.

Where features or aspects of the disclosure are described in terms of Markush groups or list of alternatives, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member, or subgroup of members, of the Markush group or list of alternatives.

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in stem cell biology, cell culture, molecular genetics, immunology, immunohistochemistry, protein chemistry, and biochemistry).

Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present disclosure are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, *A Practical Guide to Molecular Cloning*, John Wiley and Sons (1984), J. Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory Press (1989), T. A. Brown (editor), *Essential Molecular Biology: A Practical Approach*, Volumes 1 and 2, IRL Press (1991), D. M. Glover and B. D. Hames (editors), *DNA Cloning: A Practical Approach*, Volumes 1-4, IRL Press (1995 and 1996), and F. M. Ausubel *et al.* (editors), *Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) *Antibodies: A Laboratory Manual*, Cold Spring Harbour Laboratory, (1988), and J. E. Coligan *et al.* (editors) *Current Protocols in Immunology*, John Wiley & Sons (including all updates until present).

In the studies described herein, the present inventors show that administration of a proper dosage of recombinant GDNF in the distal colon via rectal enema can induce permanent formation of new functioning enteric neurons and glia in otherwise aganglionic colon, and restoration of colon motility, in HSCR mouse models. Genetic lineage tracing show that SCPs
5 located in extrinsic nerve fibers are one source for these newly generated enteric neurons and glia. It is further demonstrated that GDNF can stimulate neurogenesis in cultured explants of aganglionic colon from human HSCR patients. Thus, GDNF appeared as a primary candidate for postnatal reactivation of ENS progenitors in the aganglionic zone notably because of its ability to stimulate migration and proliferation of Schwann cells in a RET-independent but GFR α 1-
10 dependent manner through its alternative receptor neural cell adhesion molecule (NCAM). These results provide evidence that administration of recombinant GDNF administered in the colon (e.g., distal colon) may be used for the treatment for enteric neuropathies (e.g., ENS defects such as HSCR) i.e. for improving one or more of the pathological features of enteric neuropathies, in human patients.

15 Accordingly, in a first aspect, the present disclosure provides a method for inducing enteric neurogenesis in an aganglionic or hypoganglionic segment of the distal colon of a human subject suffering from an enteric neuropathy (e.g., Hirschsprung disease (HSCR) or intestinal hypoganglionosis), the method comprising administering a pharmaceutical composition comprising an effective dose of a Glial cell line-Derived Neurotrophic Factor (GDNF) polypeptide
20 and a pharmaceutically acceptable carrier into the distal colon of the subject.

The present disclosure also provides a method for restoring distal colon motility and/or epithelial barrier in a human subject suffering from an enteric neuropathy (e.g., HSCR or intestinal hypoganglionosis), the method comprising administering a pharmaceutical composition comprising an effective dose of a GDNF polypeptide and a pharmaceutically acceptable carrier
25 into the distal colon of the subject.

The present disclosure also provides the use of a pharmaceutical composition comprising a human GDNF polypeptide and a pharmaceutically acceptable carrier for inducing enteric neurogenesis in an aganglionic or hypoganglionic segment of the distal colon of a human subject suffering from an enteric neuropathy (e.g., HSCR or intestinal hypoganglionosis), wherein
30 the composition is for administration into the distal colon of the subject.

The present disclosure also provides the use of a pharmaceutical composition comprising a human GDNF polypeptide and a pharmaceutically acceptable carrier for restoring distal colon motility in a human subject suffering from an enteric neuropathy (e.g., HSCR or intestinal hypoganglionosis), wherein the composition is for administration into the distal colon of
35 the subject.

The present disclosure also provides the use of a pharmaceutical composition comprising a human GDNF polypeptide and a pharmaceutically acceptable carrier for the

manufacture of a medicament for inducing enteric neurogenesis in an aganglionic or hypoganglionic segment of the distal colon of a human subject suffering from an enteric neuropathy (e.g., HSCR or intestinal hypoganglionosis), wherein the medicament is for administration into the distal colon of the subject.

5 The present disclosure also provides the use of a pharmaceutical composition comprising a human GDNF polypeptide and a pharmaceutically acceptable carrier for the manufacture of a medicament for restoring distal colon motility in a human subject suffering from an enteric neuropathy (e.g., HSCR or intestinal hypoganglionosis), wherein the medicament is for administration into the distal colon of the subject.

10 The present disclosure also provides a pharmaceutical composition for inducing enteric neurogenesis in an aganglionic or hypoganglionic segment of the distal colon of a human subject suffering from an enteric neuropathy (e.g., HSCR or intestinal hypoganglionosis), the composition comprising a human GDNF polypeptide and a pharmaceutically acceptable carrier, and wherein the pharmaceutical composition is for administration into the distal colon of the subject.

15 The present disclosure also provides a pharmaceutical composition for restoring distal colon motility in a human subject suffering from an enteric neuropathy (e.g., HSCR or intestinal hypoganglionosis), the composition comprising a human GDNF polypeptide and a pharmaceutically acceptable carrier, and wherein the pharmaceutical composition is for administration into the distal colon of the subject.

20 The term “distal colon” as used herein refers to the last three portions of the colon, namely the descending colon, the sigmoid colon and the rectum. In an embodiment, the pharmaceutical composition is administered or is for administration into the rectum and/or the sigmoid colon. In an embodiment, the pharmaceutical composition is administered or is for administration into the rectosigmoid region, which comprises the last part of the sigmoid colon
25 and the beginning of the rectum. The skilled person would understand that the pharmaceutical composition may be administered directly into the distal colon, or may be administered at a site away from the distal colon but using suitable means to provide delivery of the pharmaceutical composition (and more specifically of the human GDNF polypeptide) into the distal colon. For example, the pharmaceutical composition may comprise a coating that is specifically degraded
30 under the conditions (e.g., pH, enzymatic environment, bacterial environment, etc.) of the distal colon, and thus pharmaceutical composition may be administered in another region of the gastrointestinal system but the human GDNF polypeptide will only be released once the pharmaceutical composition reaches the colon, and more specifically the distal colon. Approaches for colon specific drug delivery are well known in the art (see, e.g., Philip *et al.*, *Oman Med J.* 2010 Apr; 25(2): 79–87; Lee *et al.*, *Pharmaceutics.* 2020 Jan; 12(1): 68), and include pH-dependent systems
35 (e.g., using pH-dependent polymers), receptor-mediated systems, magnetically-driven systems, delayed or time-dependent systems, microbially triggered drug delivery systems (e.g., comprising

sugar-based polymers that may be degraded by enzymes produced by the colon microflora such as glucoronidase, xylosidase, arabinosidase, galactosidase), pressure controlled colonic delivery capsule (drug release induced by the higher pressures encountered in the colon), osmotic controlled drug delivery, as well as any combinations of these approaches (e.g., colon targeted delivery system (CODESTM) using a combined approach of pH dependent and microbially triggered drug delivery).

The term “enteric neuropathy” as used herein refers to a disease associated with abnormalities in the ENS, including abnormal development of the ENS, e.g., abnormal number of neurons (hypoganglionosis, aganglionosis) and/or abnormal differentiation of neurons. Examples of enteric neuropathies include enteric dysganglionoses such as HSCR and intestinal hypoganglionosis. In an embodiment, the enteric neuropathy is HSCR. In another embodiment, the enteric neuropathy is intestinal hypoganglionosis.

The expression “inducing enteric neurogenesis” as used herein refers to an increase in the production of enteric neurons and/or enteric glial cells relative to prior to treatment with the composition comprising a human GDNF polypeptide. The enteric nervous system comprises various types of neurones including enteric primary afferent neurons (EPANs), excitatory circular muscle motoneurons, inhibitory circular muscle motoneurons, longitudinal muscle motoneurons, ascending interneurons, descending interneurons, secretomotor and vasomotor neurons, and intestinofugal neurons, as well as enteric glial cells (EGCs) that provide structural support to neurons and contribute to neuronal maintenance, survival, and function (Costa *et al.*, *Gut* 2000;(Suppl IV) 47: iv15-iv19; De Giorgio *et al.*, *American Journal of Physiology-Gastrointestinal and Liver Physiology*, Vol. 303, No. 8: G887-G893, 2012).

The production of one or more of these cell types may be induced by the administration/use of the composition comprising a human GDNF polypeptide. In an embodiment, the production of EGCs, preferably Sox10-expressing EGCs, is induced by the administration/use of the composition comprising a human GDNF polypeptide. In an embodiment, the administration/use of the composition comprising a human GDNF polypeptide restores the enteric neurons/glial cell ratio in the colon (e.g., distal colon) of the patient. In a further embodiment, the enteric neurons/glial cell ratio in the colon (e.g., distal colon) of the patient is at least 0.5, e.g., between 0.5 and 1.5. In another embodiment, the administration/use of the composition comprising a human GDNF polypeptide restores the proportions of nitrergic (nNOS⁺) and cholinergic (ChAT⁺) neurons in the colon (e.g., distal colon) of the patient.

In another embodiment, the administration/use of the composition comprising a human GDNF polypeptide reduces the infiltration of inflammatory or immune cells (e.g., neutrophils) in the colon (e.g., distal colon). In another embodiment, the administration/use of the composition comprising a human GDNF polypeptide restores (partly or completely) the proportions of immune cells in the colon (e.g., distal colon).

The term “human GDNF polypeptide” as used herein refers to the native mature human GDNF protein, or to functional variants or fragments thereof that retain a biological activity of the native mature human GDNF protein, e.g., the ability to bind to a GDNF receptor (particularly the “rearranged during transfection” (RET) proto-oncogene and/or the Neural Cell Adhesion Molecule (NCAM) receptor) and trigger a signal in a cell expressing a GDNF receptor (e.g., RET and/or NCAM). The amino acid sequence of native human GDNF protein (isoform 1, the canonical sequence) is depicted in **FIG. 17A** (SEQ ID NO:1), with the sequence corresponding to the mature protein (residues 78-211) highlighted in bold. The GDNF precursor protein is processed to a mature secreted form that exists as a homodimer. Each GDNF monomer contains seven conserved cysteine residues, including Cys-101, which is used for inter-chain disulfide bridging, and others that are involved in the intramolecular ring formation known as the cysteine-knot configuration.

In an embodiment, the human GDNF polypeptide is a recombinant human GDNF polypeptide. The term “recombinant” when made in reference to a protein or a polypeptide refers to a protein or polypeptide molecule that is not isolated from a natural source (e.g., biological sample), e.g., which is expressed from a recombinant nucleic acid construct created by means of molecular biological techniques. Referring to a nucleic acid construct as “recombinant” therefore indicates that the nucleic acid molecule has been manipulated using genetic engineering, i.e. by human intervention. Recombinant nucleic acid constructs may for example be introduced into a host cell by transformation (e.g., transduction or transfection).

Functional variants or fragments of native mature human GDNF protein may include one or more amino acid substitutions, deletions and/or additions relative to the native mature human GDNF protein, and may have a biological activity that is lower, equivalent or higher than that of the native mature human GDNF protein. In an embodiment, the functional variant or fragment has an activity that is equivalent (e.g., between 90% to 110%) or higher (e.g., more than 110%) to that of the native mature human GDNF protein. In an embodiment, the variant comprises one or more conservative substitutions. Conservative amino acid substitutions are known in the art, and include amino acid substitutions in which one amino acid having certain physical and or chemical properties is exchanged for another amino acid that has the same chemical or physical properties. For instance, the conservative amino acid substitution can be an acidic amino acid substituted for another acidic amino acid (e.g., Asp to Glu or vice-versa), an amino acid with a nonpolar side chain substituted for another amino acid with a nonpolar side chain (e.g., Ala, Gly, Val, Ile, Leu, Met, Phe, Pro, Trp, Val, etc.), a basic amino acid substituted for another basic amino acid (Lys, Arg, etc.), an amino acid with a polar side chain substituted for another amino acid with a polar side chain (Asn, Cys, Gln, Ser, Thr, Tyr, etc.). In another embodiment, the variants can comprise the amino acid sequence of the native GDNF protein or polypeptide with at least one non-conservative amino acid substitution. Preferably, the non-conservative amino acid substitution(s)

enhance(s) the activity of the variant relative to that of the native mature human GDNF protein. In an embodiment, the human GDNF polypeptide has the ability to bind to the RET receptor. In an embodiment, the human GDNF polypeptide has the ability to bind to the NCAM receptor.

In an embodiment, the human GDNF polypeptide comprises at least 10, 15 or 20 amino acids (e.g., contiguous amino acids) from the mature human native GDNF protein. In an embodiment, the human GDNF polypeptide comprises the sequence ETTYDKILKNLSRNR (gliafin, SEQ ID NO:3), which corresponds to residues 153-167 of SEQ ID NO: 1 and is the putative binding domain of human GDNF to the NCAM receptor (see, Nielsen *et al.*, *J Neurosci.* 2009 Sep 9; 29(36): 11360–11376). In other embodiments, the human GDNF polypeptide comprises at least 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 or 100 amino acids (e.g., contiguous amino acids) from the mature human native GDNF protein.

In an embodiment, the human GDNF polypeptide comprises an amino acid sequence that is at least 50%, 60% or 70% identical to the sequence of residues 78-211 depicted in **FIG. 178A** (SEQ ID NO:1). In another embodiment, the human GDNF polypeptide comprises an amino acid sequence that is at least 80% identical to the sequence of residues 78-211 depicted in **FIG. 17A** (SEQ ID NO:1). In another embodiment, the human GDNF polypeptide comprises an amino acid sequence that is at least 85% identical to the sequence of residues 78-211 depicted in **FIG. 17A** (SEQ ID NO:1). In another embodiment, the human GDNF polypeptide comprises an amino acid sequence that is at least 90% identical to the sequence of residues 78-211 depicted in **FIG. 17A** (SEQ ID NO:1). In another embodiment, the human GDNF polypeptide comprises an amino acid sequence that is at least 95% identical to the sequence of residues 78-211 depicted in **FIG. 17A** (SEQ ID NO:1). In another embodiment, the human GDNF polypeptide comprises an amino acid sequence that is at least 98% identical to the sequence of residues 78-211 depicted in **FIG. 17A** (SEQ ID NO:1). In another embodiment, the human GDNF polypeptide comprises an amino acid sequence that is at least 99% identical to the sequence of residues 78-211 depicted in **FIG. 17A** (SEQ ID NO:1). In another embodiment, the human GDNF polypeptide comprises or consists of the sequence of residues 78-211 depicted in **FIG. 17A** (SEQ ID NO:1). "Identity" refers to sequence identity between two polypeptides. Identity can be determined by comparing each position in the aligned sequences. Methods of determining percent identity are known in the art, and several tools and programs are available to align amino acid sequences and determine a percentage of identity including EMBOSS Needle, ClustalW, SIM, DIALIGN, etc. As used herein, a given percentage of identity with respect to a specified subject sequence, or a specified portion thereof, may be defined as the percentage of amino acids in the candidate derivative sequence identical with the amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the Smith Waterman algorithm (Smith & Waterman, *J. Mol. Biol.* **147**: 195-7 (1981)) using the BLOSUM substitution matrices (Henikoff & Henikoff, *Proc. Natl. Acad.*

Sci. USA **89**:10915-9 (1992)) as similarity measures. A "% identity value" is determined by the number of matching identical amino acids divided by the sequence length for which the percent identity is being reported.

Covalent modifications of the human GDNF polypeptide are included within the scope of this disclosure. For example, the native glycosylation pattern of the human GDNF polypeptide may be modified (Beck *et al.*, *Curr. Pharm. Biotechnol.* **9**: 482-501, 2008; Walsh, *Drug Discov. Today* **15**: 773-780, 2010), and linking the human GDNF polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol (PEG), polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. The human GDNF polypeptide may comprise one or more modifications that confer additional biological properties to the polypeptide such as protease resistance, plasma protein binding, increased plasma half-life, tissue or intracellular penetration, etc. Such modifications include, for example, covalent attachment of molecules/moiety to the polypeptide such as fatty acids (e.g., C₆-C₁₈), attachment of proteins such as albumin (see, e.g., U.S. Patent No. 7,268,113); sugars/polysaccharides (glycosylation), biotinylation or PEGylation (see, e.g., U.S. Patent Nos. 7,256,258 and 6,528,485). The human GDNF polypeptide may also be conjugated to moieties to induce its multimerization or oligomerization (e.g., tetramerization), for example by fusing the human GDNF polypeptide to an oligomerization domain or to a molecule that may be oligomerized (e.g., biotin that may bind to 4 binding sites on streptavidin). The human GDNF polypeptide may also be conjugated to moieties that will target the GDNF polypeptide to the distal colon or to specific cells of the distal colon (e.g., Schwann cells and/or precursor thereof), for example using an antibody, antibody fragment or ligand that binds to a marker present on cells from the distal colon.

The human GDNF polypeptide can also be conjugated to one or more therapeutic or active agents (e.g., to a drug, or to another polypeptide to form a fusion polypeptide). Any method known in the art for conjugating the human GDNF polypeptide to another moiety (e.g., active agent) may be employed, including those methods described by Hunter *et al.* (1962) *Nature*, **144**:945; David *et al.* (1974) *Biochemistry*, **13**: 1014; Pain *et al.* (1981) *J. Immunol. Meth.*, **40**:219; Nygren, *J. Histochem. and Cytochem.*, **30**:407 (1982), and Hermanson, *Bioconjugate Techniques* 1996, Academic Press, Inc., San Diego.

In an embodiment, the effective dose of recombinant GDNF polypeptide administered or for administration to the human subject corresponds to a dose of about 5 µg to about 20 µg in a mouse pup, which is the range shown to be effective in the studies described herein. A 10 µl enema comprising a recombinant GDNF solution was administered to mouse pups. 10 µl is estimated to correspond to the volume necessary to fill the distal colon and rectum of the pups. Accordingly, administration of 5 µg GDNF in mice (pups) is achieved by administering 10 µl of a 0.5 µg/µl GDNF solution, and administration of 20 µg GDNF in mice (pups) is achieved by

administering 10 μ l of a 2.0 μ g/ μ l GDNF solution. The volume required to fill the distal colon and rectum of a human baby may be estimated using the formula: 10ml x weight of the baby (in kg). Accordingly, a dose of 5 μ g GDNF in mice corresponds to about 5 mg per kg in a human baby, and a dose of 20 μ g GDNF in mice corresponds to about 20 mg per kg in a human baby. Thus, in an embodiment, the effective dose of recombinant GDNF polypeptide administered or for administration to the human subject is about 5 mg to about 20 mg per kg, preferably about 10 mg to about 15 mg per kg. In an embodiment, the recombinant GDNF polypeptide is administered or is for administration through a 0.5 mg/ml to 2 mg/ml composition (solution or gel).

Formulations for rectal/distal colon administration may be presented as a suppository, which may be prepared by mixing a subject composition with one or more suitable non-irritating carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax, or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the appropriate body cavity and release the composition comprising GDNF. More recently, liquid suppositories have been developed. Liquid suppositories typically contain thermosensitive and/or mucoadhesive polymers such as poloxamers, Carbopol® (crosslinked polyacrylic acid polymers), sodium alginate, polycarbophil, hydroxypropyl methylcellulose (HPMC), hydroxyethyl cellulose, and methylcellulose.

Formulations for rectal/distal colon administration may also be presented as an enema, a liquid-drug solution, suspension or emulsion that is injected into the rectum and the distal colon. The liquid in which the GDNF polypeptide is diluted may be water or a saline solution, for example.

Formulations for rectal/distal colon administration may also be in the form of a rectal foam or gel. Rectal gels are semi-solid formulations that contain a solvent trapped within a polymer network to create a viscous consistency. Viscosity of the gel can be modified by the addition of co-solvents (e.g., glycerin and propylene glycol) and electrolytes. Foams comprise a hydrophilic liquid continuous phase containing a foaming agent and a gaseous dispersion phase distributed throughout. Following rectal administration, they transition from a foam state to a liquid or semi-solid state on the mucosal surface. Foaming agents are typically amphiphilic substances that are important for foam generation and stabilization. The molecules contain hydrophilic components that are soluble in the aqueous phase and hydrophobic components that form micelles to minimize contact with the aqueous phase.

Administration into the rectum/distal colon may be performed using currently available endoscopes or specialized catheters designed for rectal administration or injection into the distal colon wall of medications and liquids, which may be placed safely and remain comfortably in the rectum for repeated use.

The composition comprising GDNF polypeptide may be administered according to any suitable dosage regimen, for example four times-a-day, twice-a-day, once-a-day, twice-a-week,

once-a-week, etc. The treatment may be performed for any suitable period of time to achieve the desired effect, for example for 1 week, 2 weeks, 3 weeks or more.

In an embodiment, the above-mentioned treatment comprises the use/administration of more than one (*i.e.* a combination of) active/therapeutic agent, one of which being the above-mentioned pharmaceutical composition comprising a GDNF polypeptide. The combination of therapeutic agents and/or compositions may be administered or co-administered (*e.g.*, consecutively, simultaneously, at different times) in any conventional dosage form. Co-administration in the context of the present disclosure refers to the use of more than one therapy in the course of a coordinated treatment to achieve an improved clinical outcome. The pharmaceutical composition comprising a GDNF polypeptide described herein may be used in combination with other therapies or drugs, for example analgesics or anti-inflammatory agents. The pharmaceutical composition comprising a GDNF polypeptide described herein may also be used in combination with an agent that stimulate ENS progenitor proliferation, such as a neurotrophic molecule.

In an embodiment, the above-mentioned treatment with a composition comprising GDNF polypeptide may be performed in combination with surgery (*e.g.*, pull-through surgery of the Swenson, Soave or Duhamel type). Especially for neonates with HSCR, clinicians often recommend a trial of daily enema treatments prior to surgery. Addition of recombinant GDNF to the enema might increase the likelihood that children with HSCR responded well to pre-operative enema therapy. Accordingly, in an embodiment, the above-mentioned treatment with a composition comprising GDNF polypeptide is performed prior to pull-through surgery. Saline enemas are also commonly used in children with HSCR after pull-through surgery. Post-surgical problems in HSCR patients are believed to be due at least in part to hypoganglionosis in retained distal bowel, the so-called "transition zone". Thus, addition of recombinant GDNF to the enema may be useful to correct the hypoganglionosis in retained distal bowel after surgery. Accordingly, in another embodiment, the above-mentioned treatment with a composition comprising GDNF polypeptide is performed after pull-through surgery.

In an embodiment, the above-mentioned treatment with a composition comprising GDNF polypeptide is performed in combination with ENS stem cell-based therapies, which are being considered for the treatment of HSCR. GDNF may be a useful adjunct to these therapies to promote engraftment.

HSCR is clinically subdivided into short-segment (S-HSCR) and long-segment forms (L-HSCR). S-HSCR, which occurs in >80% of cases, means the ENS is absent from rectum and sigmoid colon. L-HSCR means longer regions of distal bowel are aganglionic. The method/use described herein is for the treatment of a human patient suffering from S-HSCR or L-HSCR. In an embodiment, the method/use described herein is for the treatment of a human patient suffering

from S-HSCR. In an embodiment, the method/use described herein is for the treatment of a human patient suffering from L-HSCR.

The HSCR patient may be an adult patient or pediatric patient. In an embodiment, the HSCR patient is a pediatric patient, preferably a patient that is less than 5, 4, 3 or 2 year-old, more preferably a patient that is less than 1 year-old or less than 6 month-old. In another embodiment, the patient is a male.

HSCR has been associated with mutations in *RET*, *EDNRB*, *SOX10*, *PHOX2B*, and *ZFHX1B*, as well as with Down syndrome or Trisomy 21 (Collagen VI-associated HSCR). There is also a significant sex difference with male to female ratio as high as 5 to 1. In an embodiment, the HSCR is Collagen VI-associated HSCR. In another embodiment, the HSCR is *EDNRB* mutation-associated HSCR. In an embodiment, the HSCR is male-biased HSCR. In an embodiment, the HSCR is a *RET* mutation-associated HSCR, e.g., HSCR associated with a mutation that reduces *RET* expression and/or activity in cells from the distal colon. The mutation may be a mutation in *RET* or in a protein involved in *RET* signaling. In an embodiment, the mutation is a mutation in the RET protein. Mutations in the RET gene on chromosome 10q11.2 have been shown to account for 50% of familial and 15-20% of sporadic cases of HSCR, most of which (~75%) were associated with L-HSCR.

In another embodiment, the HSCR is not a *RET* mutation-associated HSCR.

MODE(S) FOR CARRYING OUT THE INVENTION

The present invention is illustrated in further details by the following non-limiting examples.

Example 1: Materials and Methods

Mice. *Holstein* (*Tg[Sox3-GFP,Tyr]HolNpln*), *TashT* (*Tg[SRY-YFP,Tyr]TashTNpln*), and *G4-RFP* (*Gata4p[5kb]-RFP*) lines were as previously described (all maintained on a FVB/N genetic background) (39, 40, 54), whereas *Piebald-lethal* (*Ednrb^{S-L}*; JAX stock # 000308; C3H/HeJ-C57BL/6 mixed background) and *Dhh-Cre* (*Tg[Dhh-cre]1Mejr*; JAX stock # 012929; FVB/N background) were obtained from The Jackson Laboratory. Other mouse lines used were *R26^{[Floxed Stop]YFP}* (*Gt[ROSA]26Sor^{tm1(EYFP)Cos}*; provided by F. Costantini (Columbia University, USA) and maintained on an FVB/N background) (80).

For all enema treatments, mutant mouse pups were identified at P3 via pigmentation-based genotyping. Unless specified otherwise (see FIG. 1), 10 µl enemas consisting of a 1 µg/µl solution of recombinant human mature GDNF (Peprotech cat. # 450-10) diluted in PBS were administered daily between P4 to P8. Clinical grade GDNF (Medgenesis Therapeutix Inc., Canada) and a previously described 6XHis-tagged version (53) used for some experiments had similar efficiency. Other tested molecules (1µg/µl solution in 10µl enemas) included the serotonin receptor (5-HT4R) agonist RS67506 (R&D Systems, Cat. # 0990), Noggin (Sigma, Cat. #

SRP4675), endothelin-3 (Sigma, Cat. # E9137), serotonin (Sigma, Cat. # H9523), and L-ascorbic acid (Sigma, Cat. # A4403). Enemas were administered using a 24-gauge gavage needle (Fine Science Tools, Canada) attached to a micropipette. The head of the gavage needle was introduced in the rectum just beyond the anus (pre-lubricated with Vaseline™), and enemas were injected over the course of a few seconds. Pups were then placed back with their mother, and either sacrificed at P20 for tissue analysis or checked daily to track survival.

For EdU incorporation assays, mouse pups received 10 µl intraperitoneal injections of a 10mM EdU solution (ThermoFisher Scientific, Cat. # C10337) once a day during the 5-day (P4 to P8) GDNF enema treatment.

Tissue processing. All bowel tissues to be labelled were cut longitudinally along the mesentery, washed in PBS, pinned down on Sylgard-coated petri dishes, fixed with 4% PFA at 4°C overnight, and finally microdissected to separate longitudinal/circular muscles (containing myenteric neural ganglia) from the submucosa/mucosa layer (containing submucosal neural ganglia). For experiments involving *ex vivo* analyses of living tissues, longitudinal/circular muscles and submucosa/mucosa layers were similarly microdissected, but without fixation. Instead this procedure was performed in ice-cold oxygenated Krebs solution (NaH₂PO₄-2H₂O 0.187 g/L, NaCl 6.84 g/L, KCl 0.35 g/L, NaHCO₃ 2.10 g/L, Glucose 1.98 g/L, CaCl₂-2H₂O 0.368 g/L, MgCl₂-6H₂O 0.244 g/L). For histological analyses, PFA-fixed bowel tissues were prepared as described above but without further microdissection, and fixed full-thickness bowel segments were then embedded in paraffin and transversally sectioned at 10 µm.

Tissue labelling and imaging. For immunofluorescence staining, whole microdissected tissues were permeabilized for 2 hours in blocking solution (10% FBS and 1% Triton™ X-100, in PBS) before being sequentially incubated with specific primary (at 4°C overnight) and relevant secondary (at room temperature for 2 hours) antibodies, both diluted in blocking solution that was also used to wash tissues between all steps. All antibodies and dilution factors are listed in **Table 1**. EdU was detected using the Invitrogen Click-iT EdU Imaging Kit (ThermoFisher Scientific, Cat. # C10337) in accordance with the manufacturer's instructions. For histological analyses, cross-sections of full-thickness bowel tissues were stained with hematoxylin and eosin (H&E) as previously described (83).

Table 1: List of primary antibodies and dilution factors used for immunofluorescence

Antibody	Source	Catalog number	RRID reference	Dilution
6X His	R&D systems	MAB050	AB_357353	1:500
CalR	Swant	CG1	AB_10000342	1:500
ChAT	Millipore	AB144P	AB_2079751	1:100
GFP	Abcam	Ab290	AB_303395	1:500
HuC/D	Molecular Probes	A-21271	AB_221448	1:500
HuC/D (ANNA-1)	Gift from Vanda Lennon	---	AB_2313944	1:2000
Ki67	Abcam	ab15580	AB_443209	1:500

NCAM	Abcam	ab5032	AB_2291692	1:500
NOS1	Santa Cruz Biotechnology	sc-648	AB_630935	1:200
RET	R&D systems	MAB718	AB_2232594	1:500
SOX10	Santa Cruz Biotechnology	sc-17342	AB_2195374	1:200
SubP	Abcam	ab67006	AB_1143173	1:500
TH	Abcam	ab137869	---	1:500
TUJ1	Covance	MRB-435P	AB_663339	1:1000
VIP	Abcam	ab8556	AB_306628	1:500

All immunofluorescence images were acquired with either a 20X or a 60X objective on a confocal microscope (either Nikon A1R or Zeiss 710), except for H&E-stained sections that were imaged with a 10X objective using an Infinity-2 camera (Lumenera Corporation) mounted on a Leica DM 2000 microscope (Leica Microsystems Canada). Image analysis was performed with ImageJ, using the “multi-point” function for cell counting, and the “polygon selection” function for calculation of surface area.

In vivo and ex vivo analysis of colonic motility. *In vivo* analysis of distal colonic motility in P20 mice was performed using the bead latency test. Mice were anesthetized with 2% isoflurane and a 2 mm glass bead (Sigma, Cat. # 1.04014) was inserted into the distal colon with a probe over a distance of 0.5 cm from the anus. Each mouse was then isolated in its cage without access to food and water, and monitored for the time required to expel the glass bead, which was taken as a proxy for distal colonic transit. The maximal time allowed to expel the bead was 30 minutes.

For *ex vivo* analysis of colonic motility, strips of living muscles from most distal colon (1cm from the anus) of P20 mice were prepared as described above, and attached in the longitudinal direction in a Schuler organ bath (Harvard apparatus) filled with oxygenated Krebs solution. Muscle strips were initially stretched with a preload of 2 g of tension for 60 min, and contraction/relaxation of longitudinal muscles was then continuously recorded with a myograph (Narco Biosystems Inc., Model F-60) coupled to a computer equipped with the BIOPAC student Lab 4.0.2 software (BIOPAC Systems Inc.). Electrical field stimulation (EFS) was applied with a voltage stimulator (BIOPAC Systems Inc., Model BSL MP36/35) connected to electrodes, using parameters that activate enteric neurons without directly activating muscles (12 V, 20 Hz, 10s train duration, and 300 μ s stimulus pulse duration). This procedure was repeated 3 times, with 10 min washout periods between stimulations. To characterize the nitrergic and cholinergic components of EFS-induced contractile responses, N-nitro-L-arginine methyl ester (L-NAME; Sigma, Cat. # N5751) and atropine (Sigma, Cat. # A01132) were added to Krebs solution at a final concentration of 0.5 μ M and 1 μ M, respectively. The area under the curve (AUC) was measured during each EFS-induced response, and data were expressed in Δ AUC (corresponding

to the difference between the AUC measured 20s after stimulation minus the AUC measured 20s before stimulation).

Ex vivo analysis of paracellular permeability. Segments of living mucosa from most distal colon (1cm from the anus) of P20 mice were prepared as described above, and mounted in Ussing chambers with 0.5 cm² exposed surface area (Warner Instruments, Model U-9926). Each chamber contained 5 ml of DMEM/F12 medium (Wisent, Cat. # 319-085-CL), which was maintained at 37°C and continuously oxygenated (95% O₂ / 5% CO₂). After a 30 min equilibration period, 200 µl of apical medium was replaced with 200 µl of a 1 mg/ml solution of fluorescein isothiocyanate-conjugated dextran 4kDa (FD4; Sigma, Cat. # 60842-46-8). Fluorescence intensity of basolateral aliquots of 150 µl, reflecting paracellular transit from the luminal surface, was then measured every 30 min over a period of 3 hours, using a fluorimeter (TECAN, Model Infinite M1000). Fluorescence intensity was finally converted in amount of FD4 by comparison to a standard curve, and the average value for the 3-hour period was used to calculate paracellular permeability, which was expressed in ng of FD4 per surface of mucosa area per min (ng/cm²/min).

Microbiome analysis. Stool isolation, microbiome sequencing and data analysis were performed as previously described (38). Briefly, mice were sacrificed at P20 and their feces were directly collected from the colon (3 fecal pellets per mouse). Bacterial DNA was then extracted using the QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Cat. # 51604), and the V5-V6 region of the 16S rRNA gene was PCR amplified with a collection of previously described barcoded primers (84). Raw sequences generated with an Illumina MiSeq sequencer were paired and processed using the MOTHUR pipeline (85), and the BIOM package (86) was subsequently used to transfer biom files into R (87) for generating graphs of relative taxa abundance and beta diversity.

Western blot analysis. Organs from P8 or P20 mice were weighed and dissolved in RIPA buffer (25 mM Tris-HCL [pH7.6], 150mM Sodium Chloride, 1% Nonidet P-40, 1% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate, containing 1X Roche Complete protease inhibitors), using 1 mL for every 100 mg of tissue. Samples were then sonicated on ice and centrifuged at 14,000 rpm for 15 minutes at 4°C, keeping the supernatants for western blot analysis. Equal volumes of samples were electrophoretically separated in an 18% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to Immobilon-P® PVDF membranes (Bio-Rad, Cat. # 1620177). Membranes were subsequently incubated in blocking solution (5% skimmed milk and 0.1% Tween® 20, in TBS), followed by incubation with either mouse anti-GDNF (Santa Cruz Biotechnology, Cat. # sc-13147; 1:500 dilution factor) or rabbit anti-αTubulin (Abcam, Cat. # ab176560; 1:70,000 dilution factor) primary antibodies, and then relevant horseradish peroxidase-conjugated secondary antibodies, all diluted in blocking solution. Each incubation was for 60 min at room temperature, each time interspersed by 3 washes with blocking solution. Proteins were finally visualized using Immobilon western chemiluminescent HRP substrate (Millipore Sigma, Cat. # WBKLS0050) and Fusion FX imaging system (Vilber).

Ex vivo time-lapse imaging and culture of murine aganglionic colon tissues. For time-lapse imaging, strips of living muscles from the last cm of distal colon from P4 *Hol^{Tg/Tg};G4-RFP* double transgenic pups were prepared as described above, and pinned down in Sylgard-coated 35 mm ibidi μ -dishes (ibidi, Cat. # 81156). Muscle strips were cultured in suspension in DMEM/F12 medium (Wisent, Cat. # 319-085-CL) supplemented with 10% FBS and 100IU/ml antibiotic-antimycotic with or without 5 μ g/ml GDNF under standard culture conditions (37°C, 5% CO₂). After 72h of culture, each petri dish was placed in a microscope incubation chamber (Okolab) for 10 hours under the same culture conditions, and image stacks (250 μ m-thick) of RFP-labelled extrinsic nerves and SCPs were acquired every 10 min, using a 20X objective on a Nikon A1R confocal unit as previously described (39).

For *ex vivo* induction of neurogenesis, strips of living muscles from the last cm of distal colon from P4 *Hol^{Tg/Tg}* pups were prepared and cultured as described above, with the notable exceptions that culture was performed in presence of 0.5 μ M EdU and for a more extended period of time. After 96h of culture, tissues were fixed with PFA and processed for immunofluorescence and EdU labelling.

Culture of human aganglionic colon tissues. Human samples of sigmoid colon were obtained from 12 HSCR patients undergoing Swenson-type surgical resection of the aganglionic zone, as confirmed by histopathological analysis. Nine patients (7 boys and 2 girls; aged between 28 and 1638 days at the time of surgery) were recruited at the Centre Hospitalier Universitaire Sainte-Justine (Montreal, Canada) while 3 patients were recruited at the Children's Hospital of Philadelphia (2 boys and 1 girl; aged between 300 and 1177 days at the time of surgery). After the surgery, full-thickness colon tissues were placed in ice-cold Krebs solution or Belzer UW Cold Storage Solution (Bridge to Life Ltd.) and immediately brought to the relevant research laboratory. Strips of living muscles were then prepared as described above and cut in smaller pieces of 0.5 cm X 0.5 cm. At least one of these small pieces was immediately fixed and kept aside for validation of aganglionosis via immunofluorescence, while the others were cultured for 96h as described above for inducing neurogenesis in mouse tissues. Samples from two patients (aged of 86 and 1638 days at the time of surgery) were in addition cultured for 7 days, under the same conditions. At the end of culture period, all tissues were fixed with PFA and processed for immunofluorescence and EdU labelling.

Profiling of immune cell populations in P20 colon. *Holstein* mice were treated or not with GDNF by enemas (daily administration of 10 μ g of GDNF (10 μ l at 1 μ g/ μ l) for 5 consecutive days between P4 to P8. At P20, the colon of GDNF-treated *Holstein* and control (untreated *Holstein* and WT) mice was microdissected, cleaned in cold RPMI medium to remove stool, cut into small pieces, mechanically dissociated, and filtrated (40 μ m). Dissociated cells were then recovered by centrifugation, resuspended in 1 ml of ice-cold RPMI with 2% FBS, and counted with a hemocytometer. For every experimental and control groups, 1×10^6 dissociated cells were

incubated for 1h on ice with a cocktail of 14 fluorescently labeled antibodies specific for either lymphoid or myeloid cell populations. Quantitative profiling was subsequently performed using an LSR Fortessa cytometer (BD Biosciences) and FlowJo software (FlowJo LLC).

Statistics. All experiments were performed with a minimum of three biological replicates.

Where relevant, the exact number of independent replicates (*n*) and statistical tests used to calculate *P* values are included in figures and/or legends. *P* values were determined using GraphPad Prism 6, with the exception of microbiome data that were analyzed with R software (The R Foundation for Statistical Computing, Vienna, Austria).

Example 2: GDNF enemas rescue aganglionosis in three mouse models of short-segment HSCR

It was first tested if GDNF enemas in young mice could enhance enteric neuron numbers and restore function in distal aganglionic bowel using three mouse models of short-segment HSCR. Selected lines were *Holstein* (*Hol^{Tg/Tg}*; a model for Trisomy 21 [Collagen VI]-associated HSCR) (39), *TashT* (*TashT^{Tg/Tg}*; a model for male-biased HSCR) (40), and *Piebald-lethal* (*Ednrb^{S-/-}*; a model for *EDNRB* mutation-associated HSCR) (41). The enema volume necessary to fill whole colon, concentration of GDNF homodimer (30 kDa), treatment time window, as well as duration and frequency of therapy were first determined with *Hol^{Tg/Tg}* pups (**FIGs. 1A-D**). It was observed that administration of 100 µg of GDNF reduces survival relative to saline control (**FIG. 1C**), suggesting that such dose is toxic or worsens the disease. Administration of 1 µg or 25 µg of GDNF had no significant effect on survival, and 10 or 15 µg were shown to be optimal to improve survival. Remarkably, the selected treatment (i.e. daily administration of 10 µg GDNF in PBS as 10 µl enemas for 5 consecutive days between postnatal day [P]4 to P8) prevented death by megacolon in about half of *Hol^{Tg/Tg}* mice at P28, the maximum age of survival for control *Hol^{Tg/Tg}* mice (**FIG. 2A**). Most animals surviving to P28 reached adult age after GDNF treatment and mice evaluated could reproduce (two tested breeding pairs were fertile). The few males that were allowed to survive beyond P56 (the adult reference age) eventually died from megacolon at P68, P97, P101 and P180, while females eventually died from either megacolon at P63 or dystocia at P138 and P250. Importantly, the same GDNF enema treatment also prevented premature death for more than 60% of *Ednrb^{S-/-}* mice (**FIG. 2B**) and for all male *TashT^{Tg/Tg}* pups (**FIG. 2C**). Nine GDNF-treated male *TashT^{Tg/Tg}* mice kept for over a year looked healthy without any sign of tumor or other adverse effects. Because saline enemas reduce enterocolitis in children with HSCR, separate groups of *Hol^{Tg/Tg}*, *Ednrb^{S-/-}* and *TashT^{Tg/Tg}* mice were treated with PBS via enema, but PBS alone did not enhance survival (**FIG. 2A-C**). Enema treatment of *Hol^{Tg/Tg}* mice using Noggin homodimer (46 kDa), endothelin-3 monomer (26 kDa), or the serotonin receptor (5-HT4R) agonist RS67506 (454.41 g/mol) in PBS (each at 10 µg in 10 µl PBS) also failed to increase life expectancy (**FIG. 1E**). Collectively, these data suggest that GDNF enemas can be beneficial to

mice with HSCR-like distal bowel aganglionosis due to *Holstein*, *TashT*, or *Piebald-lethal* mutations.

Variability in response to GDNF enemas was observed in mutant mouse lines that are clearly GDNF responsive (**FIGs. 2A-C**). It was tried without success to increase the overall survival rate of GDNF-treated *Hol^{Tg/Tg}* animals either by replacing standard chow with a gel diet (**FIG. 1F**) or by combining GDNF with other molecules that have been shown to stimulate ENS progenitor proliferation like vitamin C (44), serotonin (45) or endothelin-3 (46) (**FIG. 1G**). Gel diet extended life expectancy of control *Hol^{Tg/Tg}* mice by 5 days on average, but did not further increase survival in the GDNF enema group (**FIG. 1F**). It is possible that GDNF enema responsiveness depends to some degree on bowel injury or inflammation that also reduces life expectancy in HSCR mouse models without GDNF treatment (47).

To determine how GDNF enemas enhanced survival of HSCR mouse models, the hypothesis that enhanced survival was caused by postnatal enteric neurogenesis in distal colon aganglionic was tested. P20 animals were analyzed because *Hol^{Tg/Tg}* mice generally reach this stage even without enema treatment (**FIG. 2A**). HuC/D⁺ myenteric neurons were abundant in WT distal colon and absent from the last cm of *Hol^{Tg/Tg}* colon (**FIG. 2D**). SOX10⁺ enteric glia were also abundant within WT myenteric ganglia. In contrast, in *Hol^{Tg/Tg}* distal colon, SOX10⁺ cells were mainly found within thick extrinsic nerve fibers (**FIG. 2D**) where SOX10⁺ SCPs reside (29). Remarkably, distal colon from GDNF-treated *Hol^{Tg/Tg}* animals had numerous HuC/D⁺ neurons and SOX10⁺ glia organized into ganglia between circular and longitudinal smooth muscles (**FIG. 2D**). These myenteric ganglia were primarily adjacent to extrinsic nerve fibers with SOX10⁺ cells. Tuj1 labeling further revealed that GDNF-induced neural ganglia formed interconnected networks in both myenteric and submucosal plexuses (**FIG. 3**). Quantification of myenteric neuron density in whole colon of *Hol^{Tg/Tg}* and male *TashT^{Tg/Tg}* mice showed GDNF effects are most prominent in distal colon (i.e. final 3 cm), with minor effects in proximal colon (**FIGs. 2D and 5A-C**). In the mid-colon of GDNF-treated *Hol^{Tg/Tg}* mice, the increased neuron density (**FIG. 2D**) was mainly due to an enlargement of pre-existing myenteric ganglia, as evidenced by a higher proportion of large ganglia (i.e. >50 neurons) (**FIG. 4A**). In the most distal colon, where untreated *Hol^{Tg/Tg}* mice are normally devoid of enteric neurons, GDNF-treated *Hol^{Tg/Tg}* mice had an average neuron density that was 40% that of WT mice ($5.2 \pm 2.2\%$ vs $13.1 \pm 3.6\%$) (**FIG. 2D**). Furthermore, while all GDNF-treated *Hol^{Tg/Tg}* mice had neurons in the distal colon at the time of dissection, some mice had very low neuron density in distal colon (2.3% or less) and these mice often had megacolon (**FIG. 1H**). Remarkably, in GDNF-treated *TashT^{Tg/Tg}* males, neuron density in the most distal colon increased from 36% ($4.7 \pm 3.8\%$ vs $13.1 \pm 3.6\%$ of neuronal density) to 108% of WT levels ($14.2 \pm 4.3\%$ vs $13.1 \pm 3.6\%$ of neuronal density) (**FIG. 4B-C**) suggesting that it is possible in some models to completely restore normal enteric neuron numbers by GDNF enemas.

Importantly, EdU labeling confirmed that GDNF induced proliferation of neuron and glia progenitors during the 5-day treatment from P4 to P8. Immunofluorescent staining of P20 *Hol^{Tg/Tg}* colon from mice that received daily EdU injections during the GDNF treatment period revealed many EdU⁺ HuC/D⁺ (presumptive neurons) and EdU⁺ SOX10⁺ (presumptive glia or neuron/glia progenitors) in both myenteric and submucosal ganglia (FIGs. 2E-F and 5A-C). The percentage of EdU⁺ cells was quite variable from ganglion to ganglion (FIG. 5C). Only a few ganglia were fully populated by EdU⁺ neurons, and such ganglia were always very small (i.e. composed of 3 neurons). Collectively, these results suggest that GDNF enemas induce proliferation of progenitors in distal colon and that some of dividing cells cluster into new ganglia that express neuronal and glial markers.

Example 3: GDNF-induced ENS is morphologically and functionally similar to WT

Focusing on the *Hol^{Tg/Tg}* model, it was next determined to what extent GDNF-induced ENS in the distal colon resembles WT at P20. Average neuron-to-glia ratio within GDNF-induced myenteric ganglia (0.77 ± 0.09 neurons/glia) was statistically similar to WT (0.96 ± 0.06 neurons/glia, $P=0.16$) (FIG. 6A). Relative proportions of major myenteric neuron subtypes, including ChAT⁺ (choline acetyltransferase) and nNOS⁺ (neuronal nitric oxide synthase) neurons, was also very similar to WT (FIGs. 6B-C). Moreover, many other neuronal subtypes were detected including TH⁺ (tyrosine hydroxylase) dopaminergic neurons, CalR⁺ (calretinin) excitatory motor neurons, VIP⁺ (vasoactive intestinal peptide) inhibitory motor neurons, and SubP⁺ (substance P) excitatory motor neurons (FIG. 6C). Interestingly, in proximal and mid colon of *Hol^{Tg/Tg}* mice (FIGs. 7A-B), GDNF treatment also corrected the imbalance of nitrergic (increased) and cholinergic (decreased) neuron subtypes that is observed upstream of the aganglionic segment in both HSCR mouse models and human patients (38, 48-51).

To evaluate function of P20 GDNF-induced myenteric ganglia, colonic motility was analyzed *in vivo* using the bead latency test. Control *Hol^{Tg/Tg}* mice did not expel a glass bead inserted into their rectum during the 30-minute observation period, but a subset of GDNF-treated *Hol^{Tg/Tg}* did expel the bead in 10-21 min, a bit slower than WT mice (range of 2-8 min) (FIG. 6D). Analysis of neuron density in these GDNF-treated *Hol^{Tg/Tg}* mice revealed a robust inverse correlation between time to expel the bead and neuron density in the distal colon (FIG. 8A). Colon motility was also evaluated *ex vivo* using strips of distal colon *muscularis externa* from P20 animals attached to force transducers in an organ bath. This system allows electric field stimulation-induced contractions of WT colon muscles to be slightly increased by inhibition of nitric oxide synthase with L-NAME (nitro-L-arginine methyl ester), which can then be robustly counteracted by inhibition of cholinergic signaling with atropine (muscarinic receptor antagonist). Similar to *in vivo* data, colon muscle strips from GDNF-treated *Hol^{Tg/Tg}* mice displayed one of two distinct response patterns. Some GDNF-treated *Hol^{Tg/Tg}* colon responses were similar to WT (4/7

mice) while others responded similar to untreated *Hol^{Tg/Tg}* (3/7 mice) (FIG. 6E and 8B). Mean area under the curve (Δ AUC) for contractile responses was graphed separately for GDNF-responsive and GDNF-unresponsive *Hol^{Tg/Tg}* mice because there appear to be two distinct groups (FIG. 6E). To indirectly test function of P20 GDNF-induced submucosal ganglia, epithelial permeability to small fluorescently labeled dextran molecules (FD4) in Ussing chambers was analyzed. Once more, colonic tissues from GDNF-treated *Hol^{Tg/Tg}* mice displayed two distinct response types, with mucosal pieces either impermeable to FD4 like WT tissues, or permeable to FD4 like control *Hol^{Tg/Tg}* tissues from mice that were not GDNF treated (FIG. 6F).

To complement ENS analyses, other HSCR-associated bowel anomalies were evaluated. *Hol^{Tg/Tg}* mouse colon had thicker smooth muscles and more neutrophils than WT mice, but GDNF-treated *Hol^{Tg/Tg}* mouse colon was similar to WT (FIGs. 6G-I and 9A-B). Similarly, stool microbiome profiling demonstrated dysbiosis in P20 *Hol^{Tg/Tg}* mouse colon, but average abundance of several bacterial genera in *Hol^{Tg/Tg}* mouse colon were indistinguishable from WT after GDNF treatment (e.g., *Desulfovibrio*, *Escherichia*, *Helicobacter*, *Mucispirillum*, *Oscillospira*, *Parabacteroides*, and *Sutterella*) (FIG. 6J). A notable exception was *Bacteroides* abundance, which was low in *Hol^{Tg/Tg}* mice and remained low after GDNF treatment. Accordingly, beta diversity analysis suggests microbial community structure is distinct among WT, *Hol^{Tg/Tg}* and GDNF-treated *Hol^{Tg/Tg}* mice (FIG. 6K).

Example 4: SCPs within extrinsic nerves are a target of GDNF in aganglionic colon

To elucidate how GDNF induces enteric neurogenesis, GDNF distribution in bowel was first evaluated via Western blot, taking advantage of size differences between recombinant (15 kDa) and endogenous (20 kDa, glycosylated) GDNF proteins in SDS-PAGE gels. Consistent with increased epithelial permeability in distal colon of *Hol^{Tg/Tg}* mice (FIG. 6F), recombinant GDNF protein was detected in GDNF-treated *Hol^{Tg/Tg}* distal colon at P8, but not in proximal colon (FIG. 10A). Surprisingly, while endogenous GDNF is normally only detected in ileum, recombinant GDNF enemas triggered robust increases in endogenous GDNF throughout the colon (FIG. 10A). Consistent with GDNF's short half-life (between 34-92h) (52), recombinant GDNF was no longer detected in colon nor in any other tissue at P20, supporting the idea that administered GDNF primarily acts during the treatment period (FIG. 11). To test for possible GDNF auto-regulatory loops and assess precise locations of recombinant GDNF during treatment, GDNF treatment of *Hol^{Tg/Tg}* mice was repeated, but now with a 6xHis-tagged version of GDNF (53) (_{His}GDNF). Time-course analysis of distal colon 2h after GDNF treatment on P4, P6 and P8 revealed recombinant GDNF accumulated over time in colon submucosa (FIG. 10B), smooth muscles, and subsets of enteric neurons (FIG. 10C and 12) of *Hol^{Tg/Tg}* mice. Interestingly, levels of the main GDNF receptor RET also increased (FIG. 10B and 12), supporting the hypothesis that GDNF-RET auto-regulatory loops are activated in GDNF-treated colon that could enhance RET signaling even if

initial RET levels were low (e.g., in patients with mutations that reduce RET levels and/or activity). Remarkably, both H_{is} GDNF and RET were detected in induced neurons close to extrinsic nerves of GDNF-treated *Hol^{Tg/Tg}* mice (FIG. 10C) and *Ednrb^{S-/S-}* mice.

It was next assessed whether nerve-associated Schwann cells could be GDNF-targeted ENS progenitors. To test this hypothesis, response of Schwann cells in extrinsic nerve fibers to GDNF was first assessed, using live explants of distal colon *muscularis externa* from P4 *Hol^{Tg/Tg};G4-RFP* double transgenic pups. In these mice, neural crest derivatives including SCPs are marked by RFP fluorescence (54). Time-lapse imaging of explants after 72h of culture in presence or absence of GDNF (5 μ g/ml) suggested GDNF stimulates both migration and proliferation of SCPs along extrinsic nerves (FIG. 10D). Impact of GDNF on proliferation of these SCPs was confirmed via immunofluorescence after 96h of ex vivo culture. Ki67⁺ SOX10⁺ double positive cells within extrinsic nerves tripled upon exposure to GDNF (5.5 $\pm$ 0.4% without GDNF vs 15.2 $\pm$ 2.1% with GDNF) (FIG. 10E, F). Although some of the GDNF-induced neurons that express RET are derived from Schwann cells, RET is possibly not needed to activate these precursors in aganglionic bowel as GDNF signaling in Schwann cells is instead mediated by NCAM (35). In line with this, the data reported in FIG. 10I shows NCAM but not RET expression in Schwann cells of extrinsic nerves in aganglionic mouse colon. These results suggest that GDNF therapy may be used even in patients having reduced RET levels and/or activity, e.g., having mutations in RET or in the RET signaling pathway.

To further demonstrate SCPs are a source of GDNF-induced neurons and glia, *in vivo* genetic cell lineage tracing with the Schwann lineage-specific Cre driver transgene *Dhh-Cre* and the *Rosa26* Cre reporter allele *R26^{[Floxed Stop]YFP}* in the *Holstein* [FVB/N] mutant background was used. Intriguingly, untreated *Dhh-Cre^{Tg/+};R26^{YFP/+}* and *Hol^{Tg/Tg};Dhh-Cre^{Tg/+};R26^{YFP/+}* animals had a lower than expected number of YFP⁺ SCP-derived neurons and glia in proximal and mid colon. While SCPs were previously reported to contribute 20% of colonic neurons in a mixed C57BL/6-129Sv genetic background at 1 month of age (29), they were found to contribute only 5-7% of myenteric neurons in a pure FVB/N genetic background at P20, and this contribution increased to 10-11% in the presence of the homozygous *Holstein* mutation (FIGs. 13A,B). Remarkably, SCP-derived (YFP⁺) cells made up 34% of distal colonic neurons in GDNF-treated *Hol^{Tg/Tg};Dhh-Cre^{Tg/+};R26^{YFP/+}* animals (FIGs. 10G,H). By daily EdU administration during GDNF treatment from P4 to P8, four subgroups of induced myenteric neurons were identified based on cellular origin (YFP fluorescence) and/or EdU incorporation (FIGs. 10G,H and 14C). While this work confirmed that *Dhh⁺* SCPs are a source of GDNF-induced neurons and glia in both myenteric (FIGs. 10G,H) and submucosal (FIG. 13C) plexus, it also revealed that a majority of induced neurons (66%) were YFP-negative, suggesting non-SCP origin (i.e. non-*Dhh*-expressing cell type(s)). Moreover, a majority of induced neurons (62%) did not incorporate EdU, regardless of cellular origin (FIGs. 10G,H), raising the possibility that neurogenesis might result from transdifferentiation (i.e., direct

differentiation of a postmitotic cell into another type of specialized cell) instead of requiring proliferating precursor cells.

Example 5: GDNF can induce new neurons in human aganglionic colon ex vivo

5 To test if GDNF could induce new enteric neurons in human tissue, an ex vivo model was needed. It was discovered that 96 hrs of GDNF treatment ex vivo induced neurons in all *Hol^{Tg/Tg}* distal colon aganglionic tissues, but neurogenesis was much less efficient than *in vivo*. Only 3 to 67 neurons were seen in these 1 cm-long explants (FIGs. 14A and 15A). Moreover, induced neurons rarely clustered into ganglia and such ganglia were always very small (FIG. 10 14B). In marked contrast to widespread EdU incorporation into SCPs (FIG. 14C and 15), EdU incorporation in induced neurons was also minimal (less than 20% of HuC/D⁺ cells in explant cultures) (FIGs. 14B,C). Control aganglionic tissues from P4 *Hol^{Tg/Tg}* mice cultured without GDNF remained devoid of neurons (FIGs. 14A,C and 15), supporting the idea that GDNF could induce neurogenesis in aganglionic distal colon ex vivo, but less efficiently than occurred with GDNF 15 enemas *in vivo*.

Finally, it was tested if GDNF could induce neurogenesis in aganglionic human colon muscle from children who had Swenson pull-through surgery to resect aganglionic distal bowel. The cohort consisted of 12 children. Epidemiologic characteristics were typical of HSCR (i.e., mostly sporadic, male-biased, short-segment) (Table 2). Patients who underwent Soave surgery 20 were not enrolled because *muscularis externa* is not resected from the most distal colon with this approach. For each subject, full-thickness resected aganglionic colon was collected on the day of surgery and immediately micro-dissected to remove mucosal and submucosal layers. Remaining *muscularis externa* was cut into smaller pieces and cultured with or without GDNF. Exposure to GDNF for 96h markedly increased the proportion of EdU⁺ SCPs in 9/9 human tissues where EdU 25 was added to media (FIG. 14D,E). Most importantly, new neurons expressing HuC/D, β III-Tubulin (Tuj1), RET, PGP9.5 and PHOX2B were also detected in three HSCR explants (FIGs. 14F,G and 16). These three explants were from the youngest children of our cohort (28 to 44 days old) (FIG. 14G and Table 2). Two of these young children had sporadic HSCR with unknown genetic causes. The third child had a MEN2A syndrome-associated *RET* mutation (Table 2). For older 30 children, it was tested whether longer GDNF treatment might be beneficial. Very interestingly, culturing distal colon from older children for 7 days (n=2; aged of 86 and 1638 days at the time of surgery) allowed identification of neurons that incorporated EdU in association with extrinsic nerve fibers (FIG. 14H). Collectively, this data provides compelling evidence that the observations made in mice may be extended to humans.

35

Table 2. Overview of HSCR colon samples used for ex vivo preclinical testing of GDNF therapy.

Age at surgery (days)	Genetic status	Sex	Clinical Classification	Extent of aganglionosis (cm)	Number of neurons	EdU ⁺ SCPs (%)
28	Sporadic, unknown mutation	M	Short segment disease	5	31	38
36	Sporadic, unknown mutation	M	Short segment disease	25	27	33
44	MEN2a syndrome, <i>RET</i> mutation	M	Short segment disease	6	15	44
80	Sporadic, unknown mutation	M	Short segment disease	7	3	34
85	Sporadic, unknown mutation	M	Short segment disease	6	1	32
86	Sporadic, unknown mutation	M	Short segment disease	7	0	46
249	Mowat-Wilson syndrome, <i>ZFHX1B</i> mutation	F	Short segment disease	30	0	32
300	Data not available	M	Data not available	Data not available	0	Not quantified
344	Sporadic, unknown mutation	M	Short segment disease	26	0	Not quantified
349	Sporadic, unknown mutation	M	Short segment disease	9	0	43
1177	Bardet-Biedl Syndrome, <i>BBS1</i> mutation	F	Long segment disease	40	0	Not quantified
1638	Sporadic, unknown mutation	F	Short segment disease	8	0	50

Example 6: Effect of GDNF on immune cell populations in the colon of *Holstein* mice

Multi-color flow cytometry analysis shows that the abnormal proportions of immune cells observed in the colon of untreated *Hol^{Tg/Tg}* mice become generally normalized back to WT immune cell proportions upon GDNF treatment. For a majority of markers, density plots shows either a complete (B cells, CD73⁺ T cells, CD44⁺CD62L⁺ T cells, RORy⁺ T cells, CX3CR1⁺MHC-II⁺ macrophages) or at least partial (CD4⁺ T cells, CD39⁺ T cells, CD25⁺ T cells, Ly-6C⁺CD64⁺ monocytes, F4-80⁺MHC-II⁺ macrophages, CD11b⁺CD103⁻ macrophages) rescue. The results are summarized in **Table 3**.

Table 3. Immune cell proportions in WT mice and *Hol^{Tg/Tg}* mice treated or not with GDNF

Cell type	% in WT	% in <i>Hol^{Tg/Tg}</i> mice (- GDNF)	% in <i>Hol^{Tg/Tg}</i> mice (+ GDNF)
CD4 ⁺ T cells	83.7	34.5	65.4
CD8 ⁺ T cells	8.47	18	15.6
B cells (CD19 ⁺)	84.6	6.98	84.6

CD39 ⁺ T cells	0.19	6.47	1.66
CD73 ⁺ T cells	31.1	56.7	30.4
CD44 ⁺ CD62L ⁺ T cells	17.6	5.30	15.9
RORy ⁺ Th17 cells	0.18	0.26	0.18
CD25 ⁺ T _{reg} cells	2.67	21	7.74
F4-80 ⁺ MHC-II ⁺ macrophages	0.43	3.72	2
Ly-6C ⁺ CD64 ⁺ monocytes	17	78.3	38
CD11b ⁺ CD103 ⁻ macrophages	0.67	34.1	5.16
CX3CR1 ⁺ MHC II ⁻ macrophages	0.34	0.53	0.31

Although the present invention has been described hereinabove by way of specific embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims. In the claims, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to". The singular forms "a", "an" and "the" include corresponding plural references unless the context clearly dictates otherwise.

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WHAT IS CLAIMED IS:

1. A method for inducing enteric neurogenesis in an aganglionic or hypoganglionic segment of the distal colon of a human subject suffering from an enteric neuropathy, the method comprising administering a pharmaceutical composition comprising an effective dose of a recombinant Glial cell line-Derived Neurotrophic Factor (GDNF) polypeptide and a pharmaceutically acceptable carrier into the distal colon of the subject.
2. The method of claim 1, wherein the GDNF polypeptide comprises an amino acid sequence having at least 70% identity with amino acids 78-211 of SEQ ID NO:1.
3. The method of claim 2, wherein the GDNF polypeptide comprises an amino acid sequence having at least 90% identity with amino acids 78-211 of SEQ ID NO:1.
4. The method of claim 3, wherein the GDNF polypeptide comprises an amino acid sequence having at least 95% identity with amino acids 78-211 of SEQ ID NO:1.
5. The method of claim 4, wherein the GDNF polypeptide comprises amino acids 78-211 of SEQ ID NO:1.
6. The method of any one of claims 1 to 5, wherein the effective dose of recombinant GDNF polypeptide administered to the human subject corresponds to a dose of about 5 µg to about 20 µg in a mouse pup.
7. The method of any one of claims 1 to 6, wherein the pharmaceutically acceptable carrier comprises a saline solution or a gelling agent.
8. The method of any one of claims 1 to 7, wherein the pharmaceutical composition is administered rectally through enema.
9. The method of any one of claims 1 to 7, wherein the pharmaceutical composition is administered by injection into the distal colon wall.
10. The method of any one of claims 1 to 9, wherein the pharmaceutical composition is administered once-a-day up to four times a day.
11. The method of any one of claims 1 to 10, wherein the pharmaceutical composition is administered for at least 2 consecutive days.
12. The method of any one of claims 1 to 11, wherein the method is performed prior to surgical removal of the aganglionic or hypoganglionic segment in the subject.
13. The method of any one of claims 1 to 11, wherein the method is performed after surgical removal of the aganglionic or hypoganglionic segment in the subject.
14. The method of claim 12 or 13, wherein the surgical removal of the aganglionic or hypoganglionic segment is through pull-through surgery.

15. The method of any one of claims 1 to 14, wherein the enteric neuropathy is intestinal hypoganglioneosis.
16. The method of any one of claims 1 to 14, wherein the enteric neuropathy is Hirschsprung disease (HSCR).
- 5 17. The method of claim 16, wherein the subject suffers from short-segment HSCR.
18. The method of claim 16 or 17, wherein the HSCR is sporadic HSCR.
19. The method of any one of claims 16 to 18, wherein the HSCR is associated with a reduced expression or activity of the RET receptor.
20. The method of claim 19, wherein the HSCR is associated with a mutation in the RET gene.
- 10 21. The method of any one of claims 1 to 20, wherein the enteric neurogenesis comprises production of enteric neurons and/or enteric glial cells.
22. The method of claim 21, wherein the neurogenesis comprises production of enteric neurons and enteric glial cells.
23. The method of claim 21 or 22, wherein the production of enteric neurons and/or enteric
15 glial cells comprises proliferation of enteric neurons and/or enteric glia progenitors.
24. The method of any one of claims 1 to 23, wherein the method corrects the imbalance of nitrergic and cholinergic neuron subtypes located upstream of the aganglionic or hypoganglionic segment.
25. The method of any one of claims 1 to 24, wherein the method restores distal colon motility
20 in the subject.
26. The method of any one of claims 1 to 25, wherein the method restores the proportions of lymphoid and/or myeloid immune cells in the distal colon of the subject.
27. The method of any one of claims 1 to 26, wherein the human subject is less than 5-year-old.
- 25 28. The method of claim 27, wherein the human subject is less than 6-month-old.
29. The method of any one of claims 1 to 28, wherein the pharmaceutical composition is administered into the rectum and/or the sigmoid colon.
30. The method of claim 29, wherein the pharmaceutical composition is administered or is for administration into the rectosigmoid region.
- 30 31. A pharmaceutical composition comprising a recombinant Glial cell line-Derived Neurotrophic Factor (GDNF) polypeptide and a pharmaceutically acceptable carrier for inducing enteric neurogenesis in an aganglionic or hypoganglionic segment of the distal colon of a human

subject suffering from an enteric neuropathy, wherein the composition is for administration into the distal colon of the subject.

32. The pharmaceutical composition for use according to claim 31, wherein the GDNF polypeptide comprises an amino acid sequence having at least 70% identity with amino acids 78-211 of SEQ ID NO:1.

33. The pharmaceutical composition for use according to claim 32, wherein the GDNF polypeptide comprises an amino acid sequence having at least 90% identity with amino acids 78-211 of SEQ ID NO:1.

34. The pharmaceutical composition for use according to claim 33, wherein the GDNF polypeptide comprises an amino acid sequence having at least 95% identity with amino acids 78-211 of SEQ ID NO:1.

35. The pharmaceutical composition for use according to claim 34, wherein the GDNF polypeptide comprises amino acids 78-211 of SEQ ID NO:1.

36. The pharmaceutical composition for use according to any one of claims 31 to 35, wherein the dose of recombinant GDNF polypeptide used corresponds to a dose of about 5 µg to about 20 µg in a mouse pup.

37. The pharmaceutical composition for use according to any one of claims 31 to 36, wherein the pharmaceutically acceptable carrier is a saline solution or a gelling agent.

38. The pharmaceutical composition for use according to any one of claims 31 to 37, wherein the pharmaceutical composition is for rectal administration through enema.

39. The pharmaceutical composition for use according to any one of claims 31 to 38, wherein the pharmaceutical composition is for administration by injection into the distal colon wall.

40. The pharmaceutical composition for use according to any one of claims 31 to 39, wherein the pharmaceutical composition is for administration once-a-day up to four times a day.

41. The pharmaceutical composition for use according to any one of claims 31 to 40, wherein the pharmaceutical composition is for administration for at least 2 consecutive days.

42. The pharmaceutical composition for use according to any one of claims 31 to 41, wherein the pharmaceutical composition is for use prior to surgical removal of the aganglionic or hypoganglionic segment in the subject.

43. The pharmaceutical composition for use according to any one of claims 31 to 41, wherein the pharmaceutical composition is for use after surgical removal of the aganglionic or hypoganglionic segment in the subject.

44. The pharmaceutical composition for use according to claim 42 or 43, wherein the surgical removal of the aganglionic or hypoganglionic segment is through pull-through surgery.

45. The pharmaceutical composition for use according to any one of claims 31 to 44, wherein the enteric neuropathy is intestinal hypoganglionosis.

5 46. The pharmaceutical composition for use according to any one of claims 31 to 44, wherein the enteric neuropathy is Hirschsprung disease (HSCR).

47. The pharmaceutical composition for use according to claim 46, wherein the subject suffers from short-segment HSCR.

10 48. The pharmaceutical composition for use according to claim 46 or 47, wherein the HSCR is sporadic HSCR.

49. The pharmaceutical composition for use according to any one of claims 46 to 48, wherein the HSCR is associated with a reduced expression or activity of the RET receptor.

50. The pharmaceutical composition for use according to claim 49, wherein the HSCR is associated with a mutation in the RET gene.

15 51. The pharmaceutical composition for use according to any one of claims 31 to 50, wherein the enteric neurogenesis comprises production of enteric neurons and/or enteric glial cells.

52. The pharmaceutical composition for use according to claim 51, wherein the enteric neurogenesis comprises production of enteric neurons and enteric glial cells.

20 53. The pharmaceutical composition for use according to claim 51 or 52, wherein the production of enteric neurons and/or glial cells comprises proliferation of enteric neuron and/or enteric glia progenitors.

54. The pharmaceutical composition for use according to any one of claims 31 to 53, wherein the pharmaceutical composition corrects the imbalance of nitroergic and cholinergic neuron subtypes located upstream of the aganglionic or hypoganglionic segment.

25 55. The pharmaceutical composition for use according to any one of claims 31 to 54, wherein the pharmaceutical composition restores distal colon motility in the subject.

56. The pharmaceutical composition for use according to any one of claims 31 to 55, wherein the pharmaceutical composition restores the proportions of lymphoid and/or myeloid immune cells in the distal colon of the subject.

30 57. The pharmaceutical composition for use according to any one of claims 31 to 56, wherein the human subject is less than 5-year-old.

58. The pharmaceutical composition for use according to claim 57, wherein the human subject is less than 6-month-old.

59. The pharmaceutical composition for use according to any one of claims 31 to 58, wherein the pharmaceutical composition is for administration into the rectum and/or the sigmoid colon.

60. The pharmaceutical composition for use according to claim 59, wherein the pharmaceutical composition is for administration into the rectosigmoid region.

5 61. Use of a pharmaceutical composition comprising a recombinant Glial cell line-Derived Neurotrophic Factor (GDNF) polypeptide and a pharmaceutically acceptable carrier for the manufacture of a medicament for inducing enteric neurogenesis in an aganglionic or hypoganglionic segment of the distal colon of a human subject suffering from an enteric neuropathy, wherein the medicament is for administration into the distal colon of the subject.

10 62. The use according to claim 61, wherein the GDNF polypeptide comprises an amino acid sequence having at least 70% identity with amino acids 78-211 of SEQ ID NO:1.

63. The use according to claim 62, wherein the GDNF polypeptide comprises an amino acid sequence having at least 90% identity with amino acids 78-211 of SEQ ID NO:1.

15 64. The use according to claim 63, wherein the GDNF polypeptide comprises an amino acid sequence having at least 95% identity with amino acids 78-211 of SEQ ID NO:1.

65. The use according to claim 64, wherein the GDNF polypeptide comprises amino acids 78-211 of SEQ ID NO:1.

66. The use according to any one of claims 61 to 65, wherein the dose of recombinant GDNF polypeptide used corresponds to a dose of about 5 µg to about 20 µg in a mouse pup.

20 67. The use according to any one of claims 61 to 66, wherein the pharmaceutically acceptable carrier is a saline solution or a gelling agent.

68. The use according to any one of claims 61 to 67, wherein the pharmaceutical composition is for rectal administration through enema.

25 69. The use according to any one of claims 61 to 67, wherein the pharmaceutical composition is for administration by injection into the distal colon wall.

70. The use according to any one of claims 61 to 69, wherein the pharmaceutical composition is for administration once-a-day up to four times a day.

71. The use according to any one of claims 61 to 70, wherein the pharmaceutical composition is for administration for at least 2 consecutive days.

30 72. The use according to any one of claims 61 to 71, wherein the pharmaceutical composition is used prior to surgical removal of the aganglionic or hypoganglionic segment in the subject.

73. The use according to any one of claims 61 to 72, wherein the pharmaceutical composition is used after surgical removal of the aganglionic or hypoganglionic segment in the subject.

74. The use according to claim 72 or 73, wherein the surgical removal of the aganglionic or hypoganglionic segment is through Swenson pull-through surgery.

75. The use according to any one of claims 61 to 74, wherein the enteric neuropathy is intestinal hypoganglionosis.

5 76. The use according to any one of claims 61 to 75, wherein the enteric neuropathy is Hirschsprung disease (HSCR).

77. The use according to claim 76, wherein the subject suffers from short-segment HSCR.

78. The use according to claim 76 or 77, wherein the HSCR is sporadic HSCR.

10 79. The use according to any one of claims 76 to 78, wherein the HSCR is associated with a reduced expression or activity of the RET receptor.

80. The use according to claim 79, wherein the HSCR is associated with a mutation in the RET gene.

81. The use according to any one of claims 61 to 80, wherein the enteric neurogenesis comprises production of enteric neurons and/or enteric glial cells.

15 82. The use according to claim 81, wherein the enteric neurogenesis comprises production of enteric neurons and enteric glial cells.

83. The use according to claim 81 or 82, wherein the production of enteric neurons and/or glial cells comprises proliferation of enteric neuron and/or enteric glia progenitors.

20 84. The use according to any one of claims 61 to 83, wherein the method corrects the imbalance of nitrergic and cholinergic neuron subtypes located upstream of the aganglionic or hypoganglionic segment.

85. The use according to any one of claims 61 to 84, wherein the medicament restores distal colon motility in the subject.

25 86. The use according to any one of claims 61 to 85, wherein the medicament restores the proportions of lymphoid and/or myeloid immune cells in the distal colon of the subject.

87. The use according to any one of claims 61 to 86, wherein the human subject is less than 5-year-old.

88. The use according to claim 87, wherein the human subject is less than 6-month-old.

30 89. The use according to any one of claims 61 to 88, wherein the medicament is for administration into the rectum and/or the sigmoid colon.

90. The use according to claim 89, wherein the medicament is for administration into the rectosigmoid region.

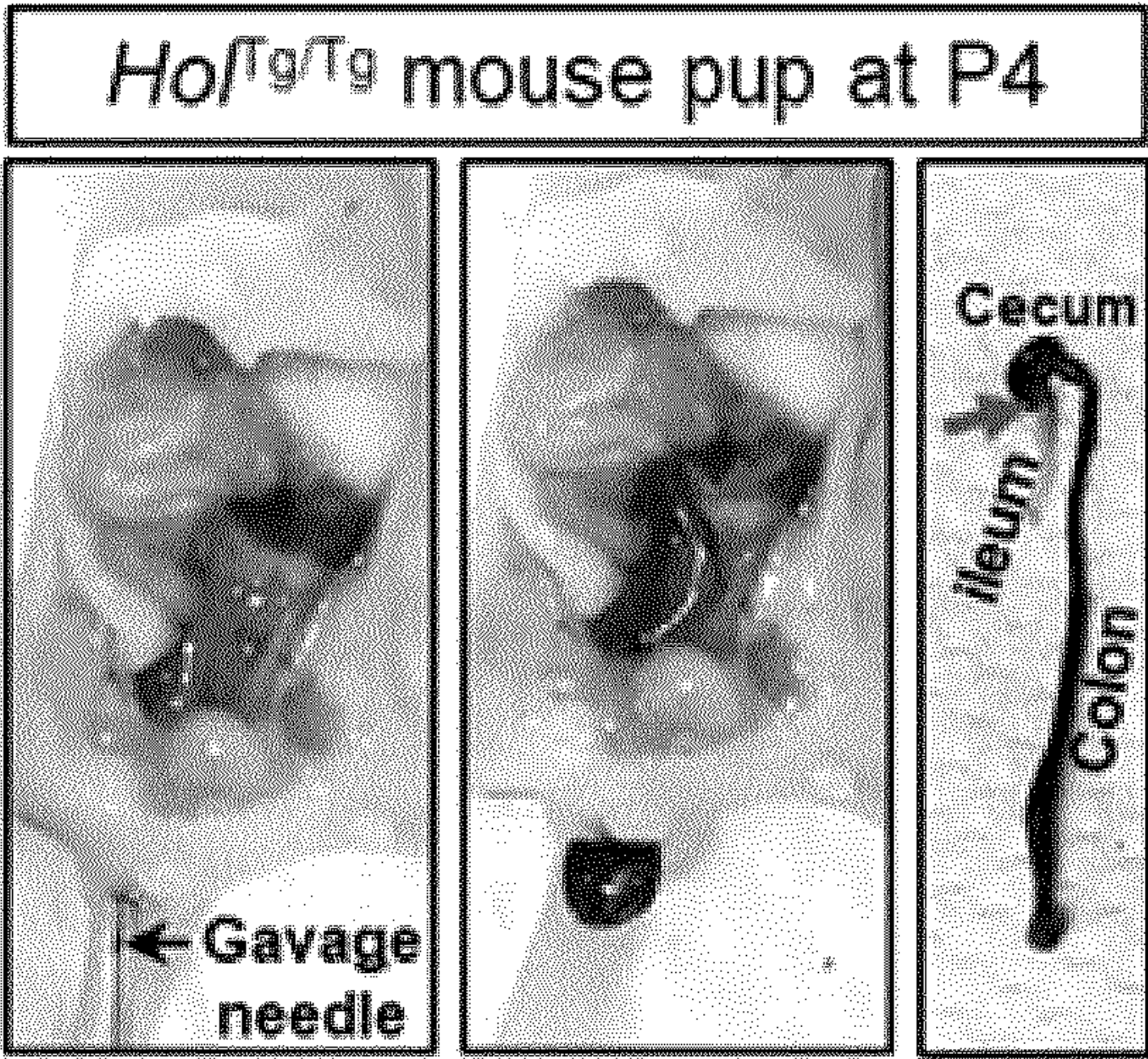


FIG. 1A

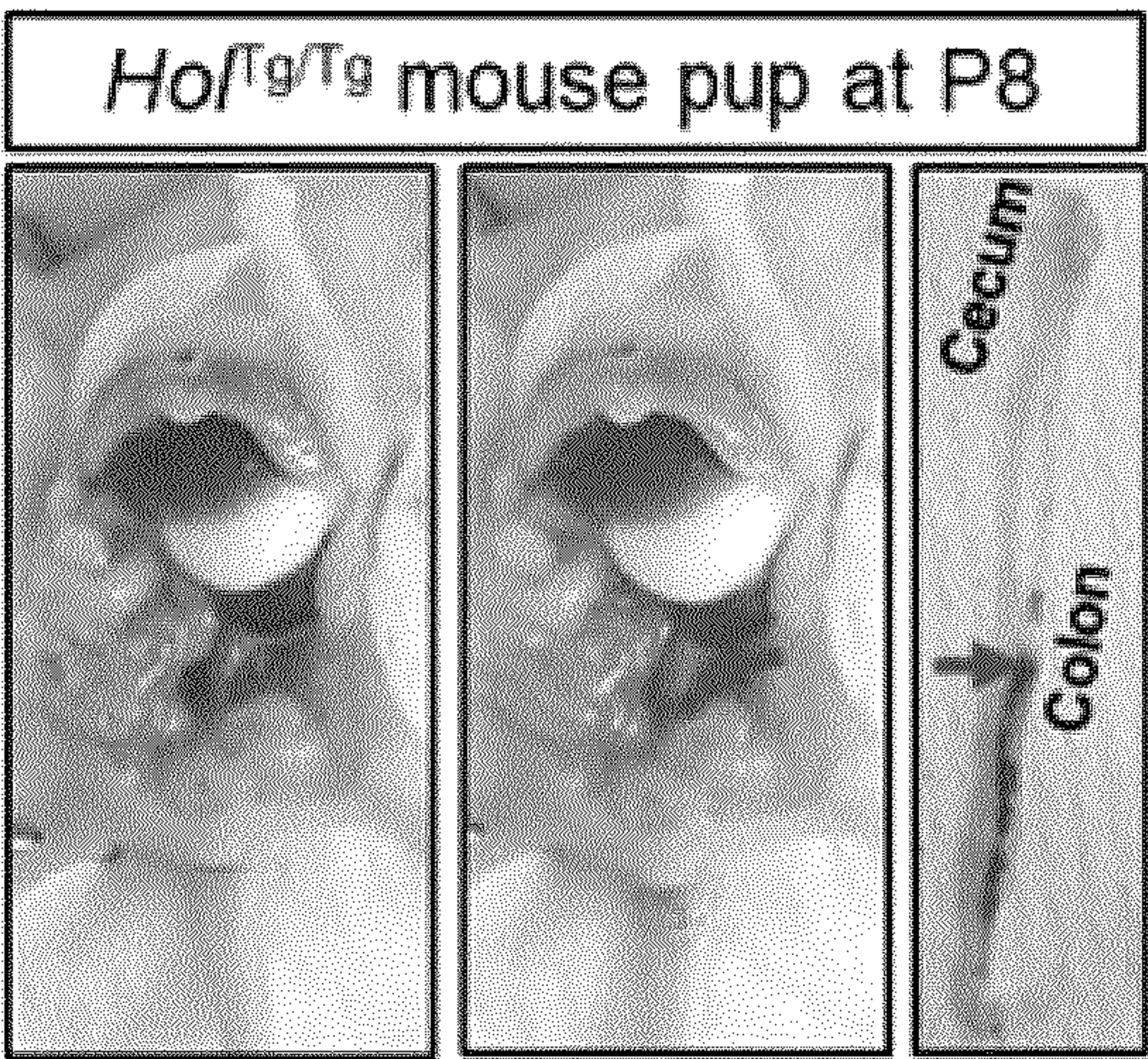


FIG. 1B

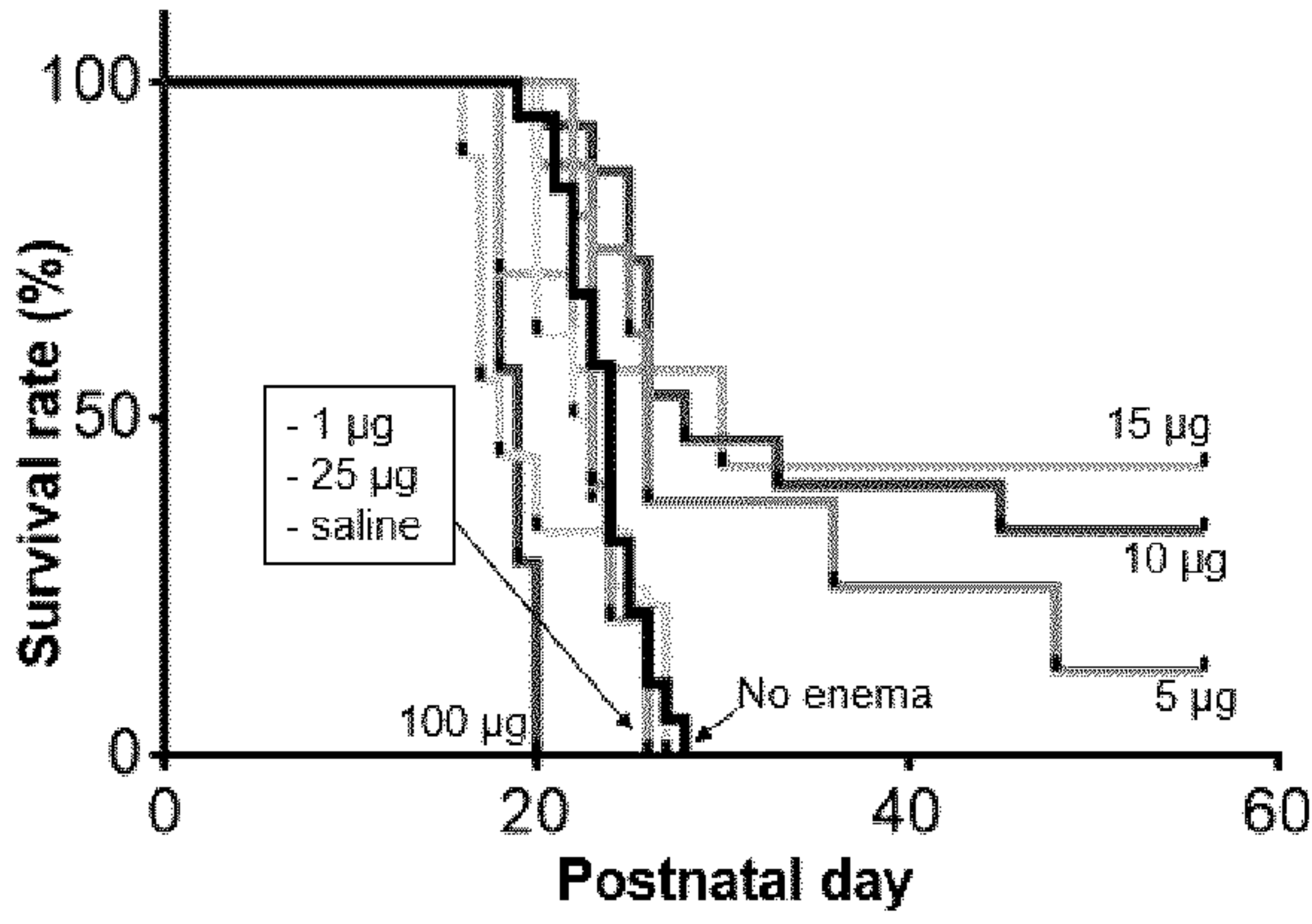


FIG. 1C

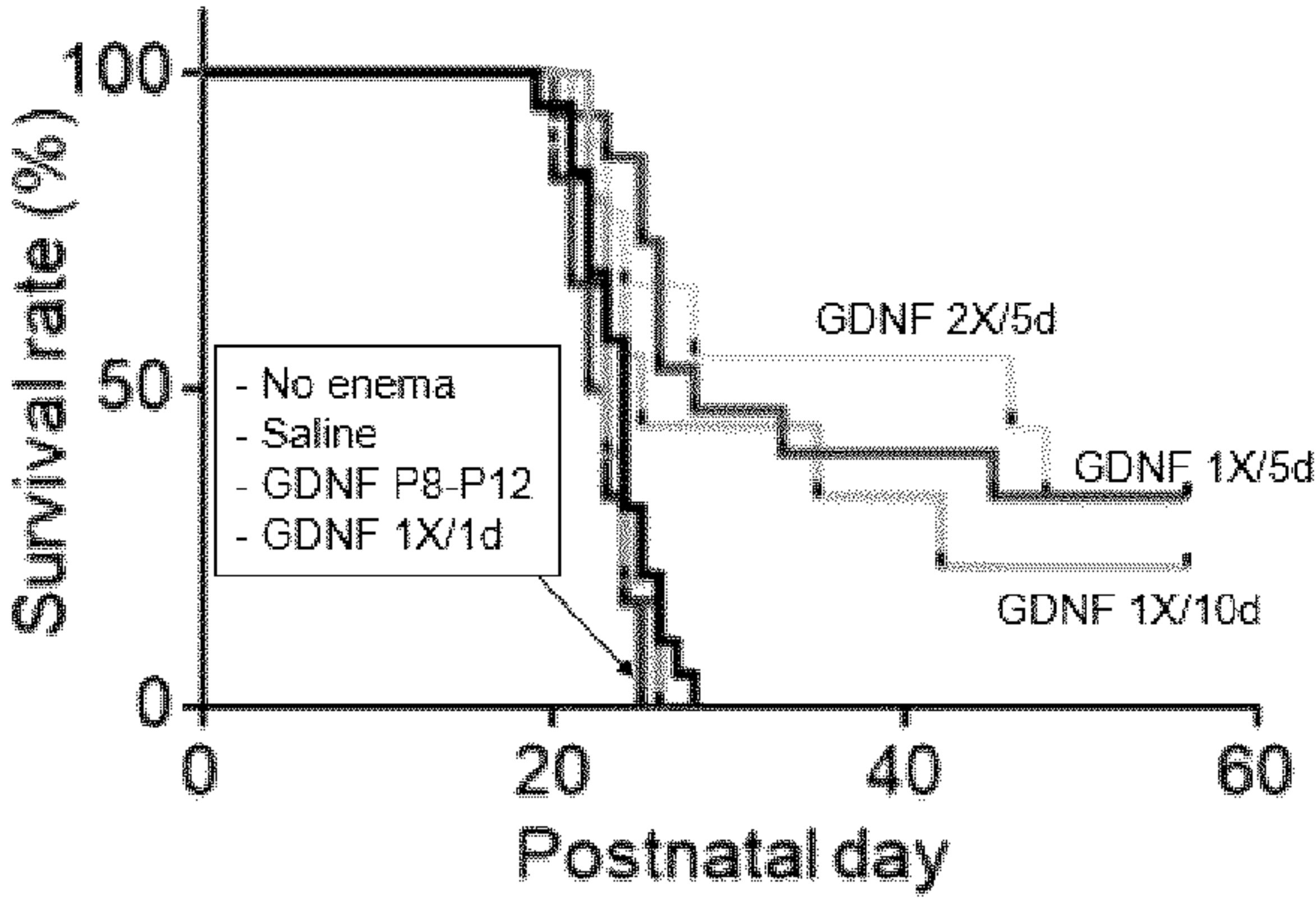


FIG. 1D

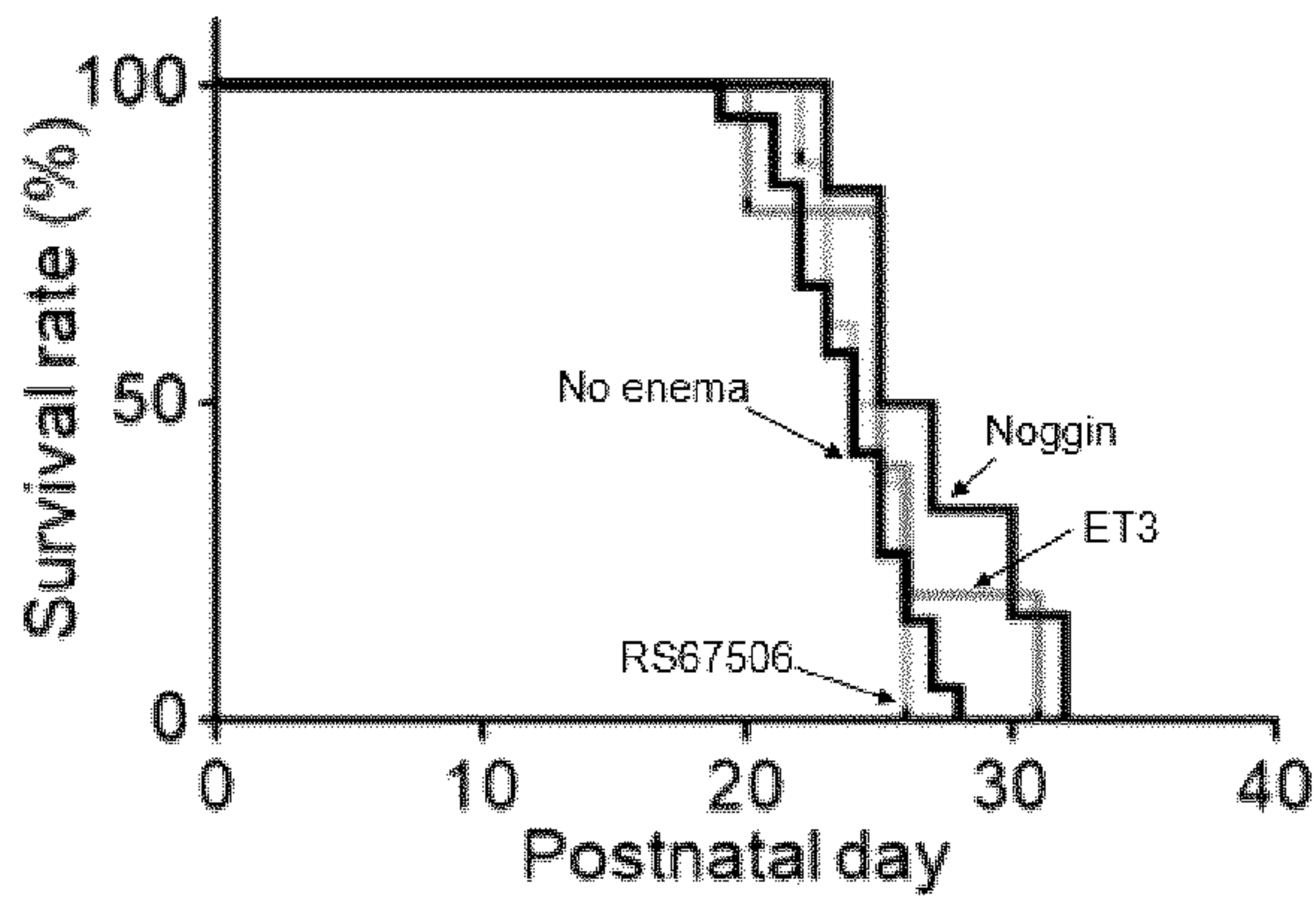


FIG. 1E

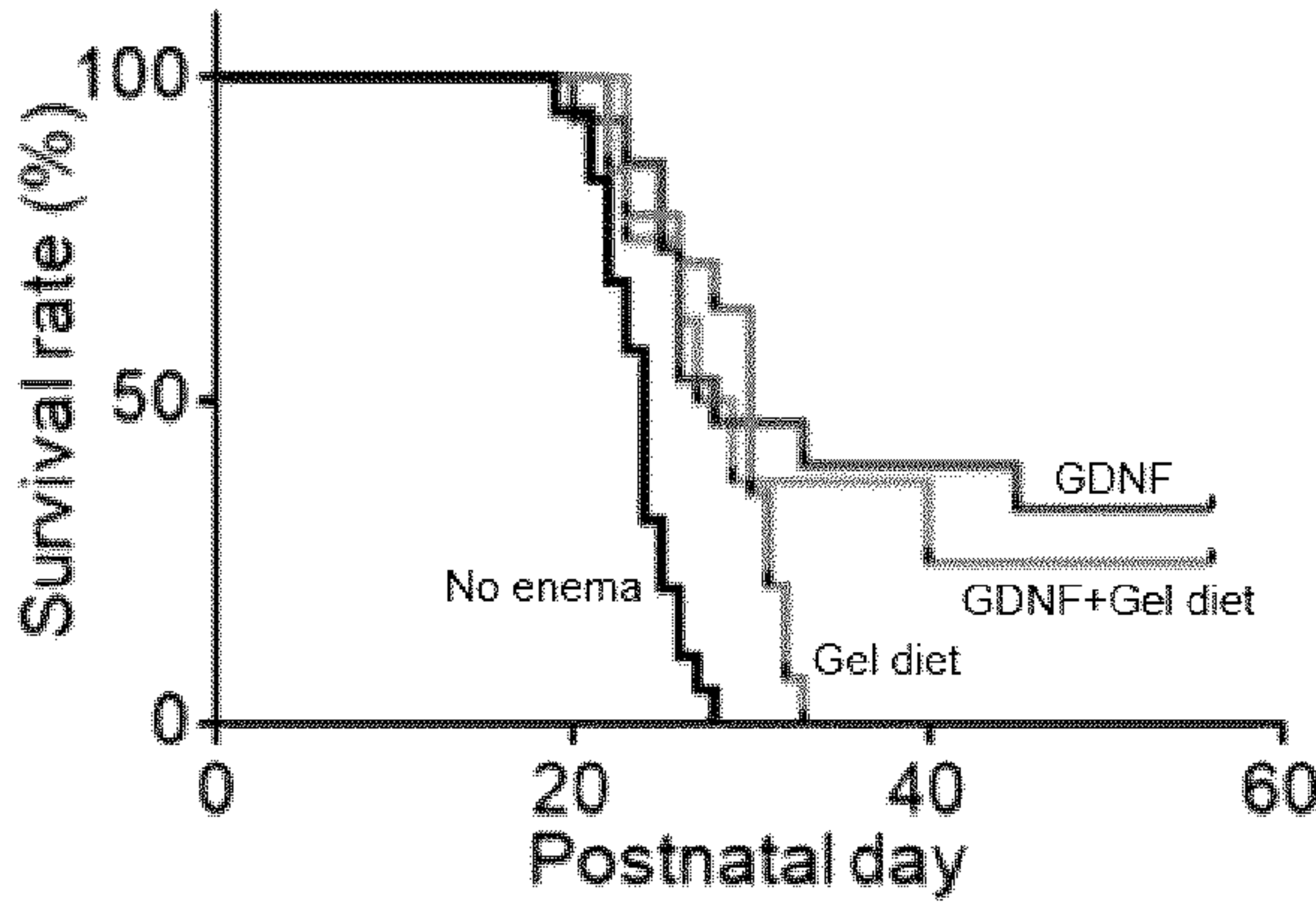


FIG. 1F

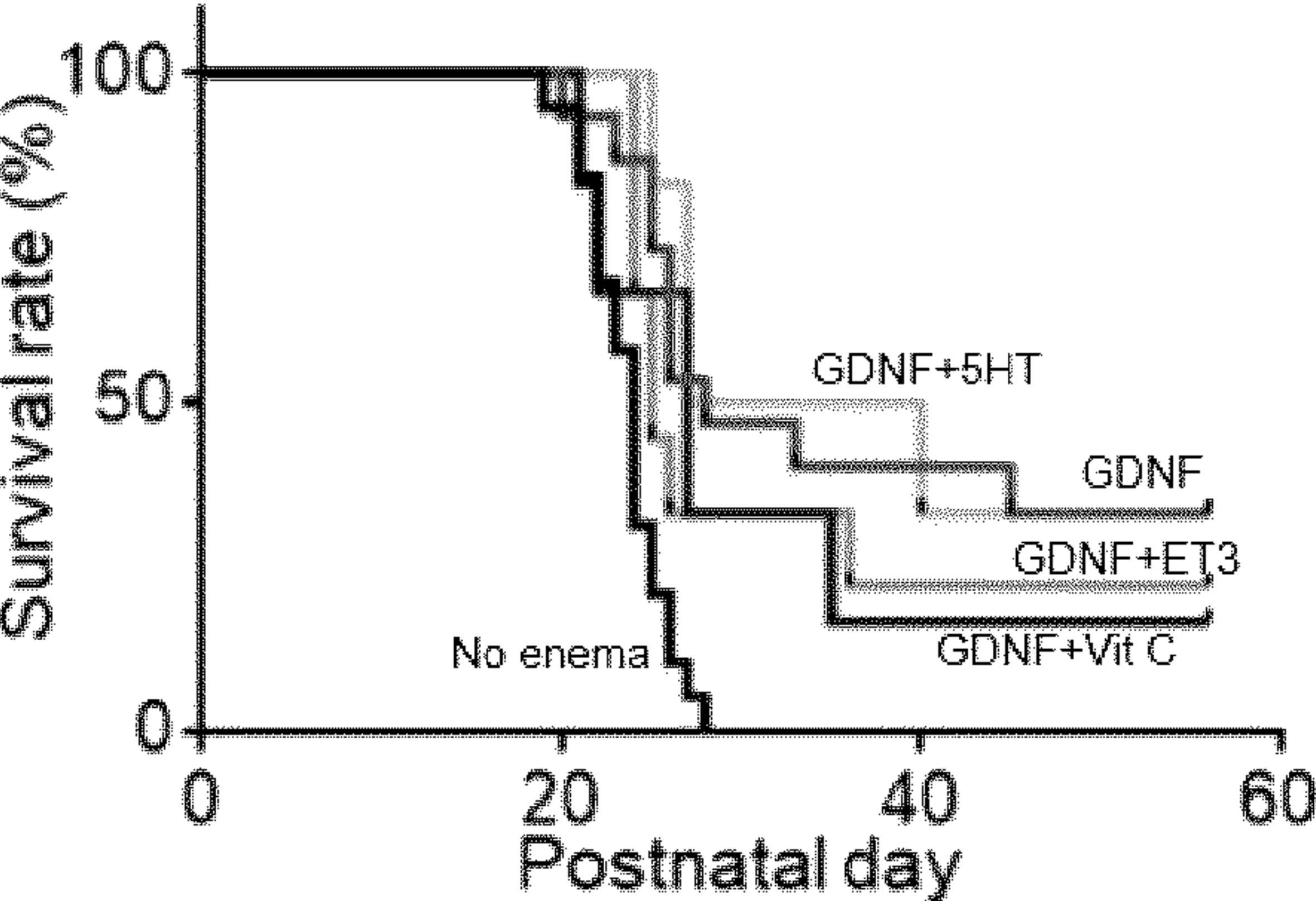


FIG. 1G

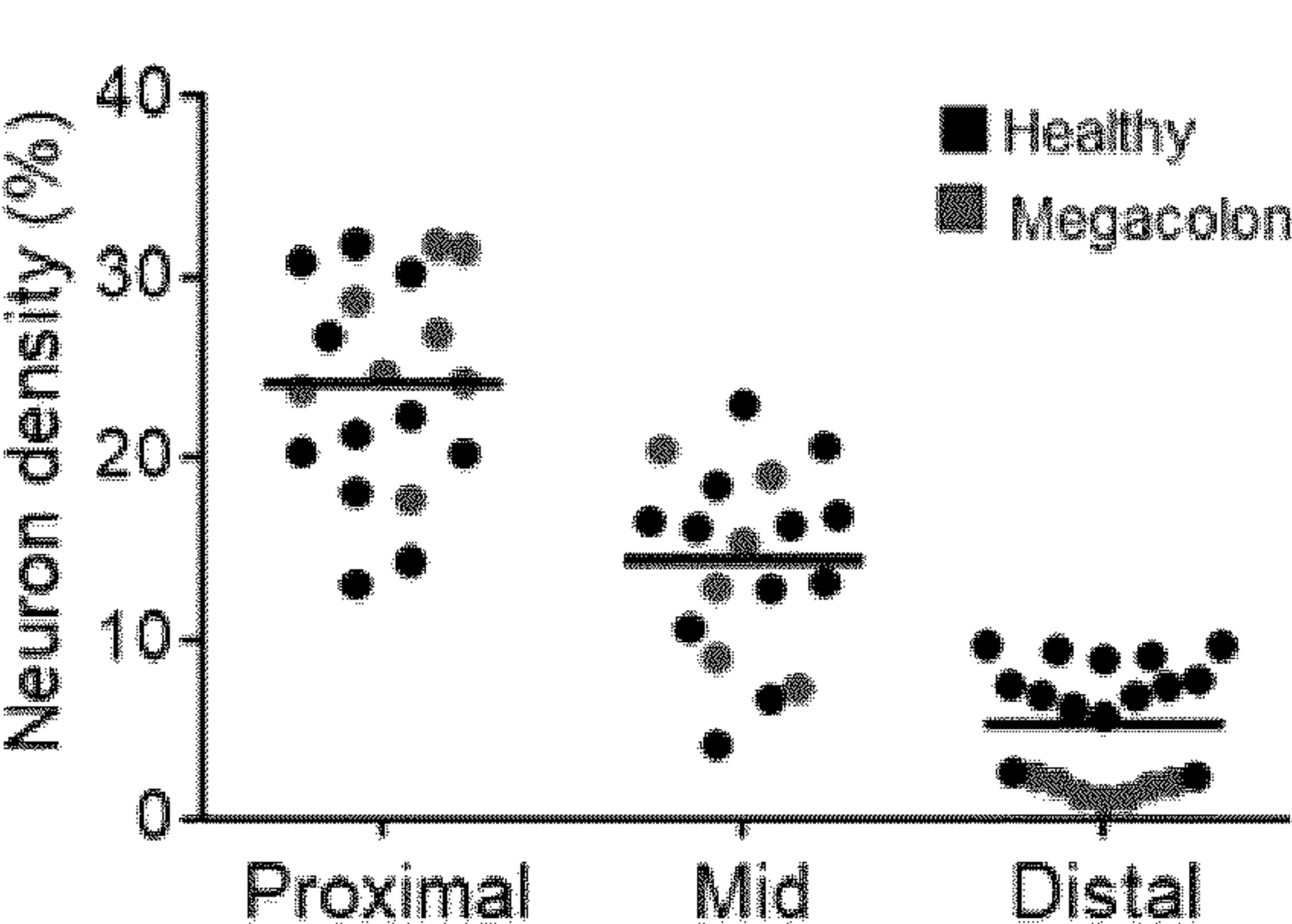


FIG. 1H

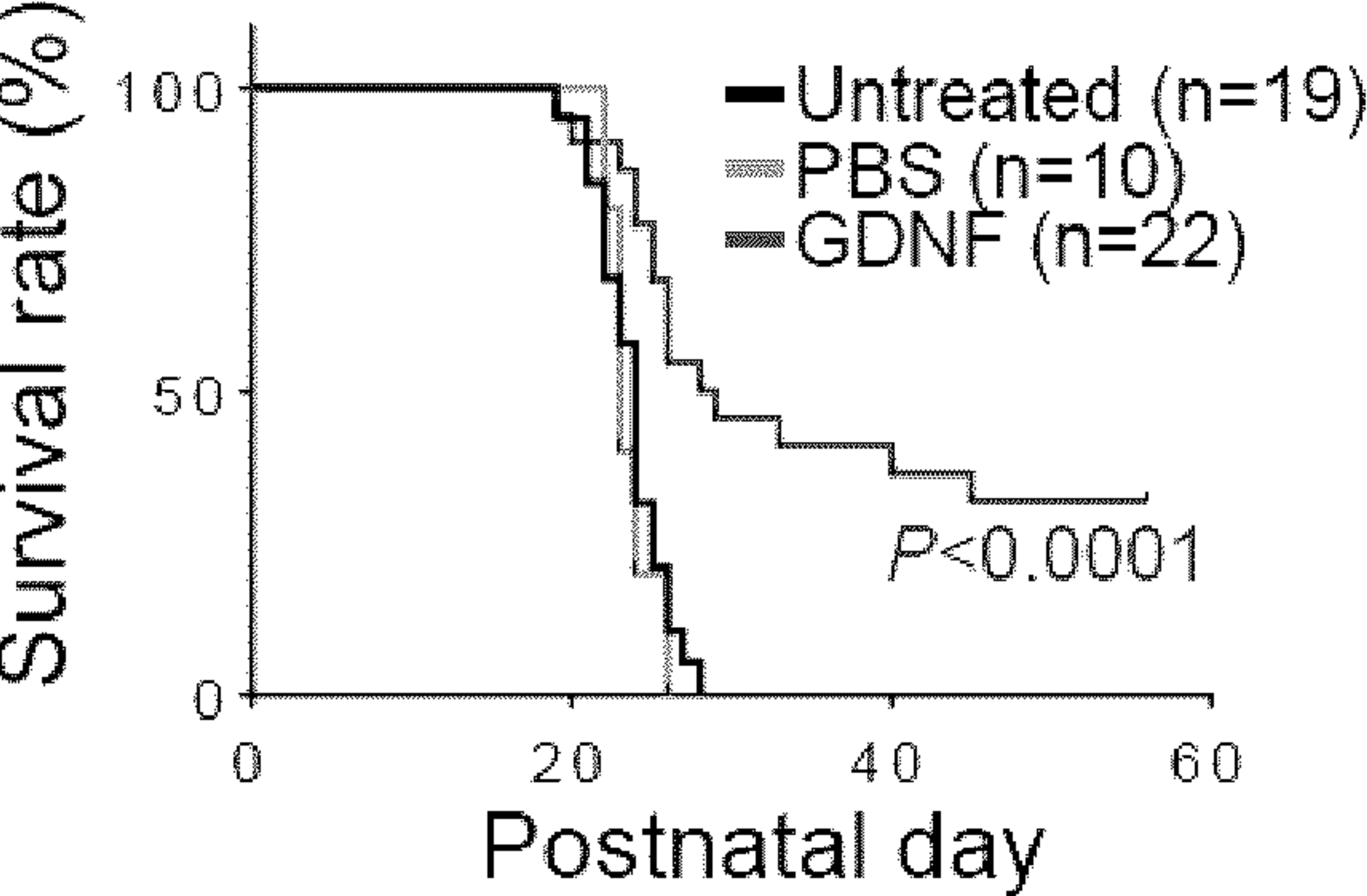


FIG. 2A

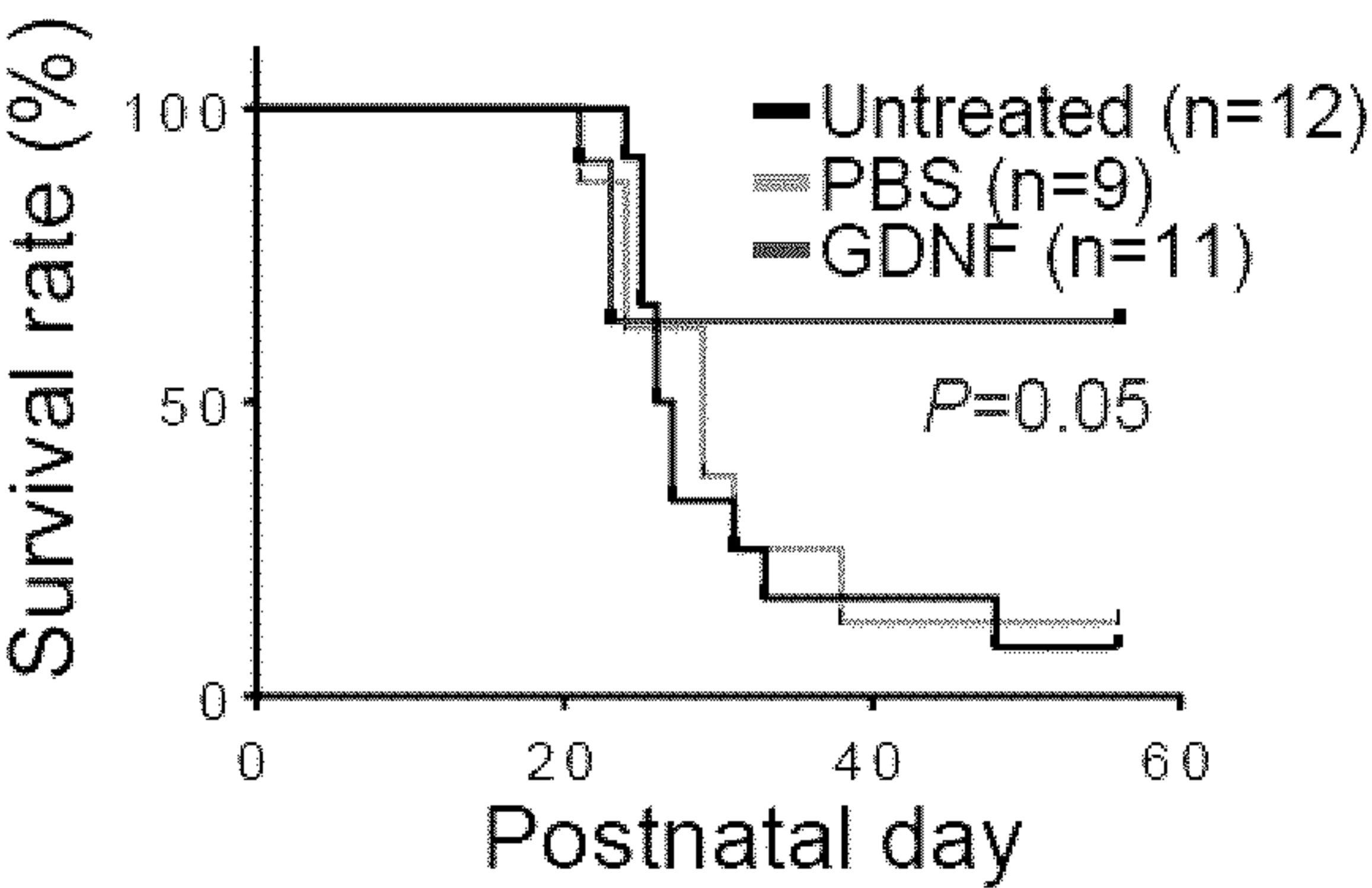


FIG. 2B

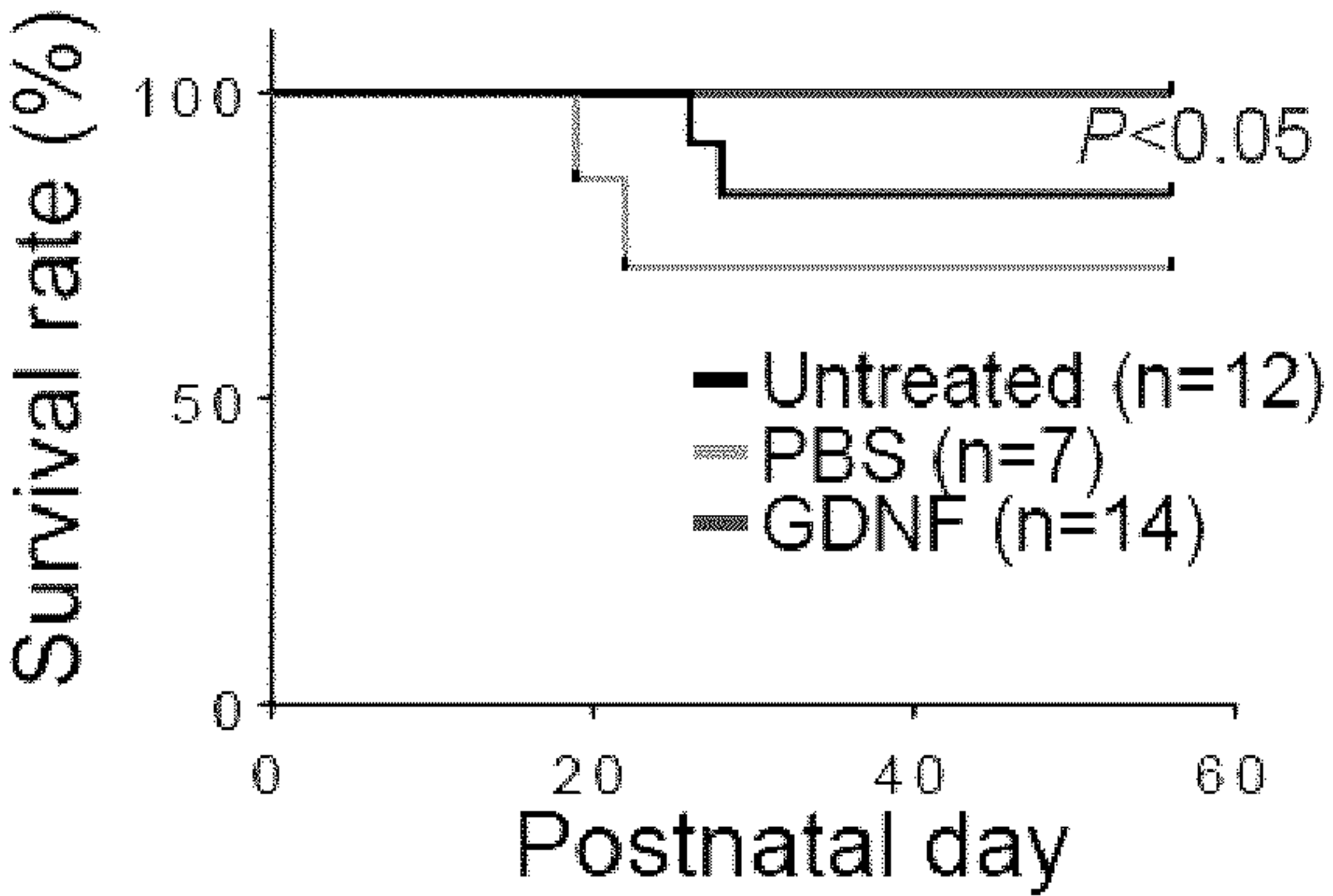


FIG. 2C

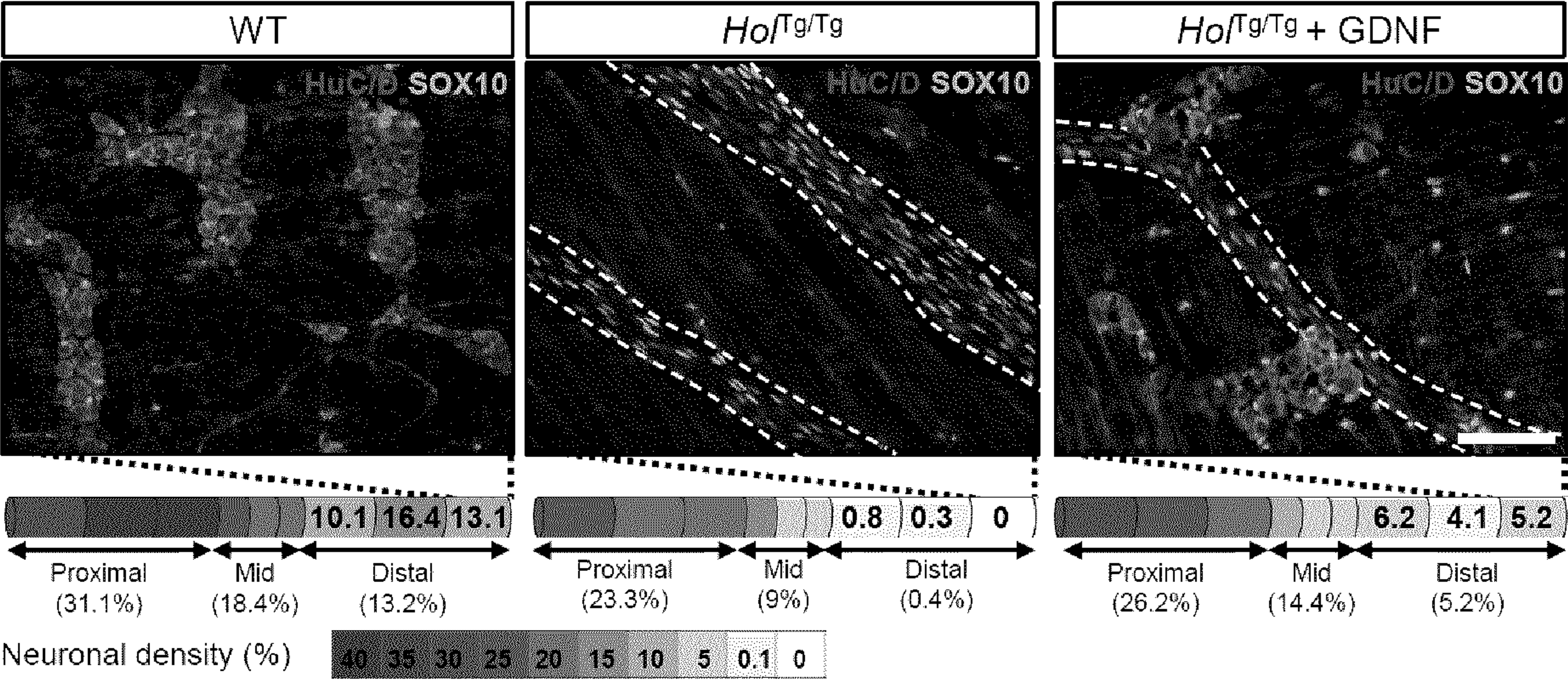


FIG. 2D

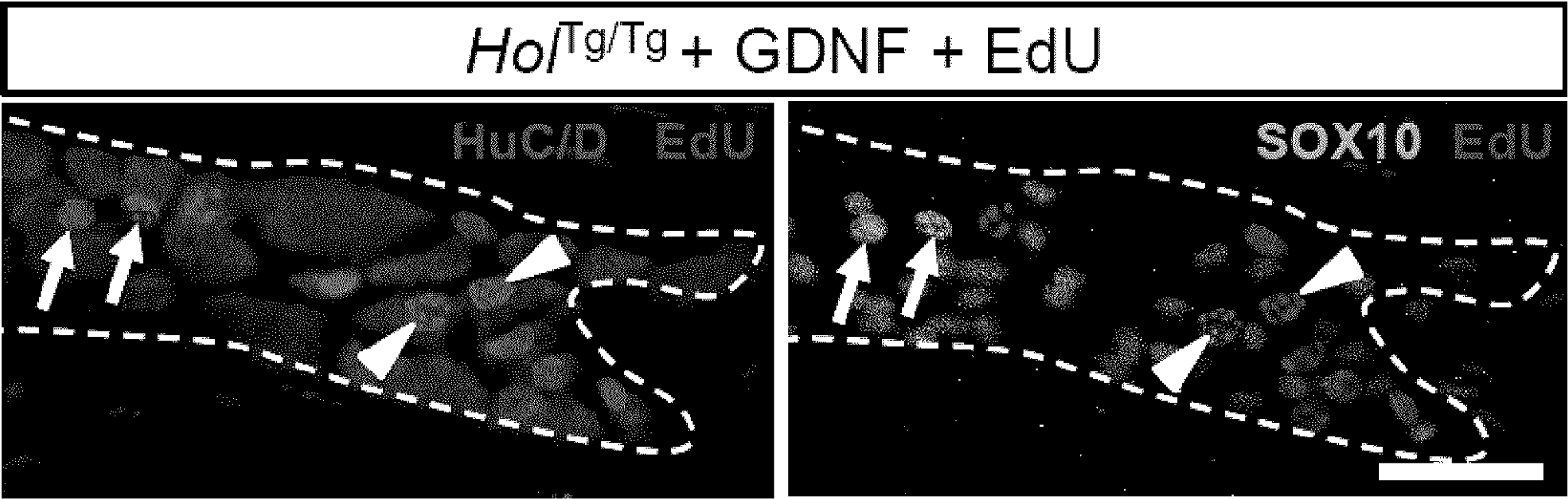


FIG. 2E

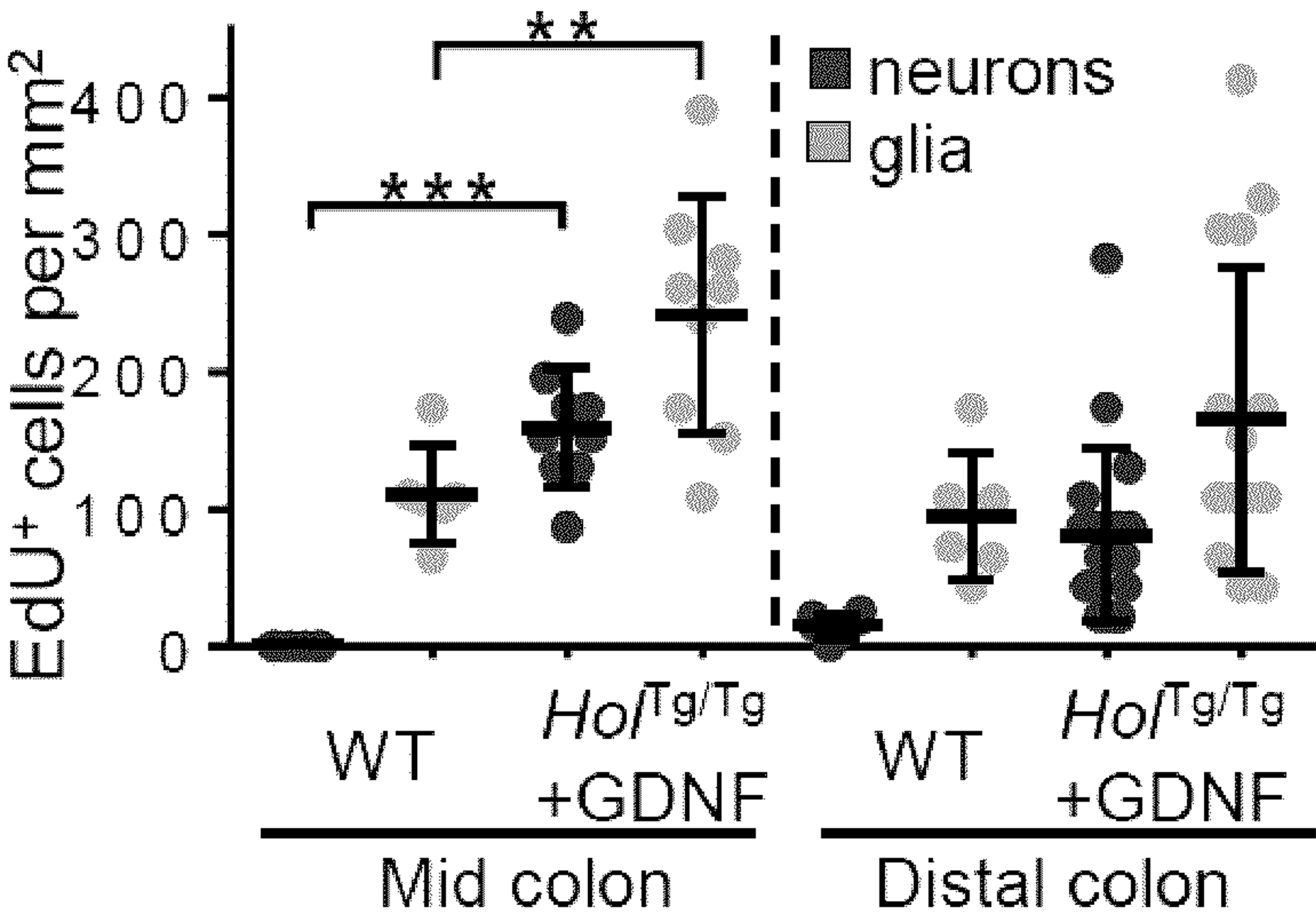


FIG. 2F

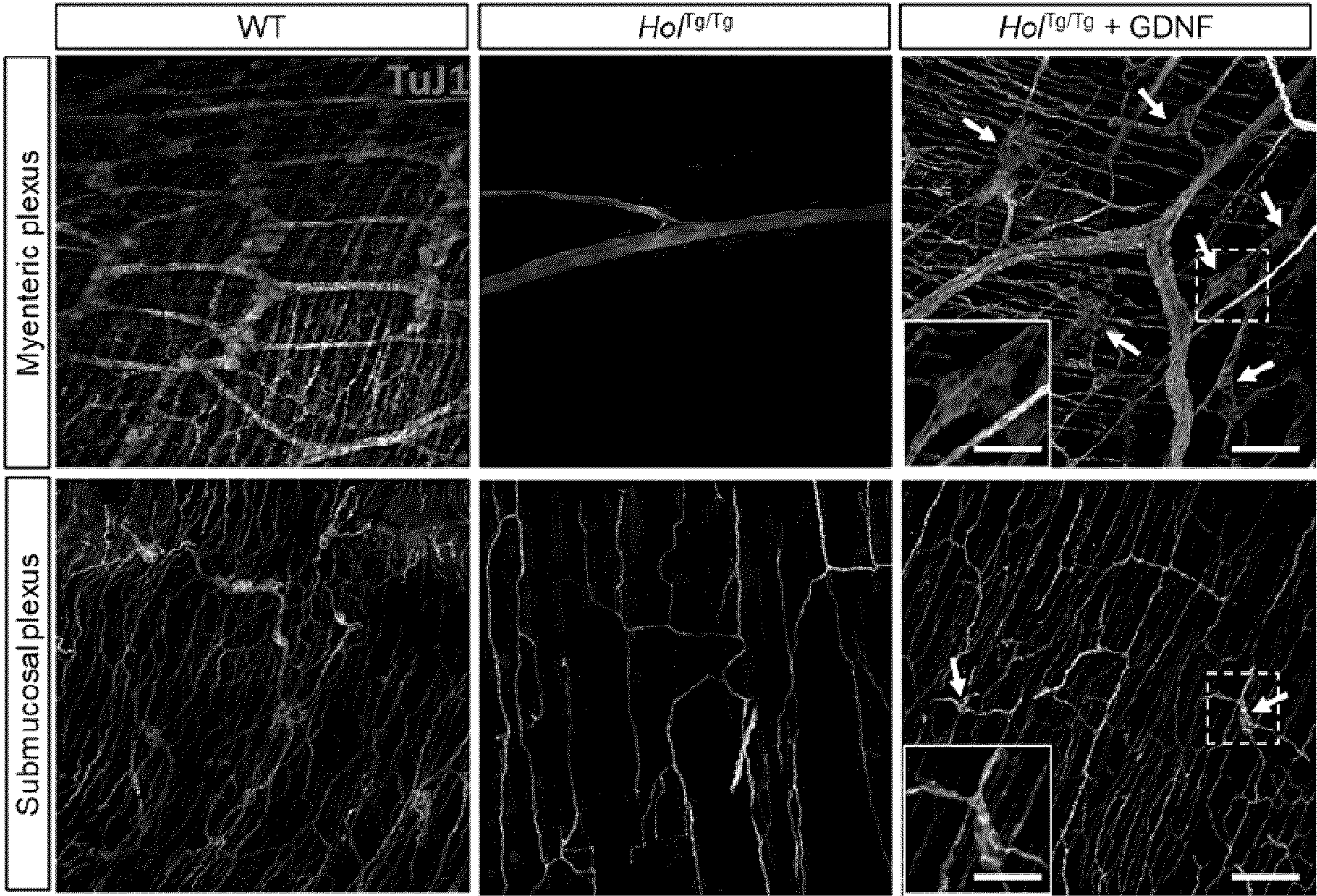


FIG. 3

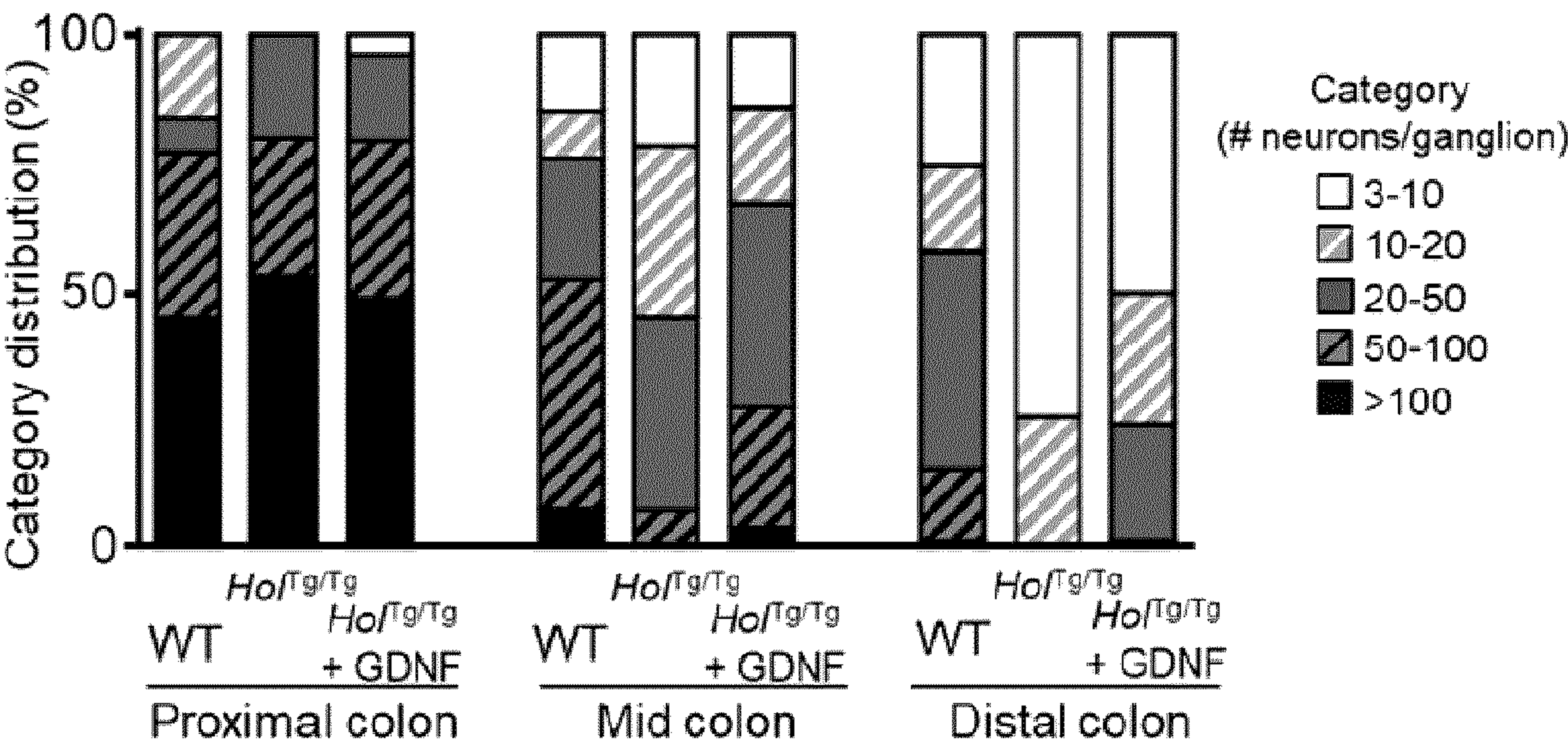


FIG. 4A



FIG. 4B

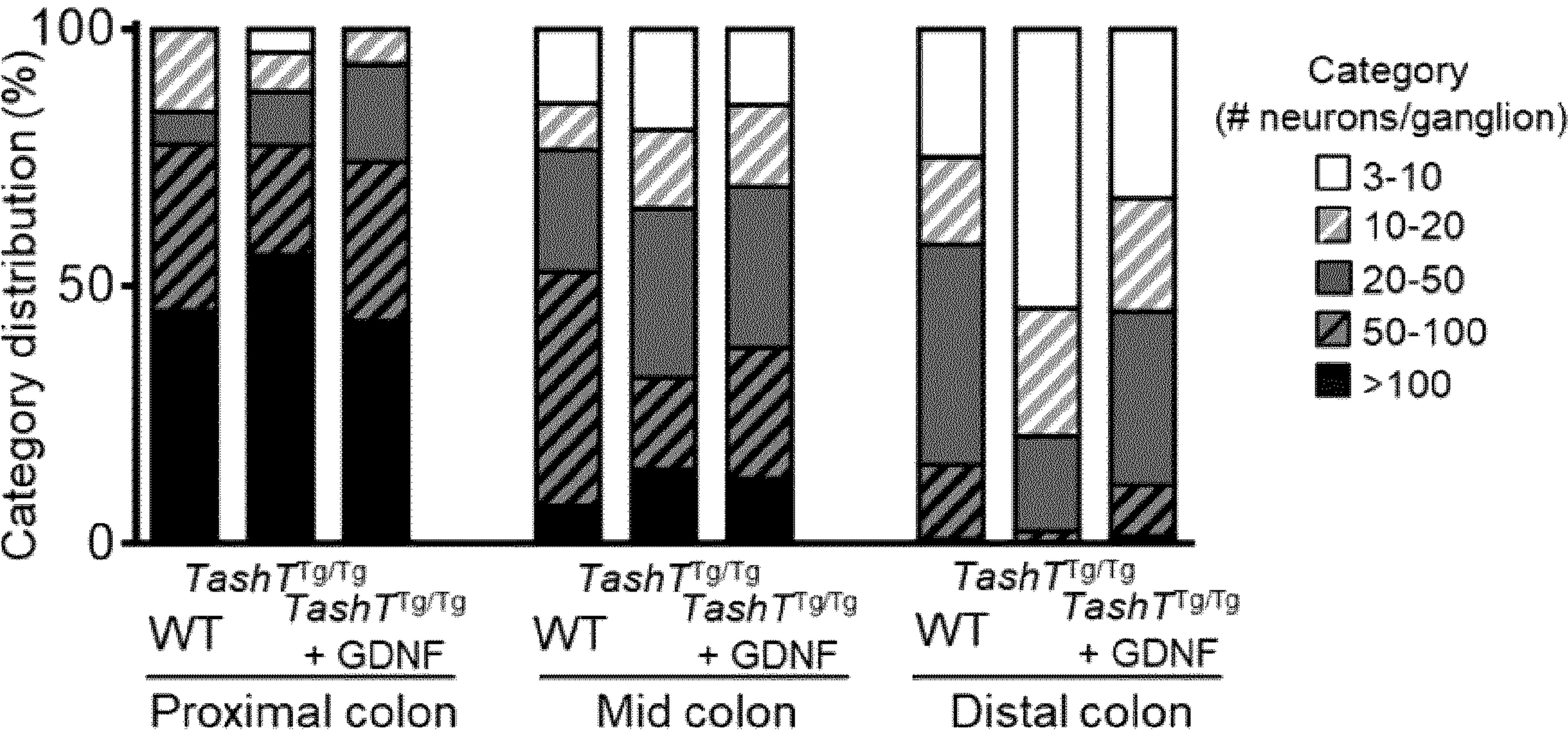


FIG. 4C

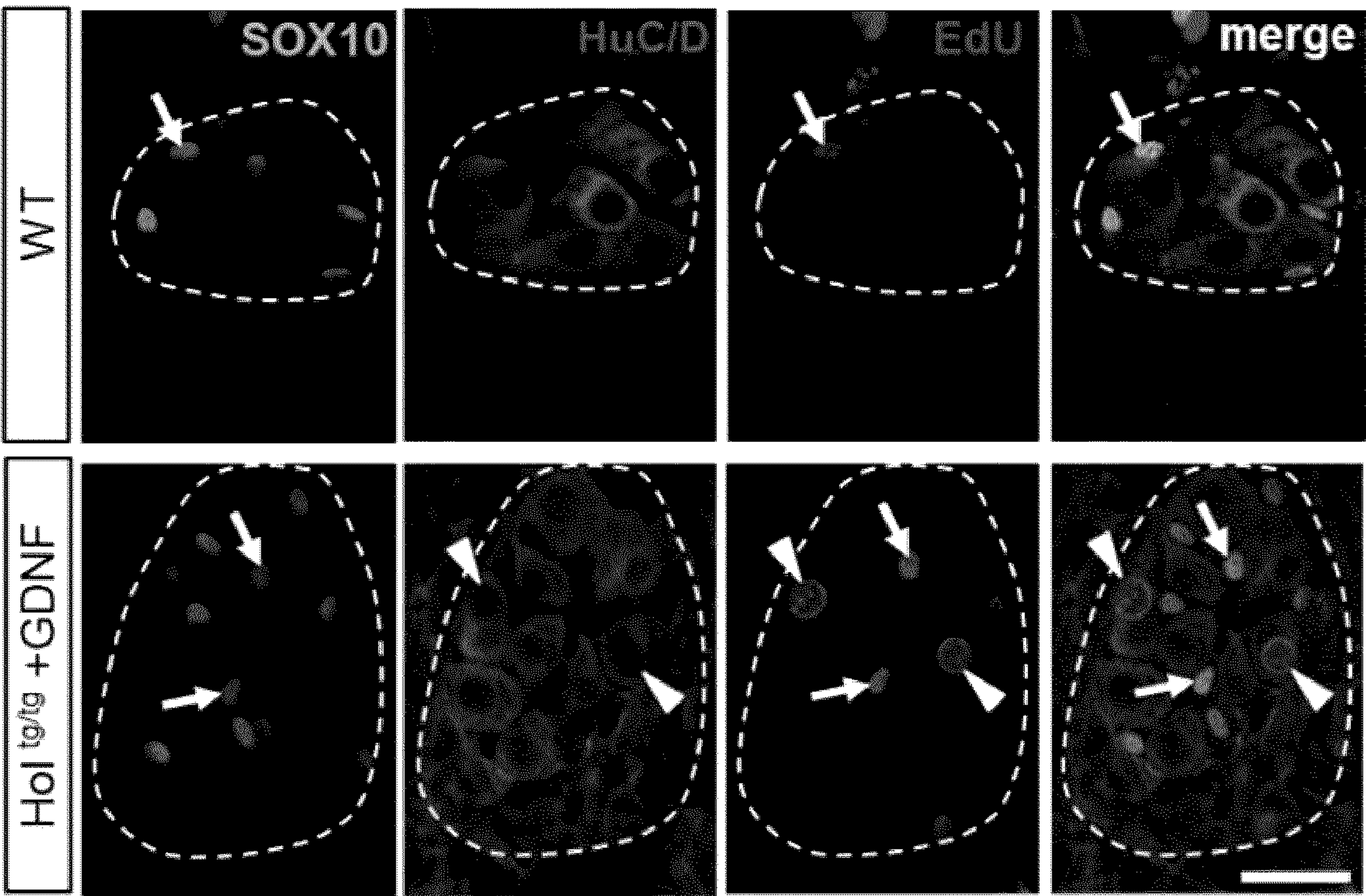


FIG. 5A

6/25

Submucosal plexus

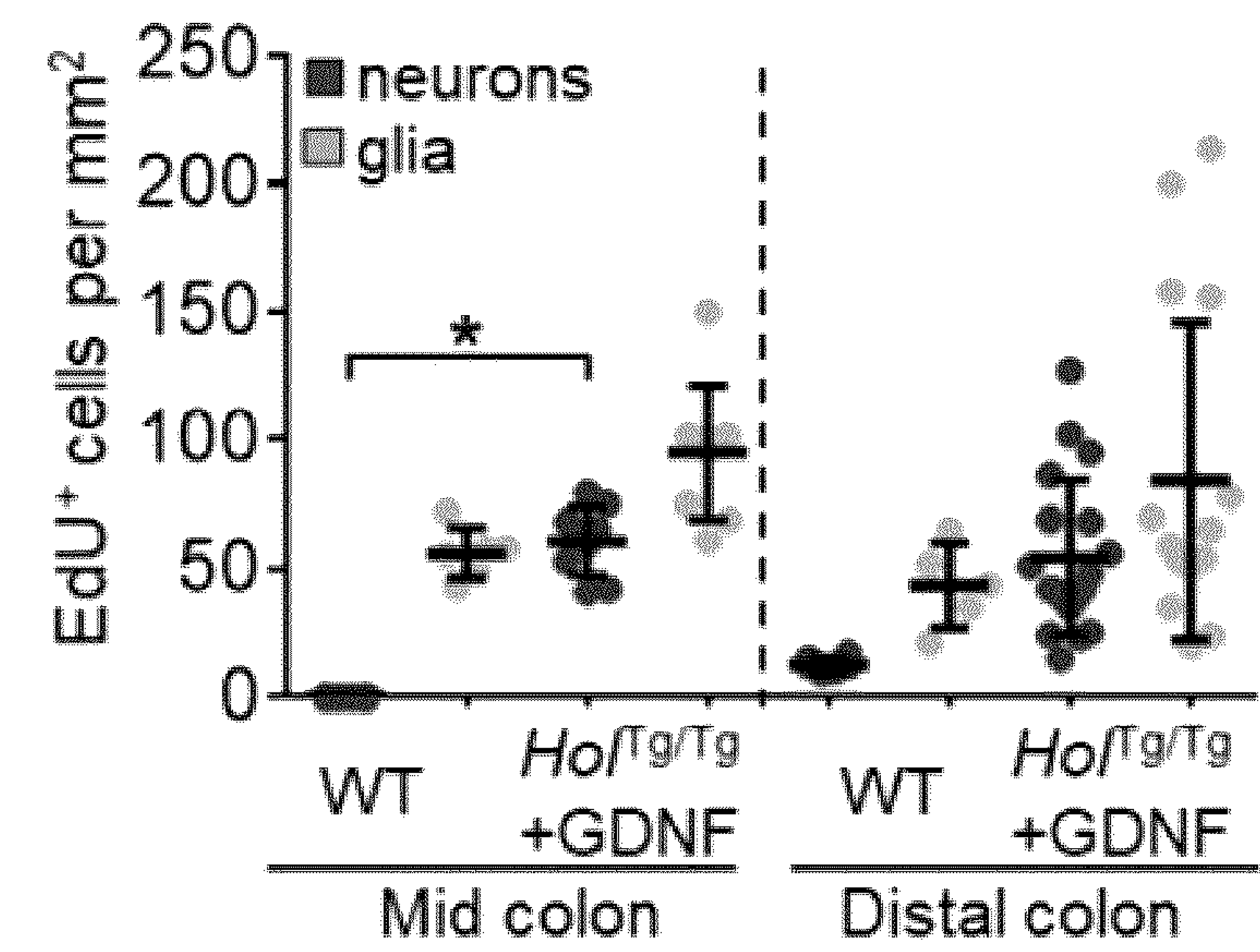


FIG. 5B

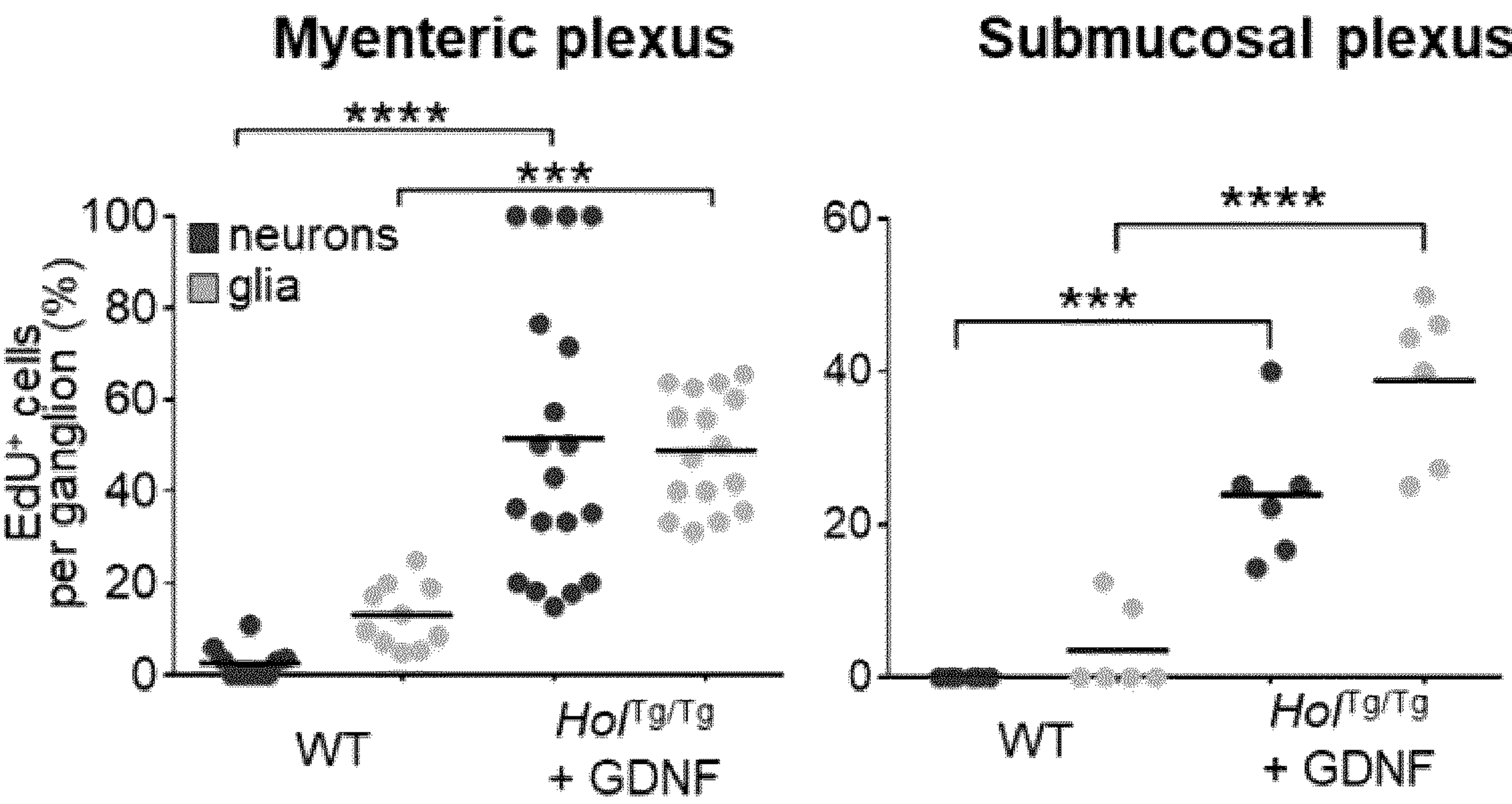


FIG. 5C

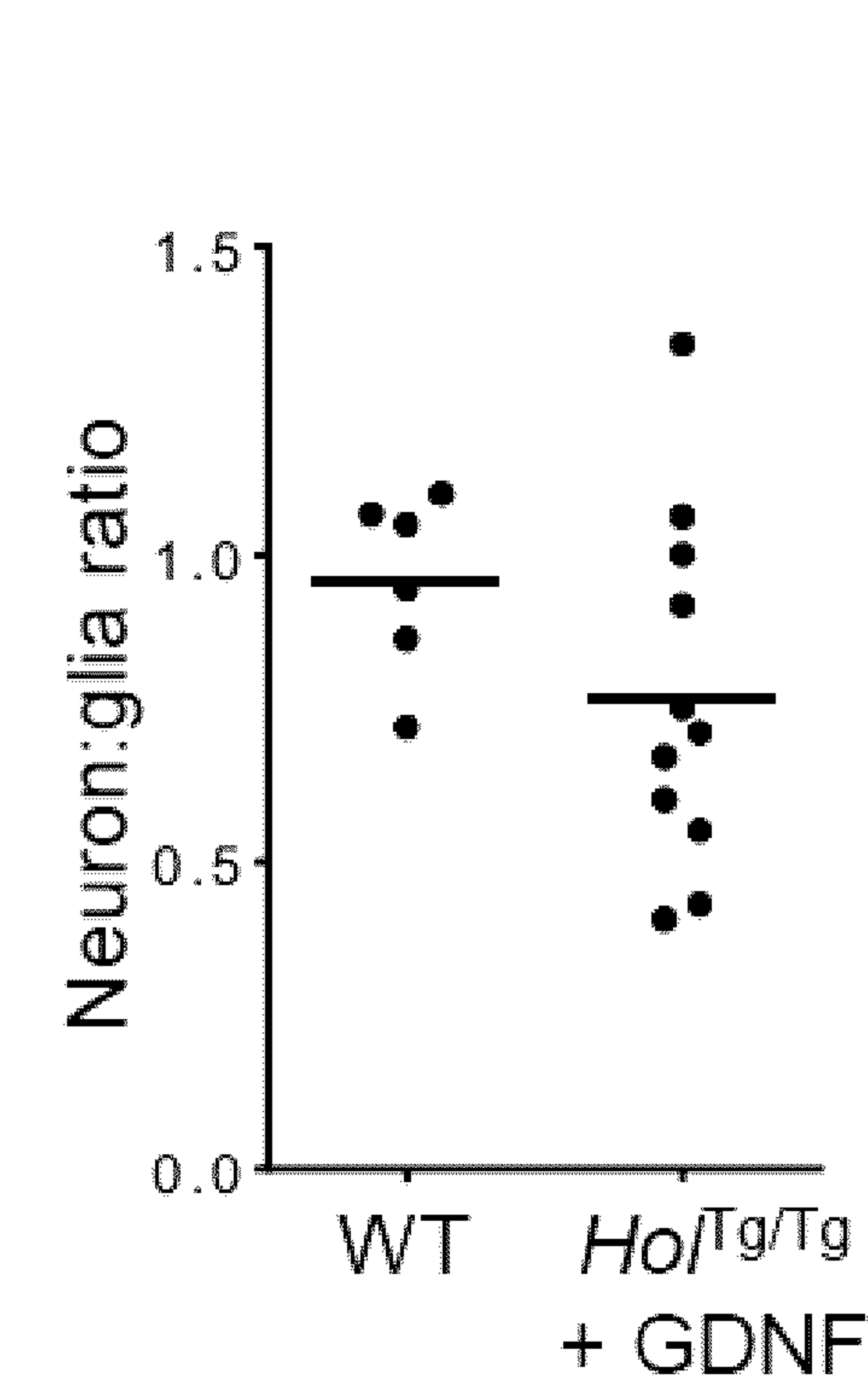


FIG. 6A

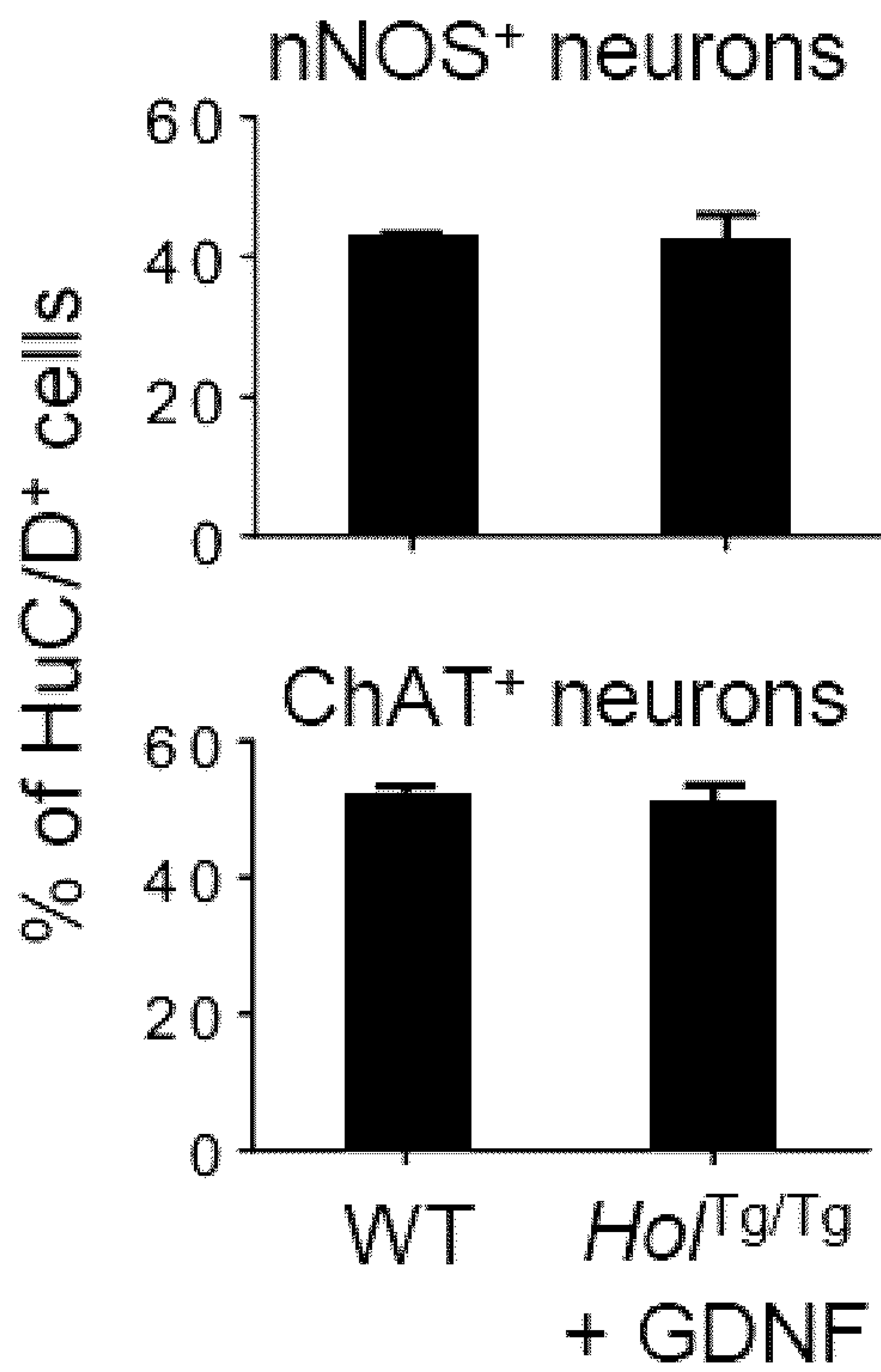


FIG. 6B

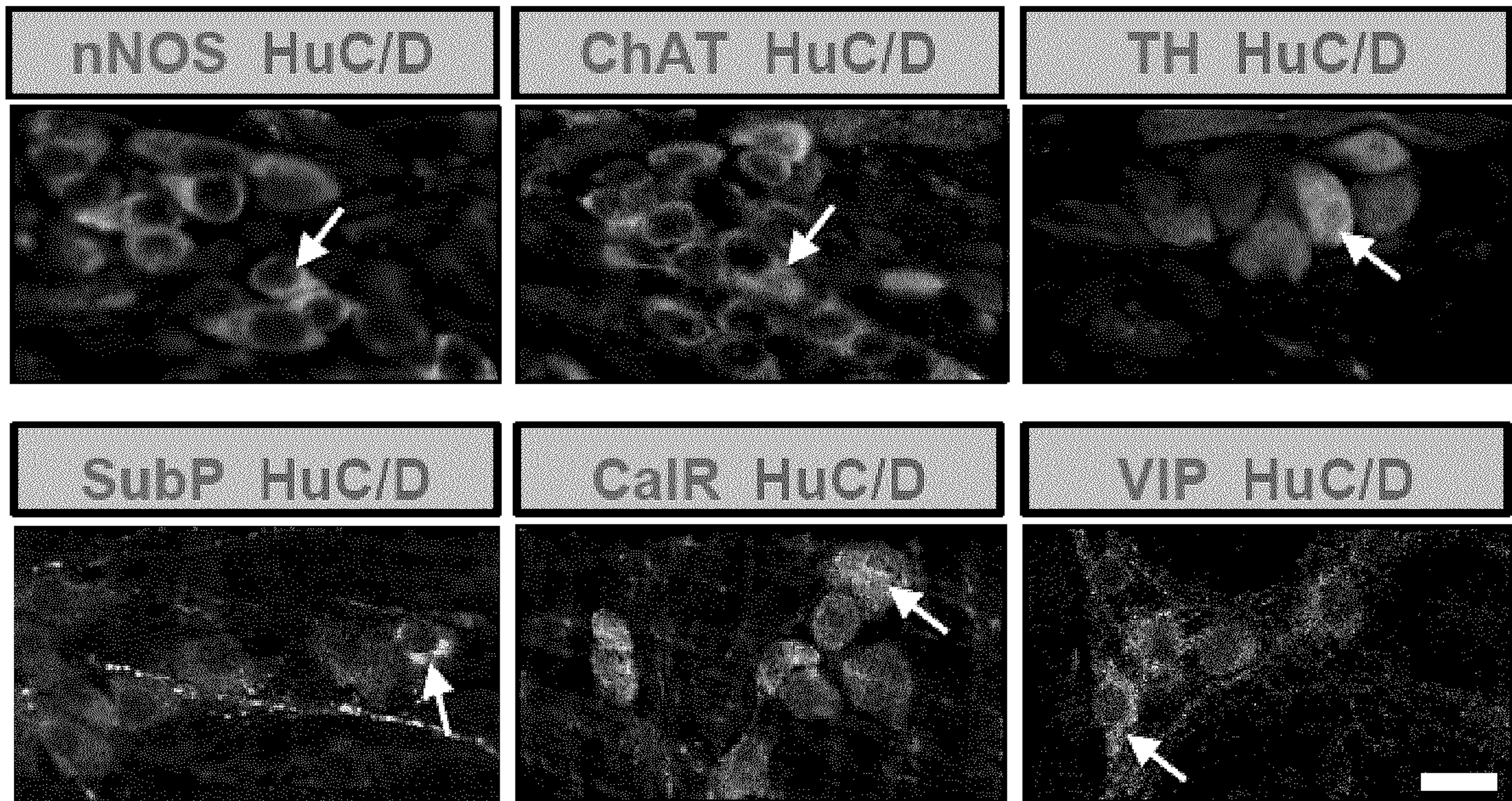


FIG. 6C

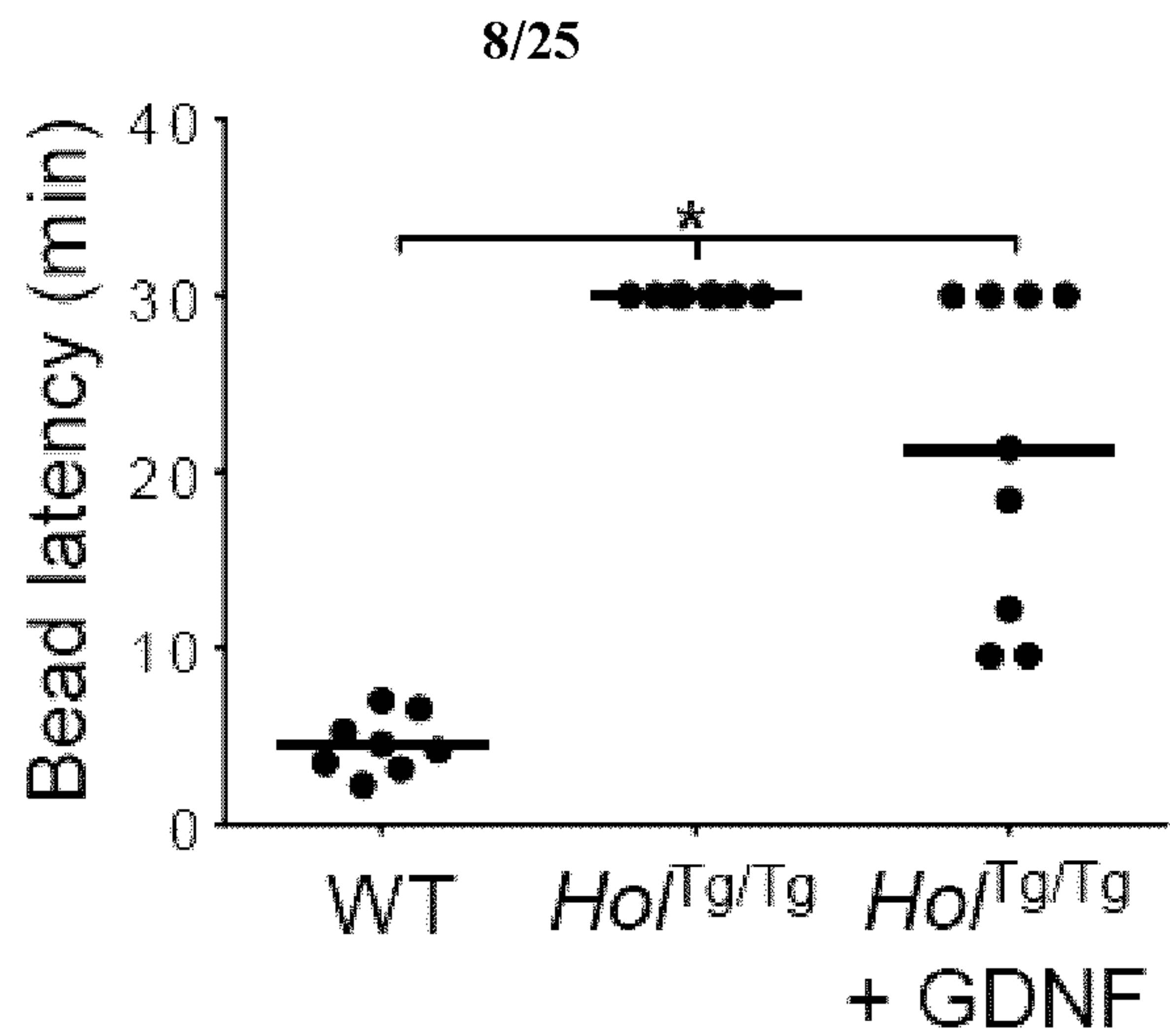


FIG. 6D

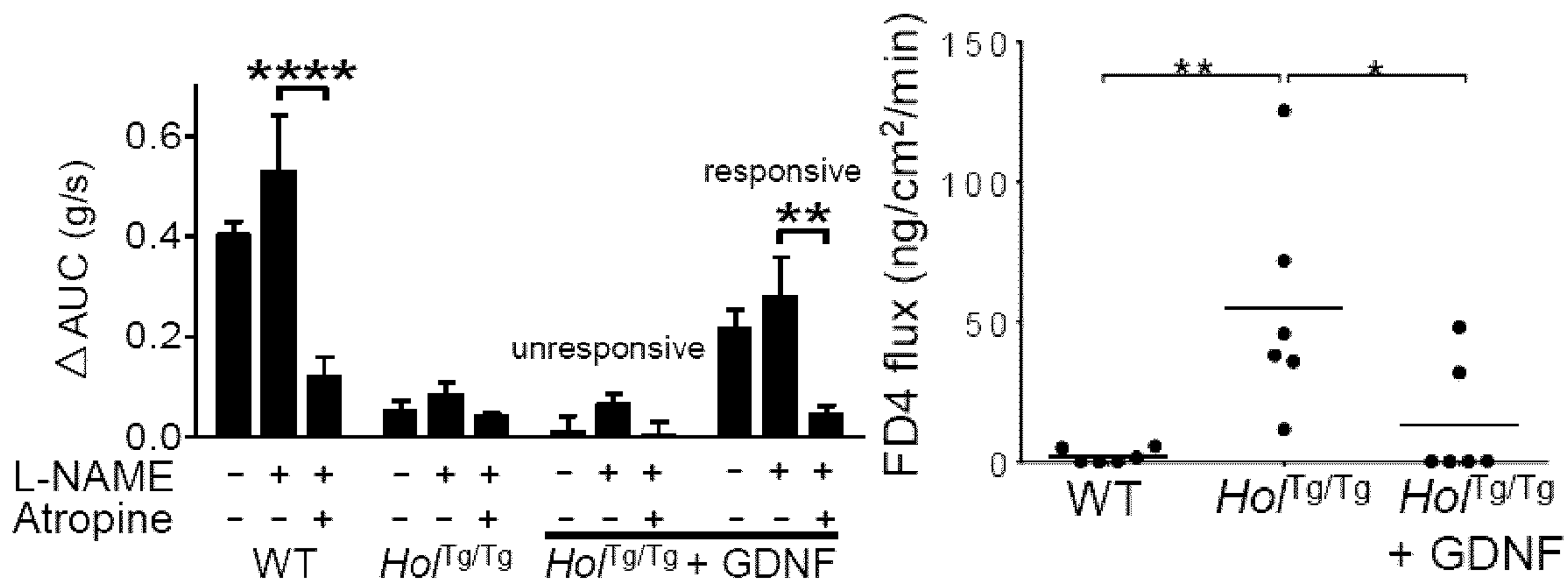


FIG. 6E

FIG. 6F

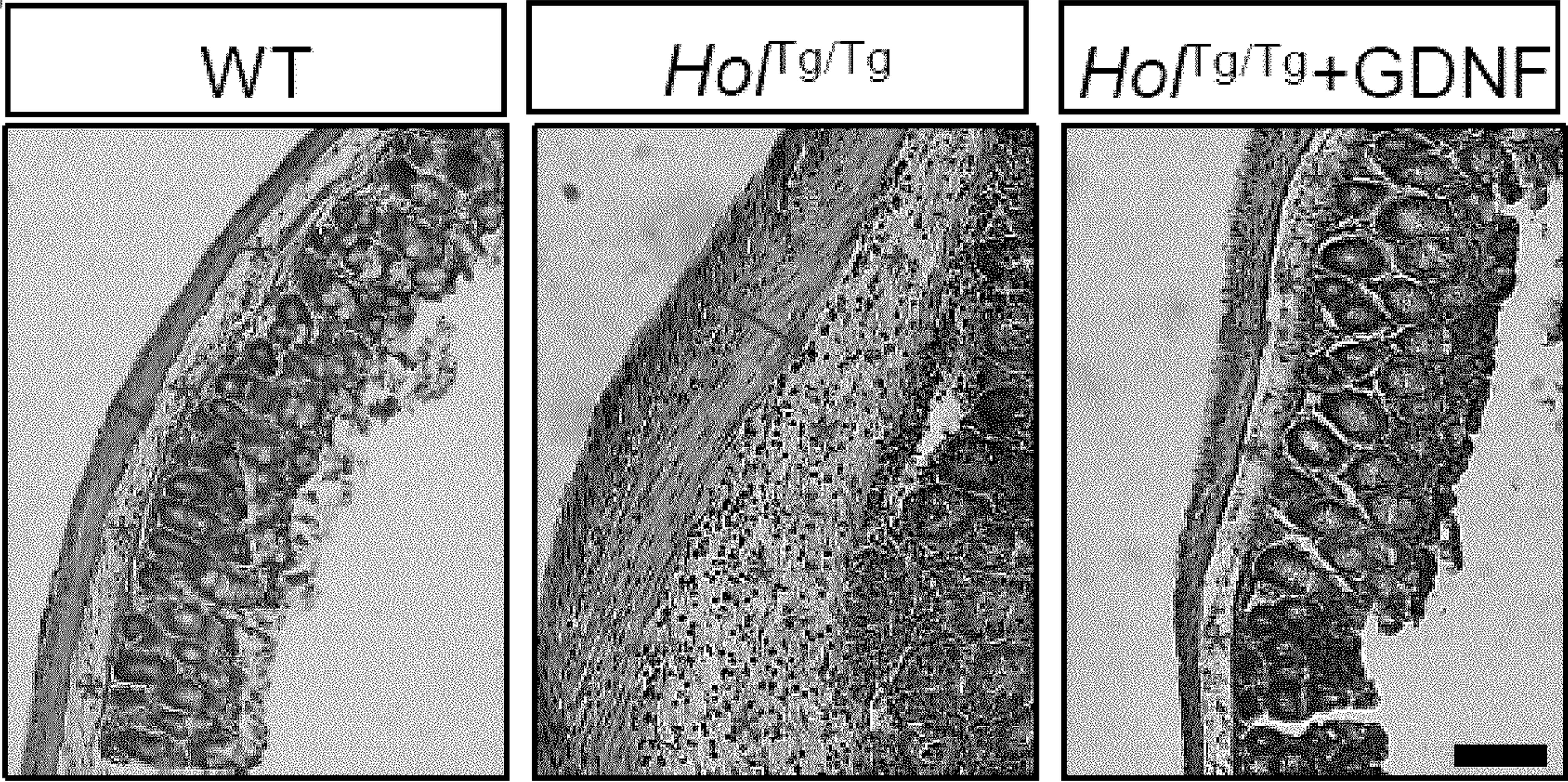


FIG. 6G

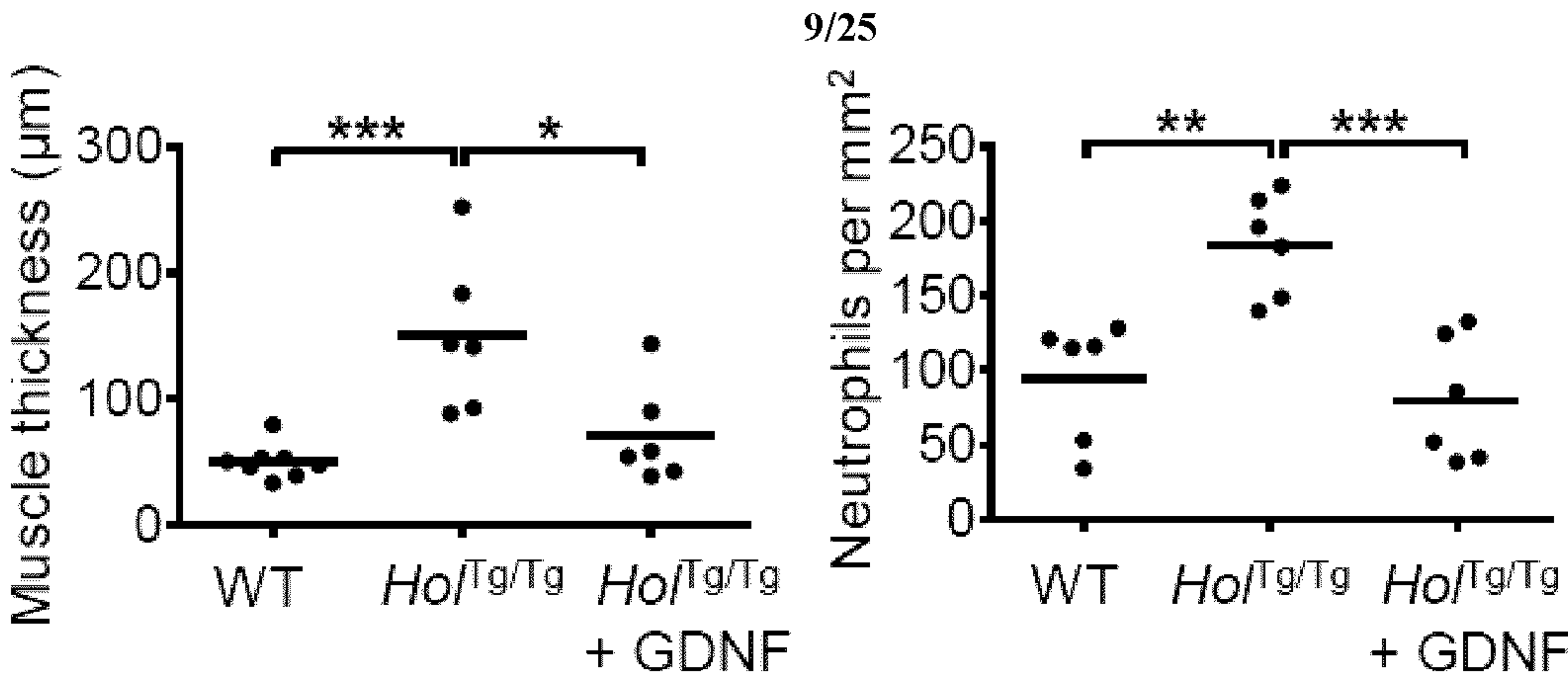


FIG. 6H

FIG. 6I

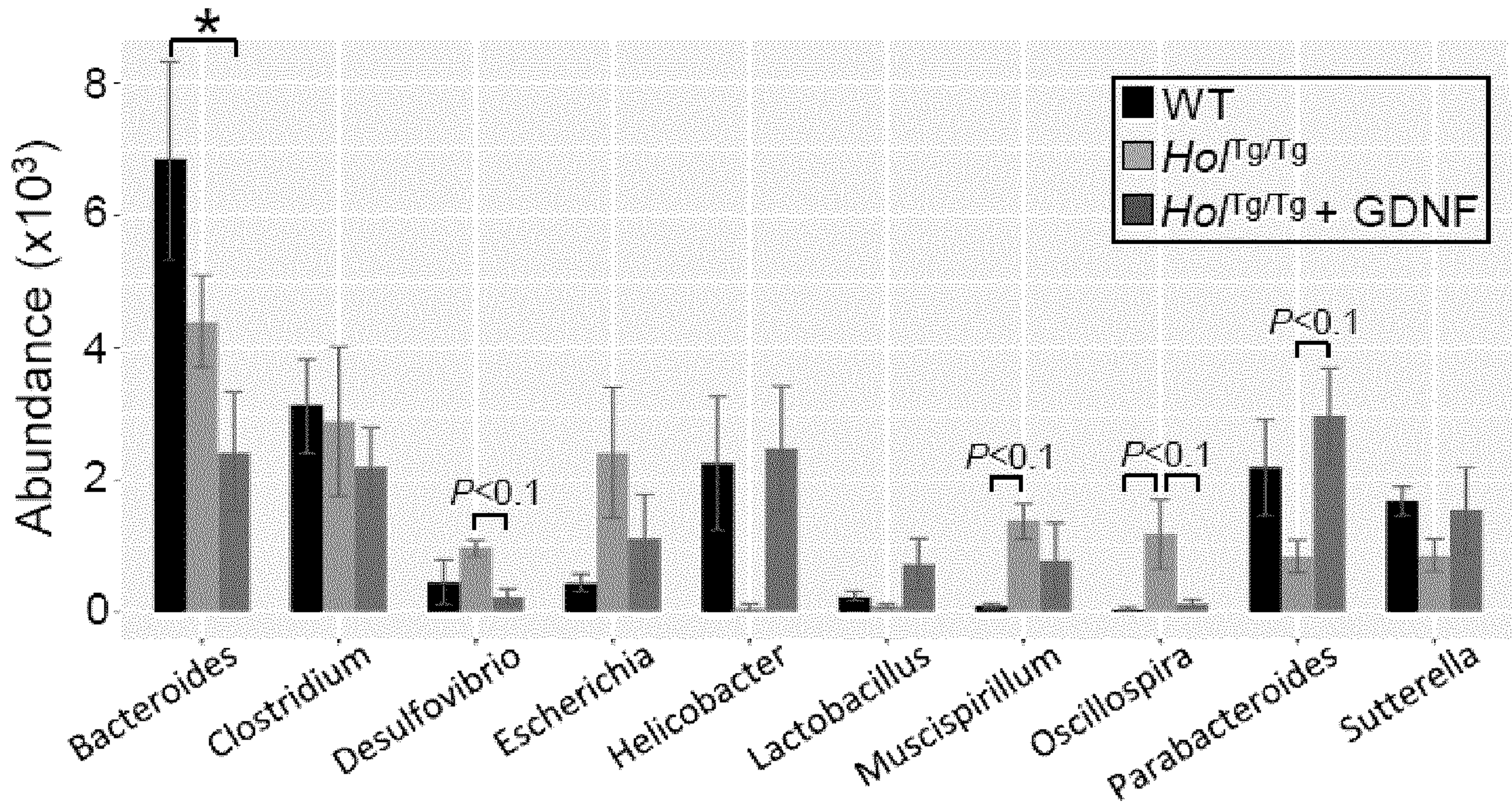


FIG. 6J

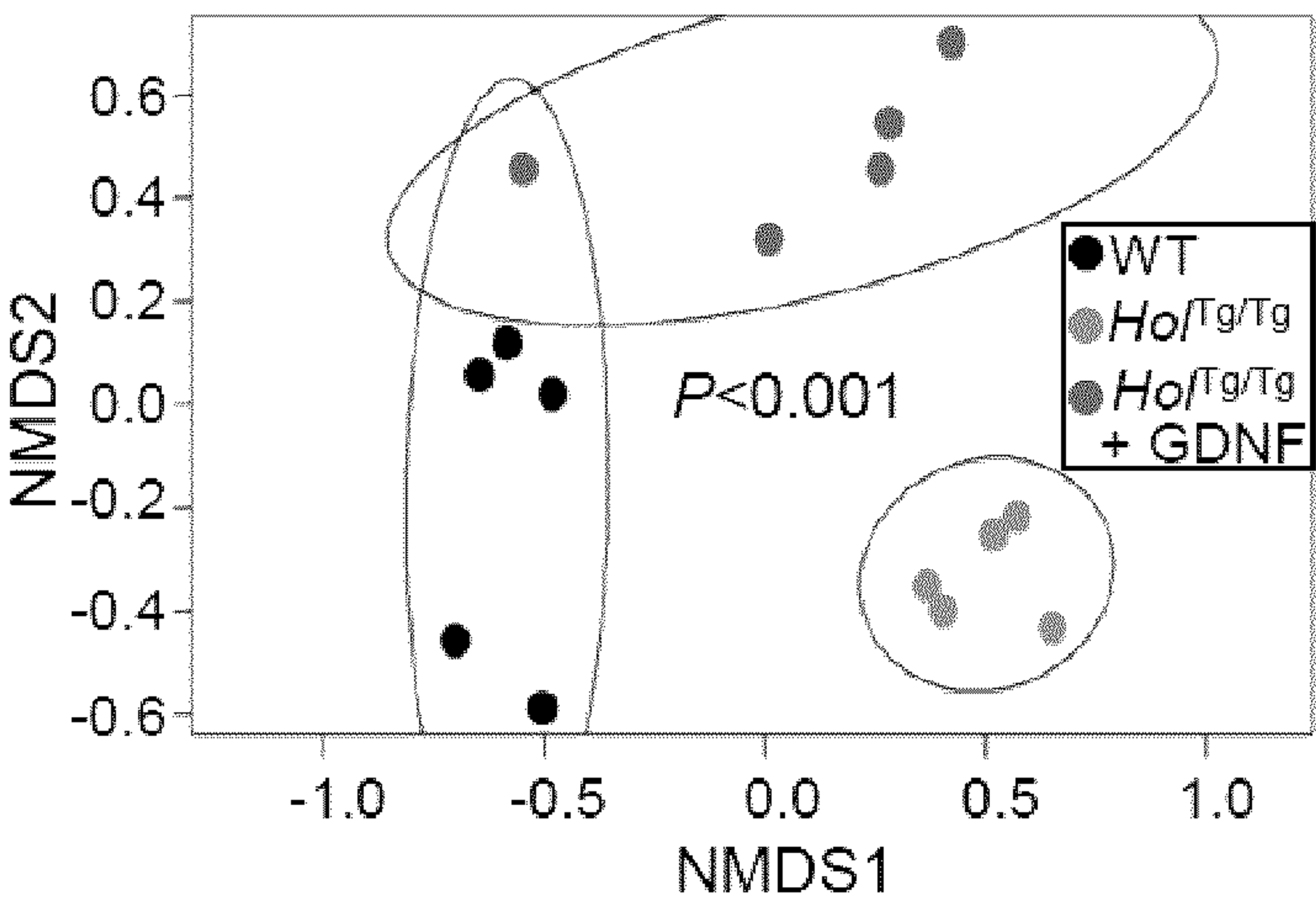


FIG. 6K

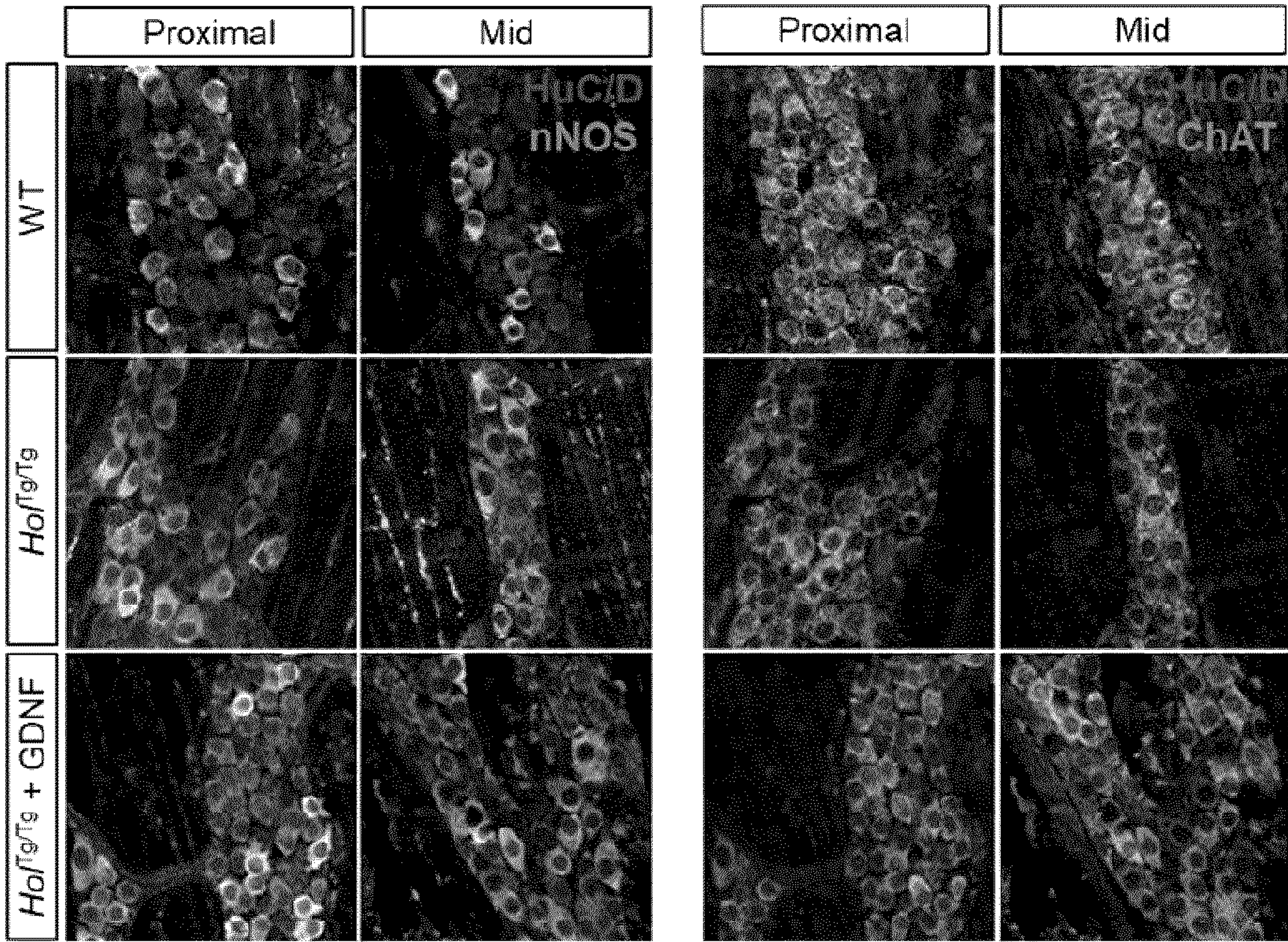


FIG. 7A

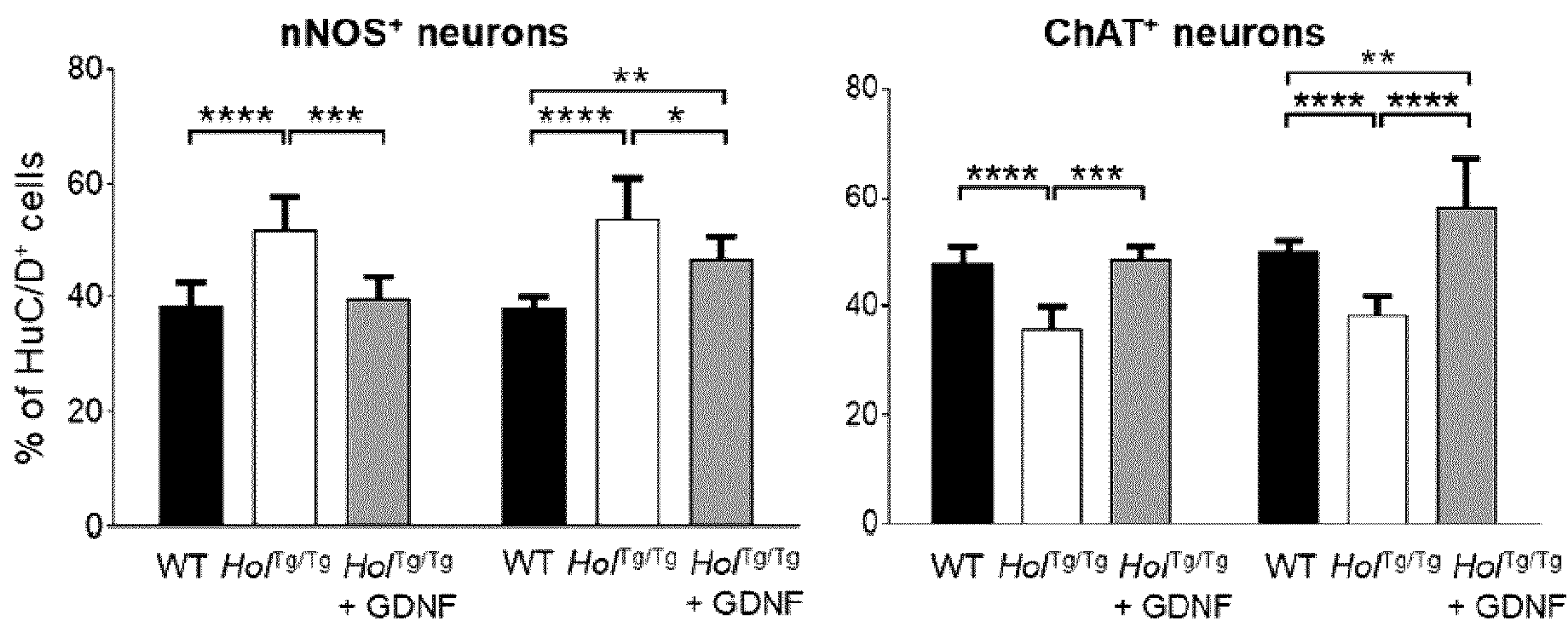


FIG. 7B

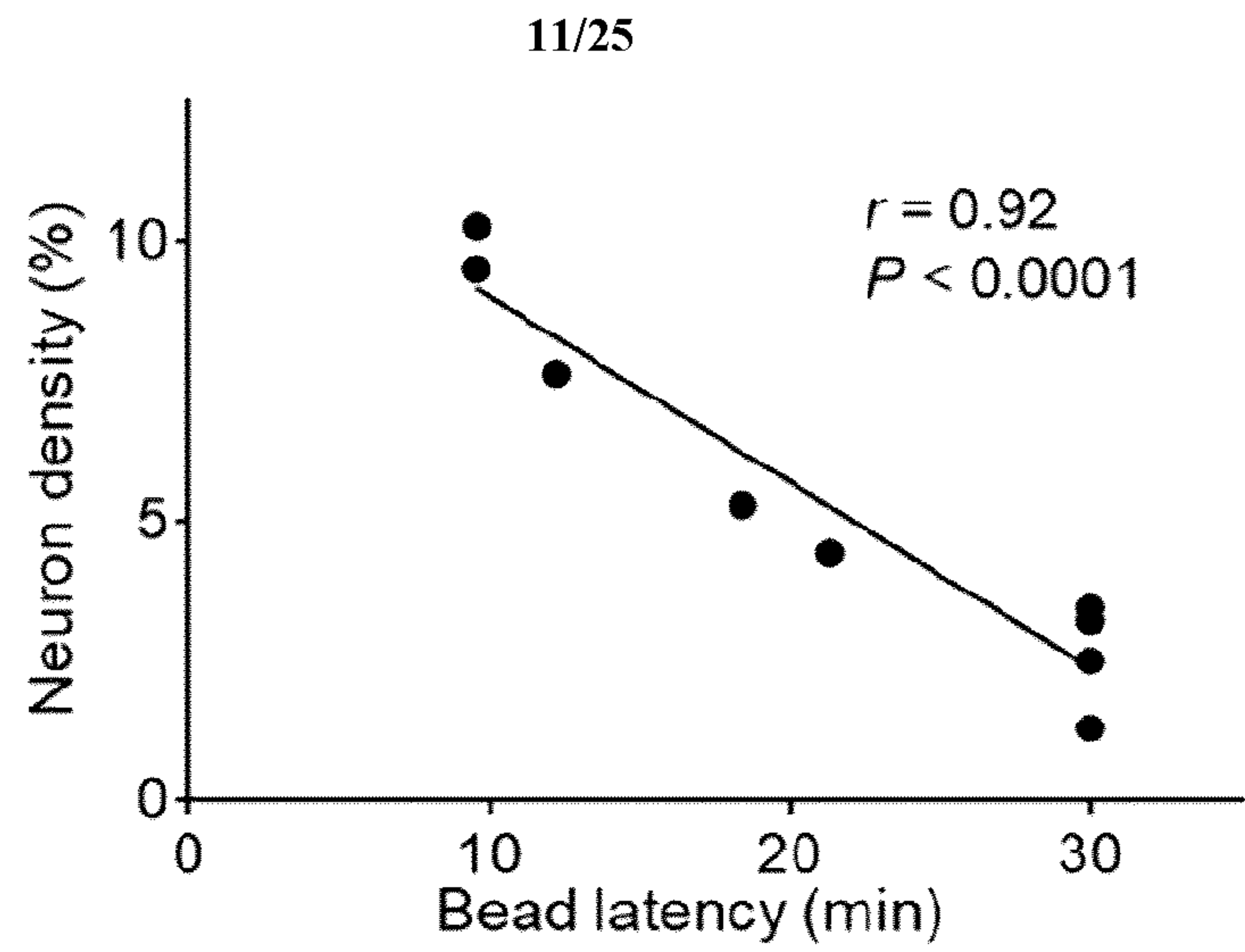


FIG. 8A

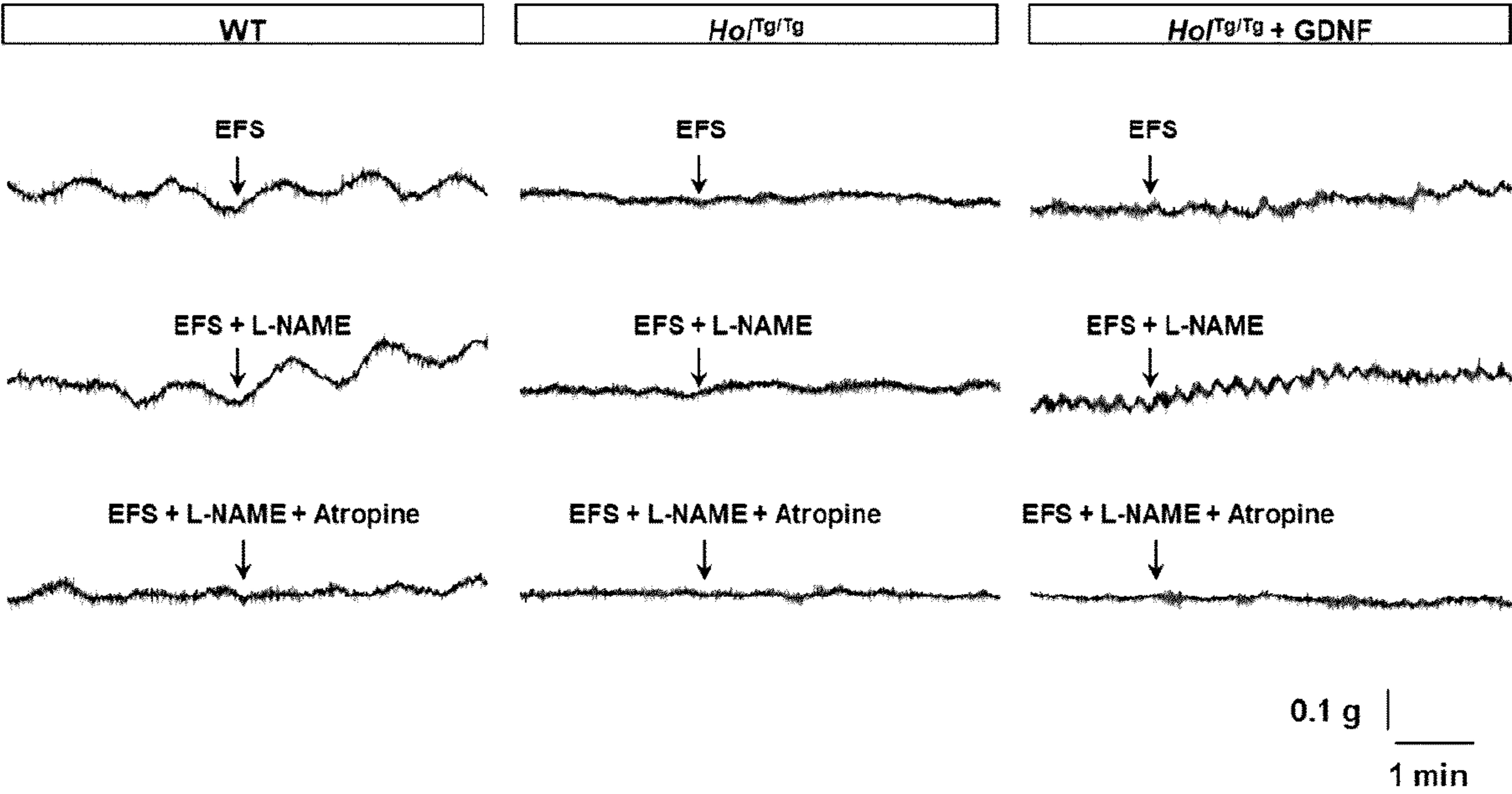


FIG. 8B

12/25

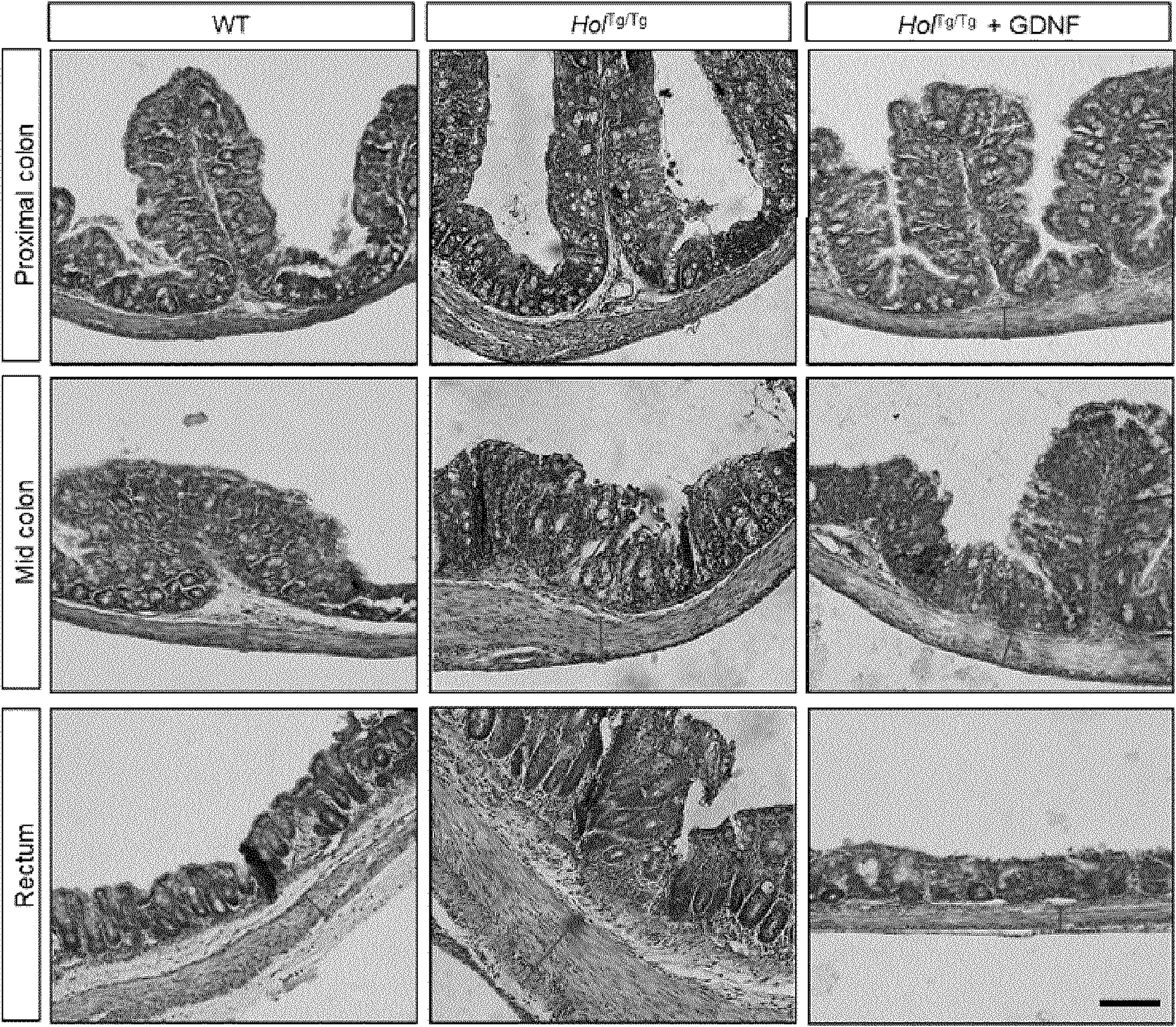


FIG. 9A

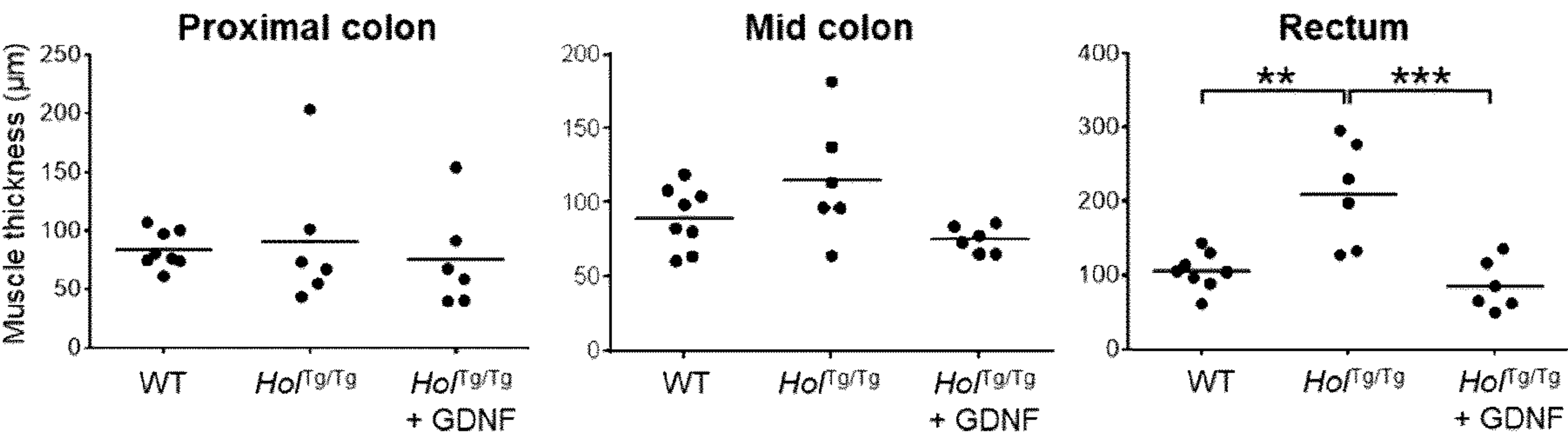


FIG. 9B

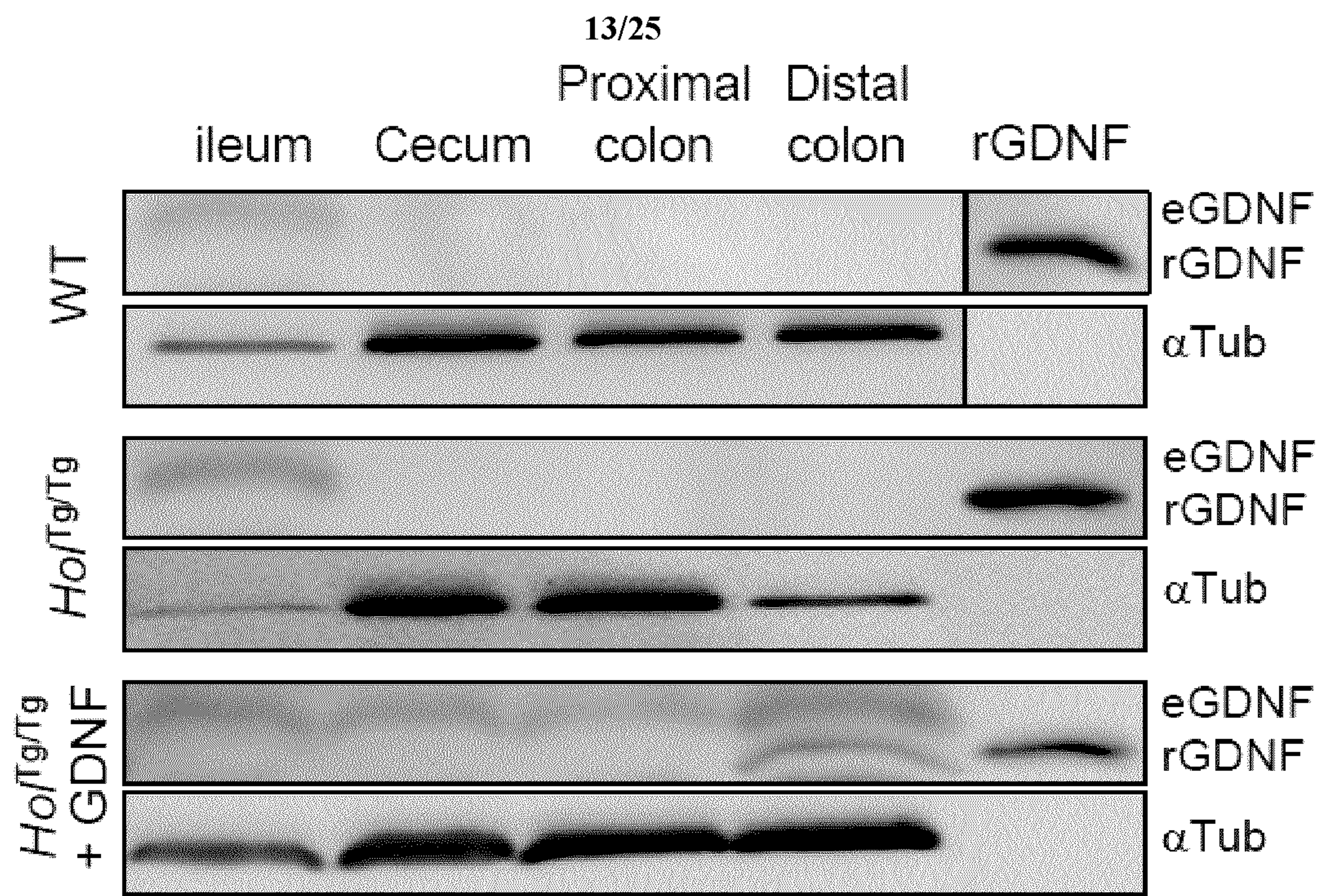


FIG. 10A

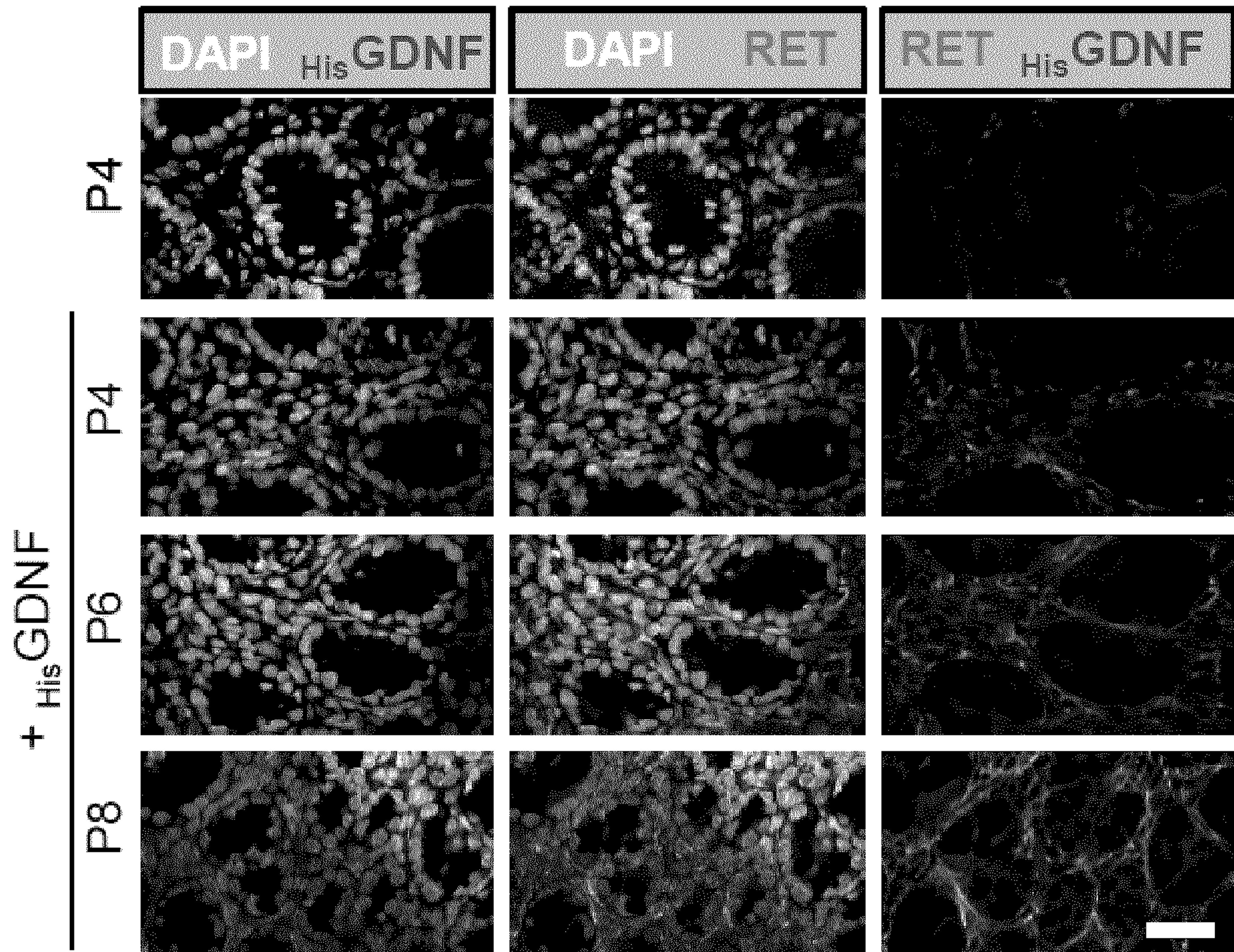


FIG. 10B



FIG. 10C

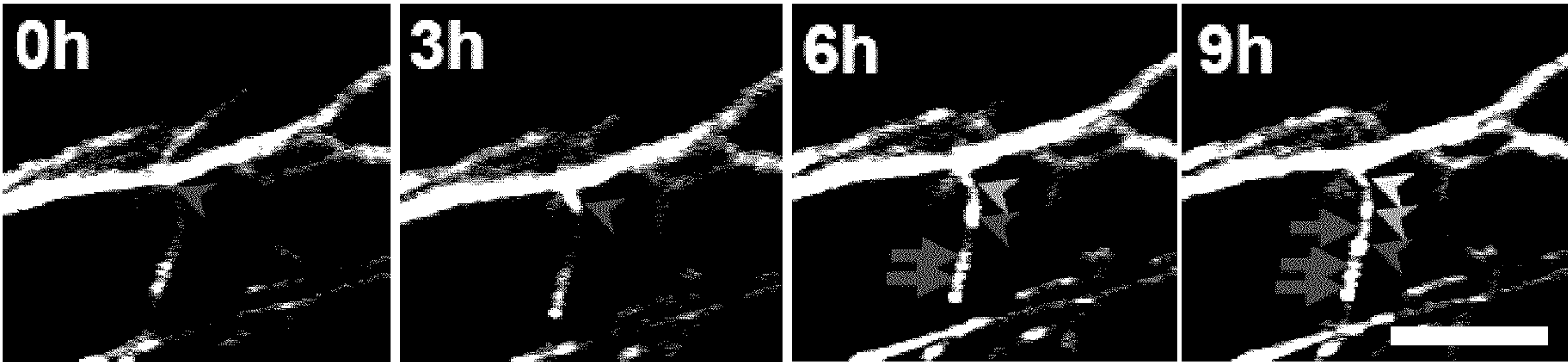


FIG. 10D

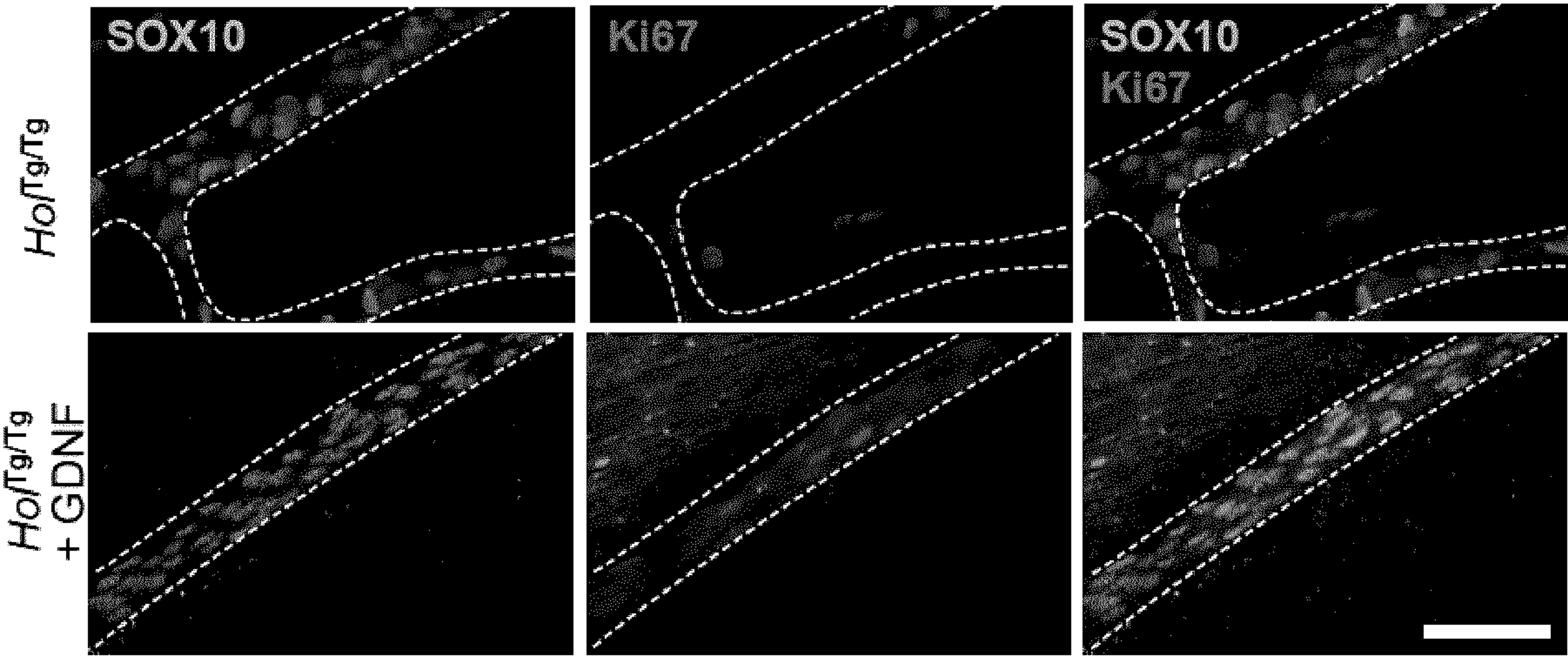


FIG. 10E

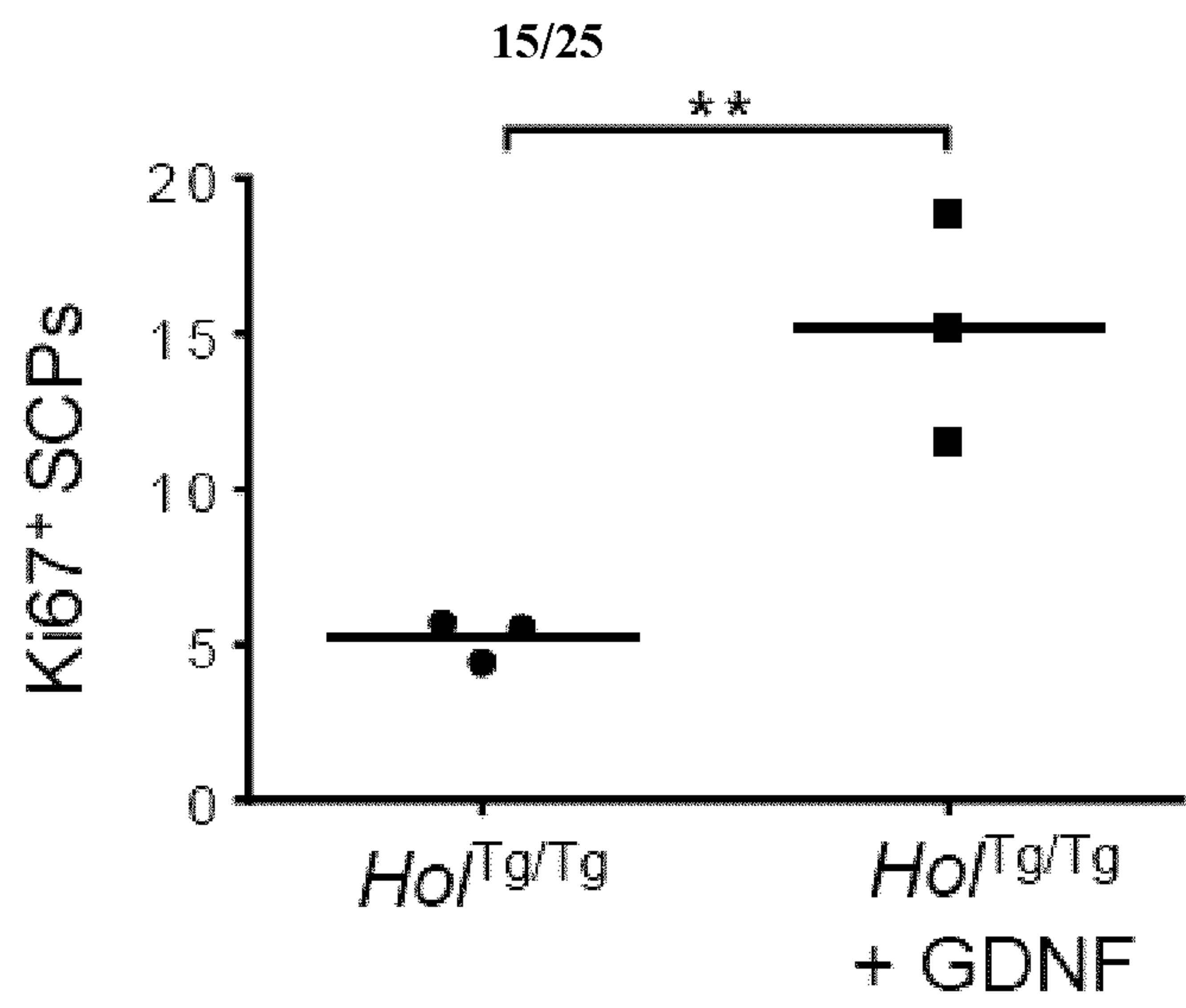


FIG. 10F

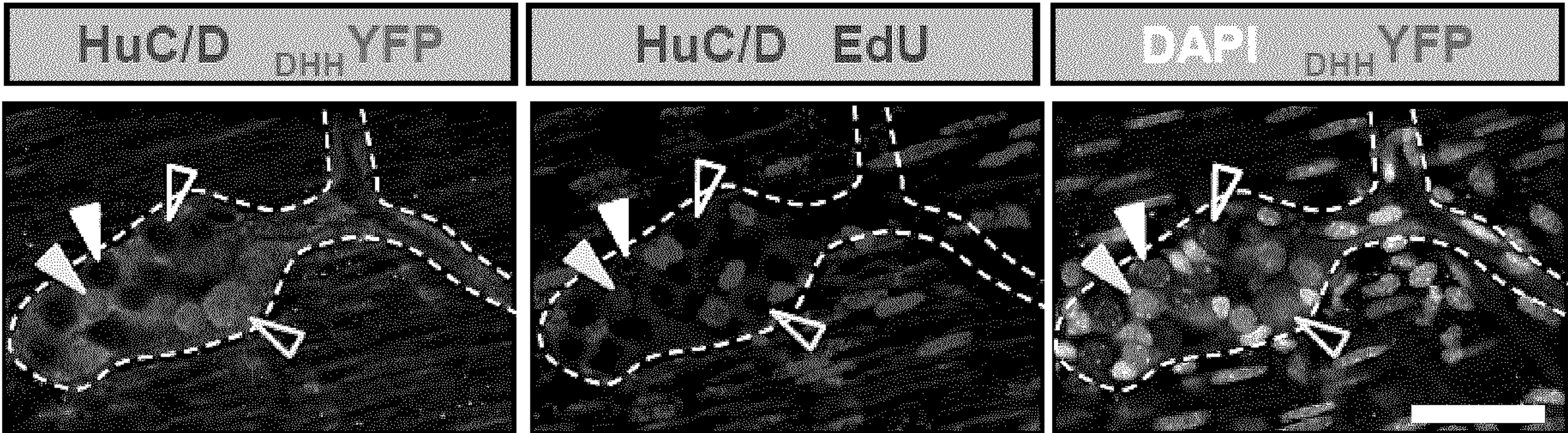


FIG. 10G

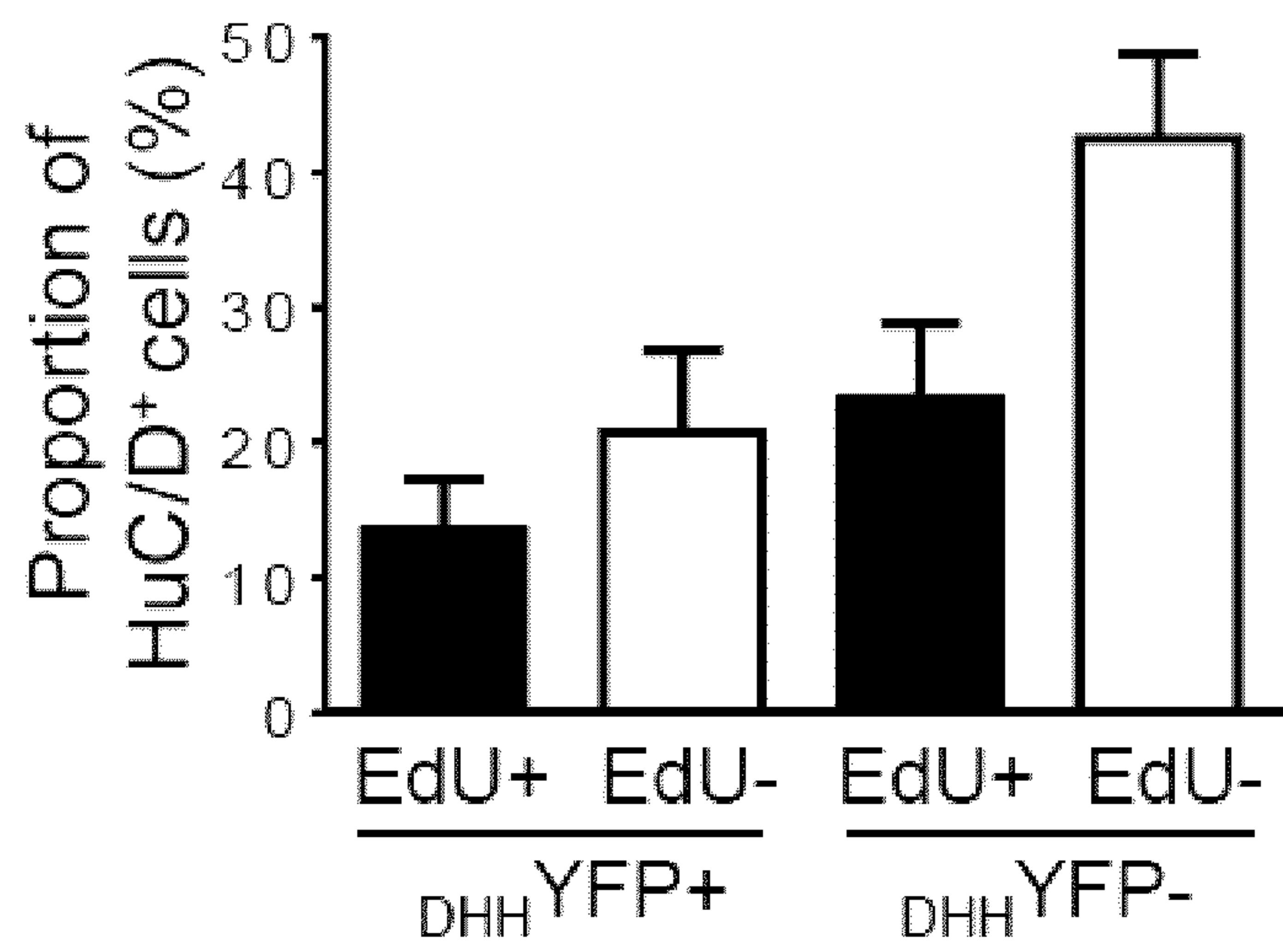


FIG. 10H

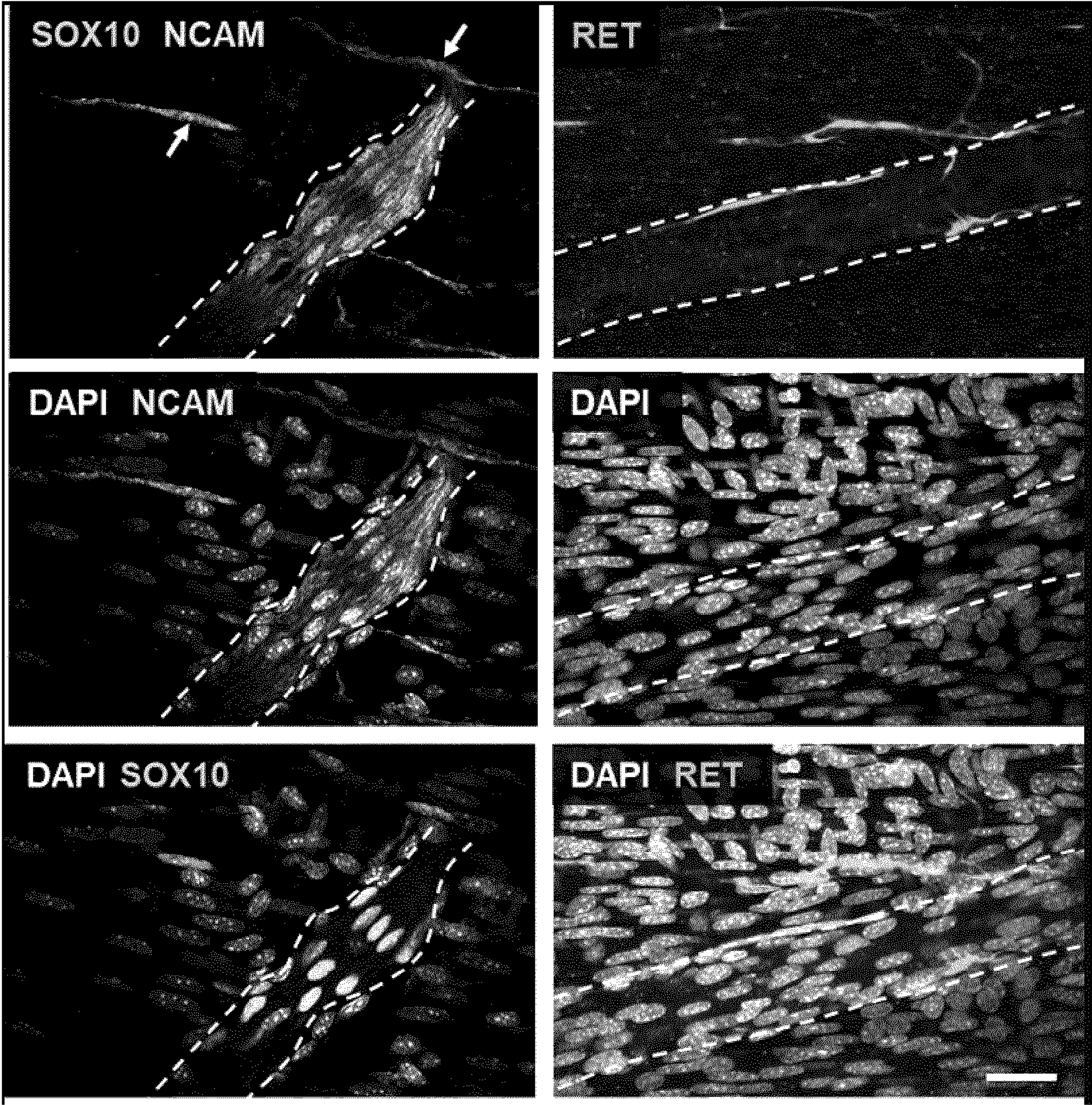


FIG. 10I

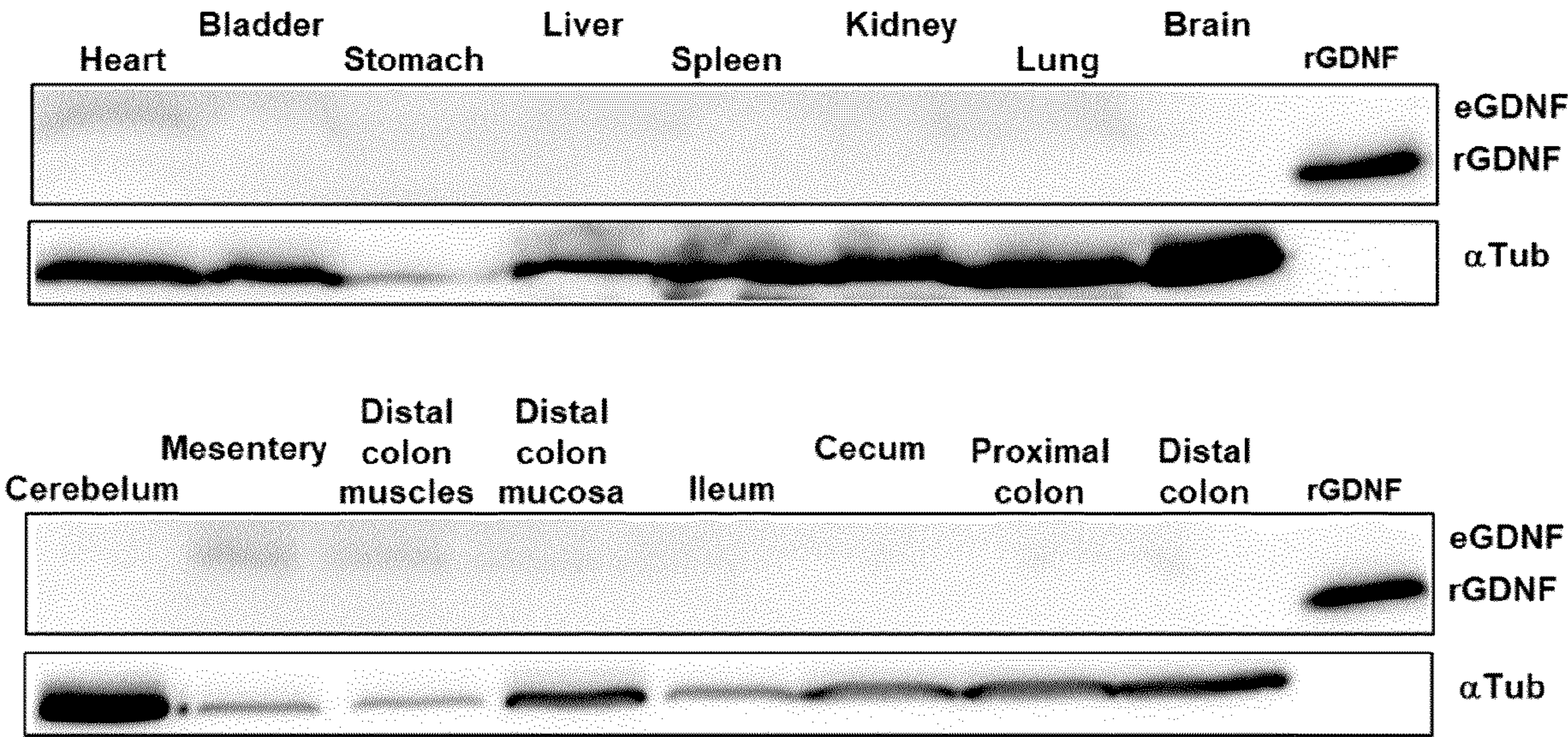


FIG. 11

17/25

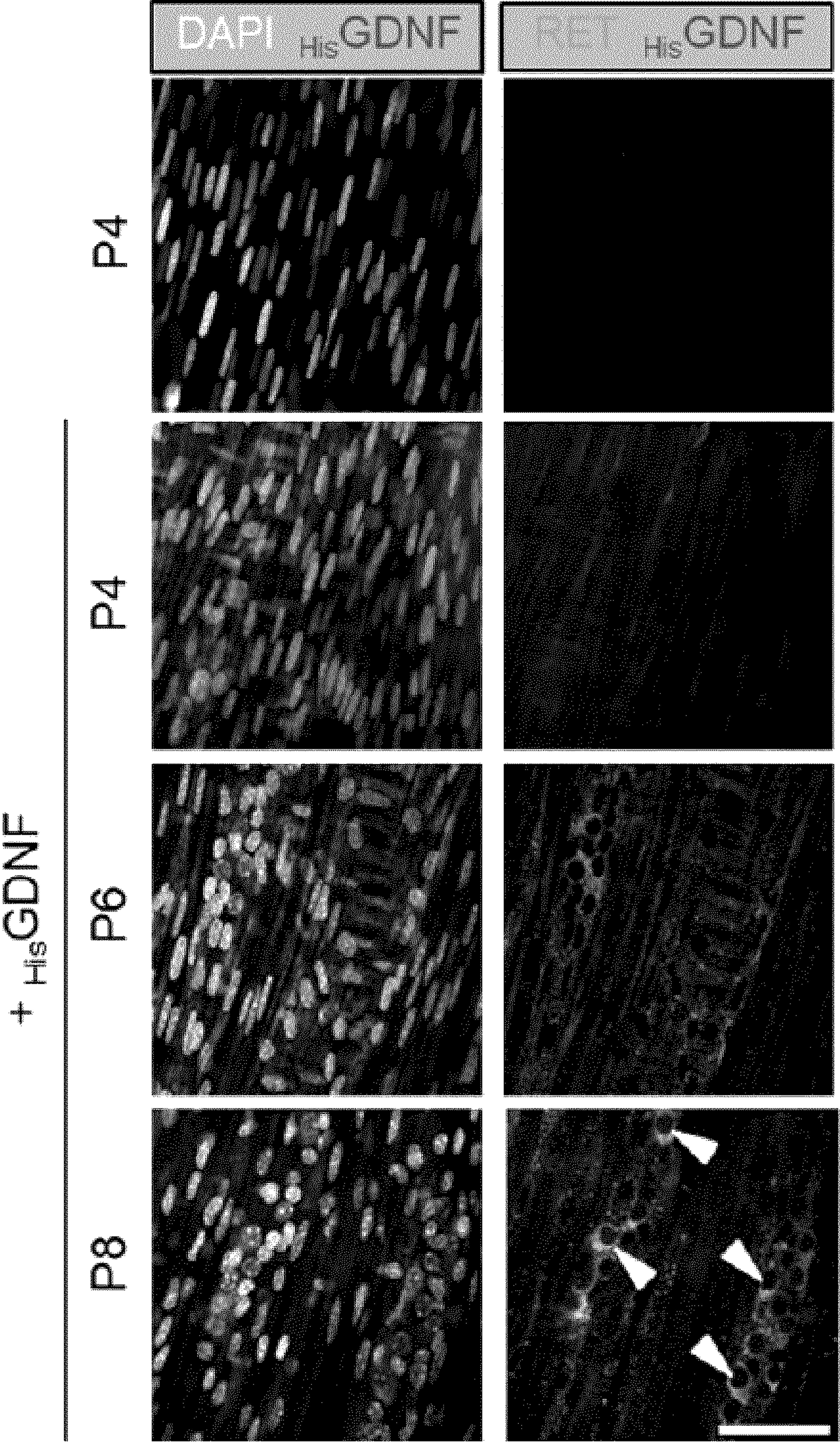


FIG. 12

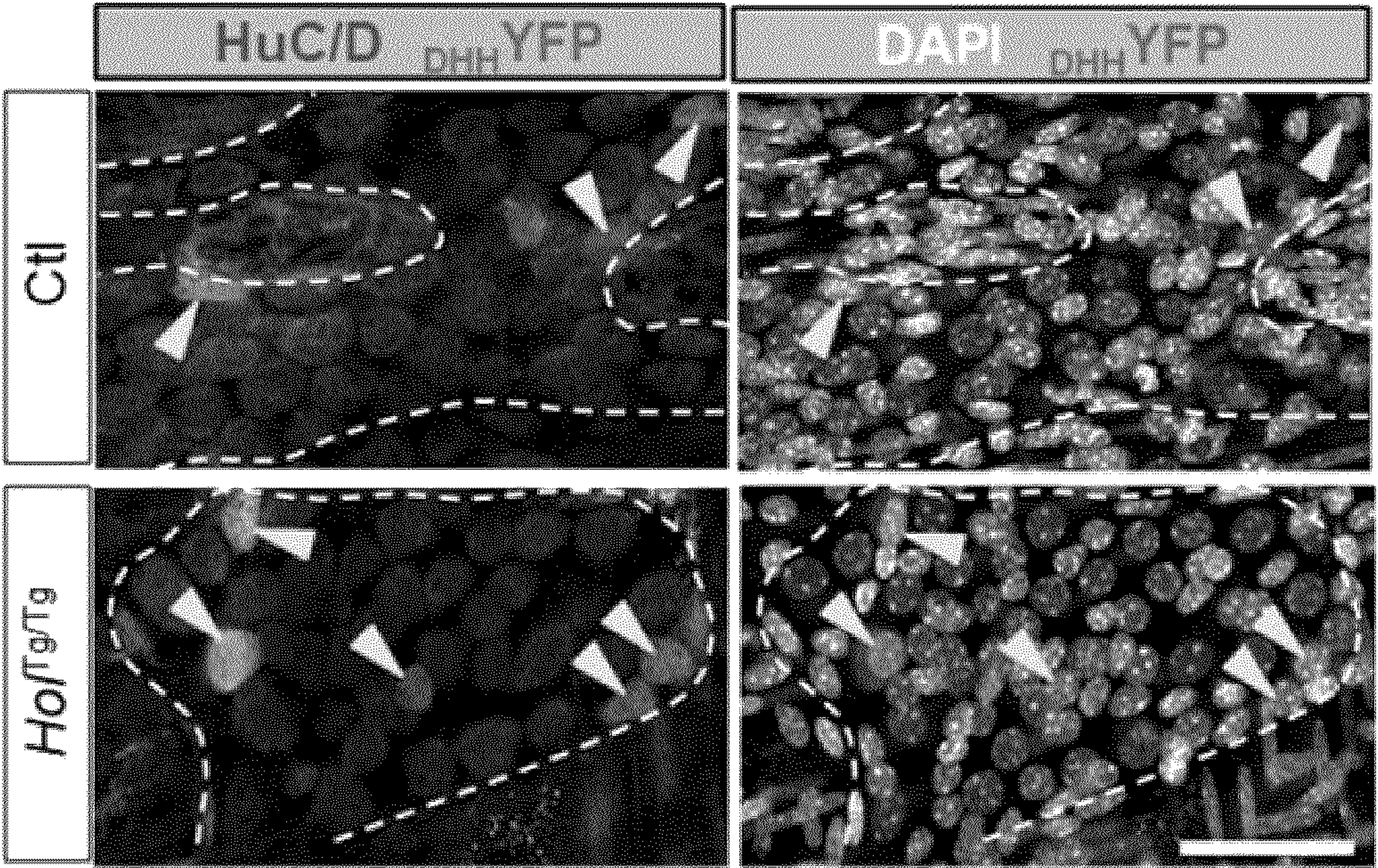


FIG. 13A

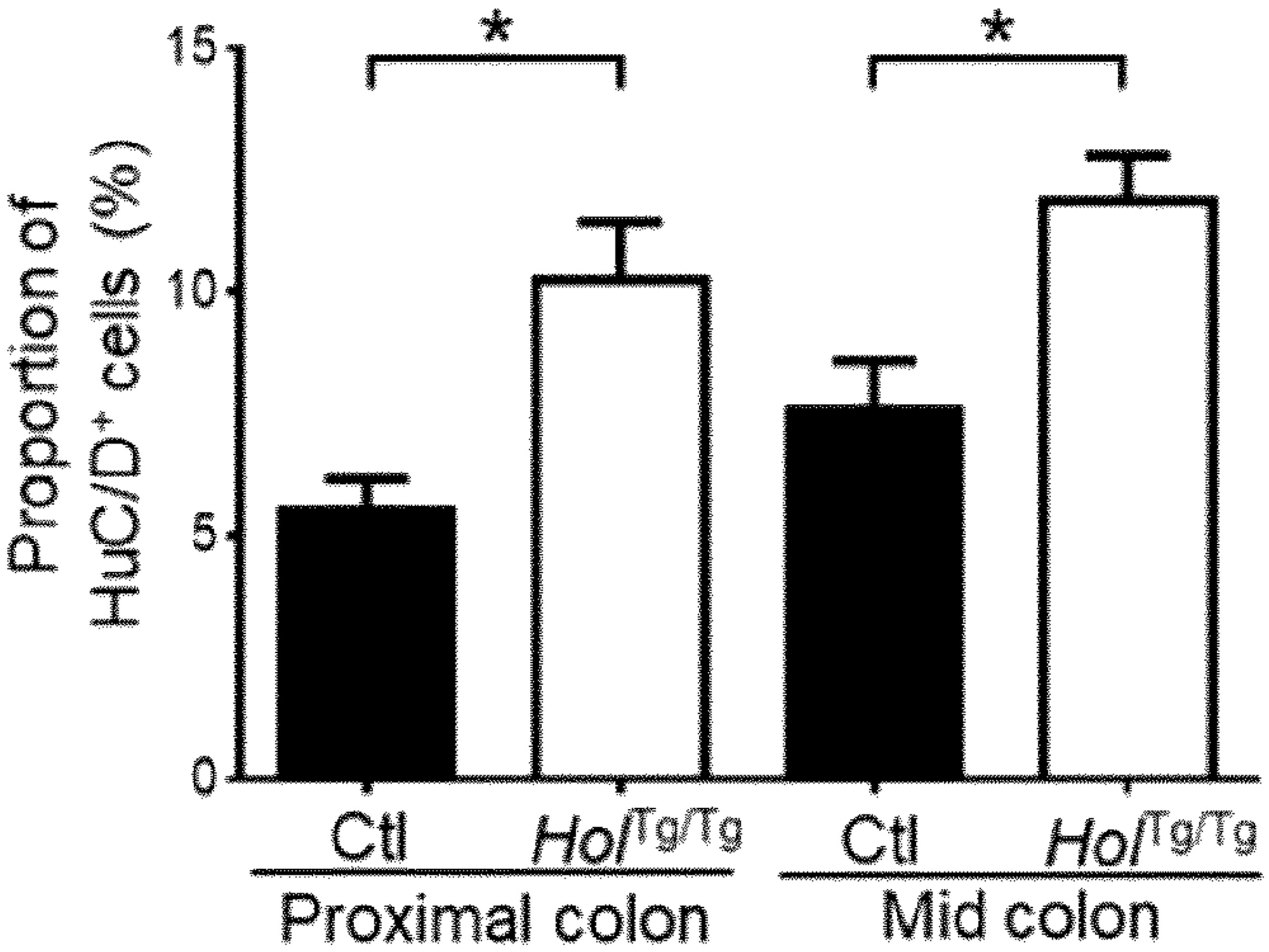


FIG. 13B

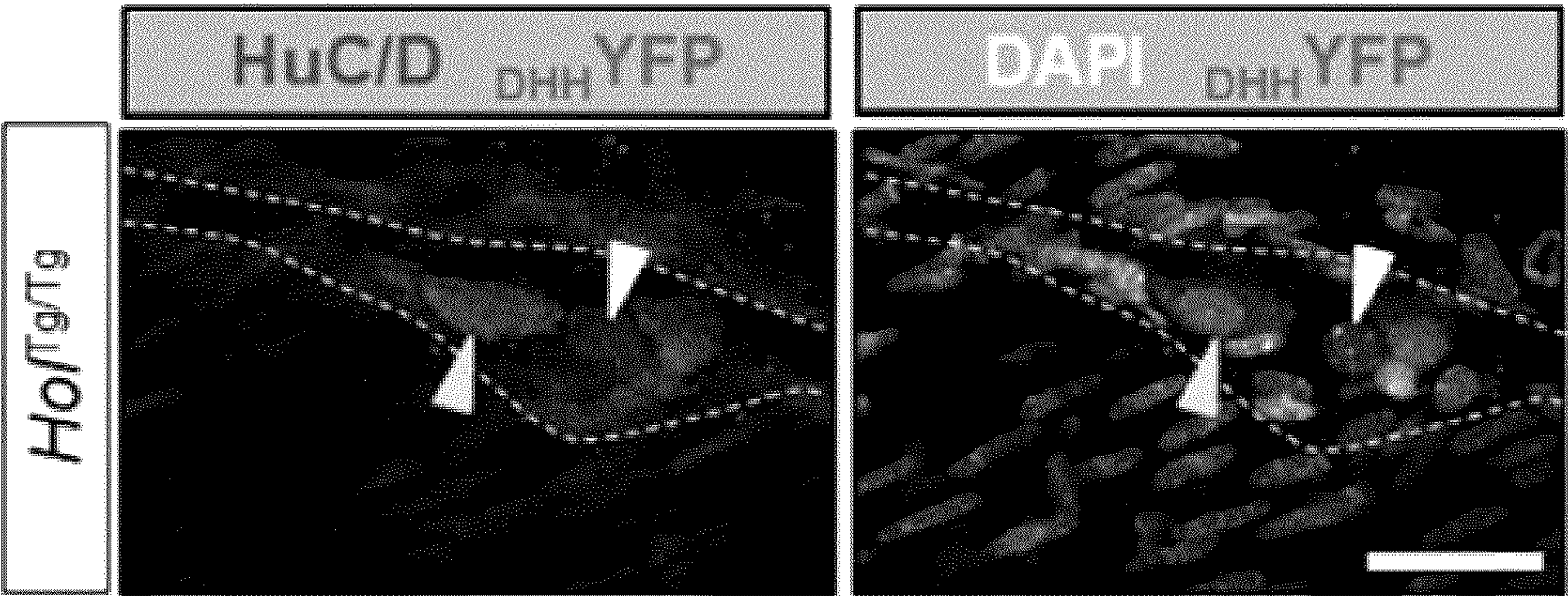


FIG. 13C

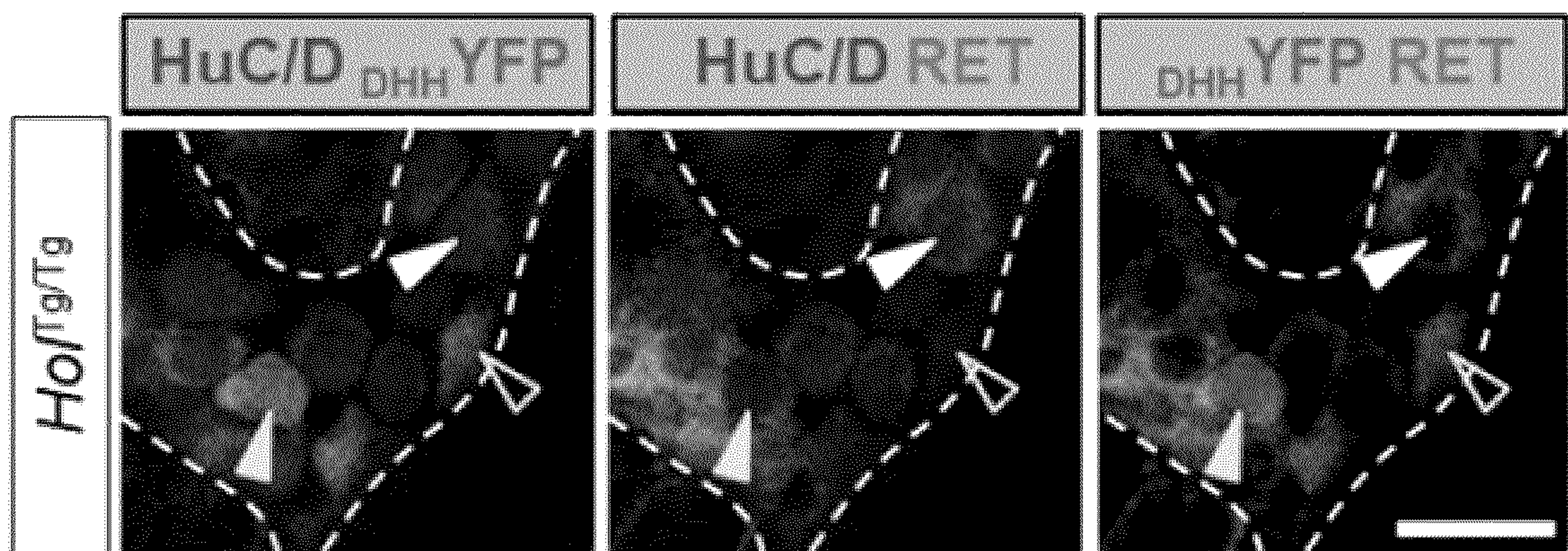


FIG. 13D

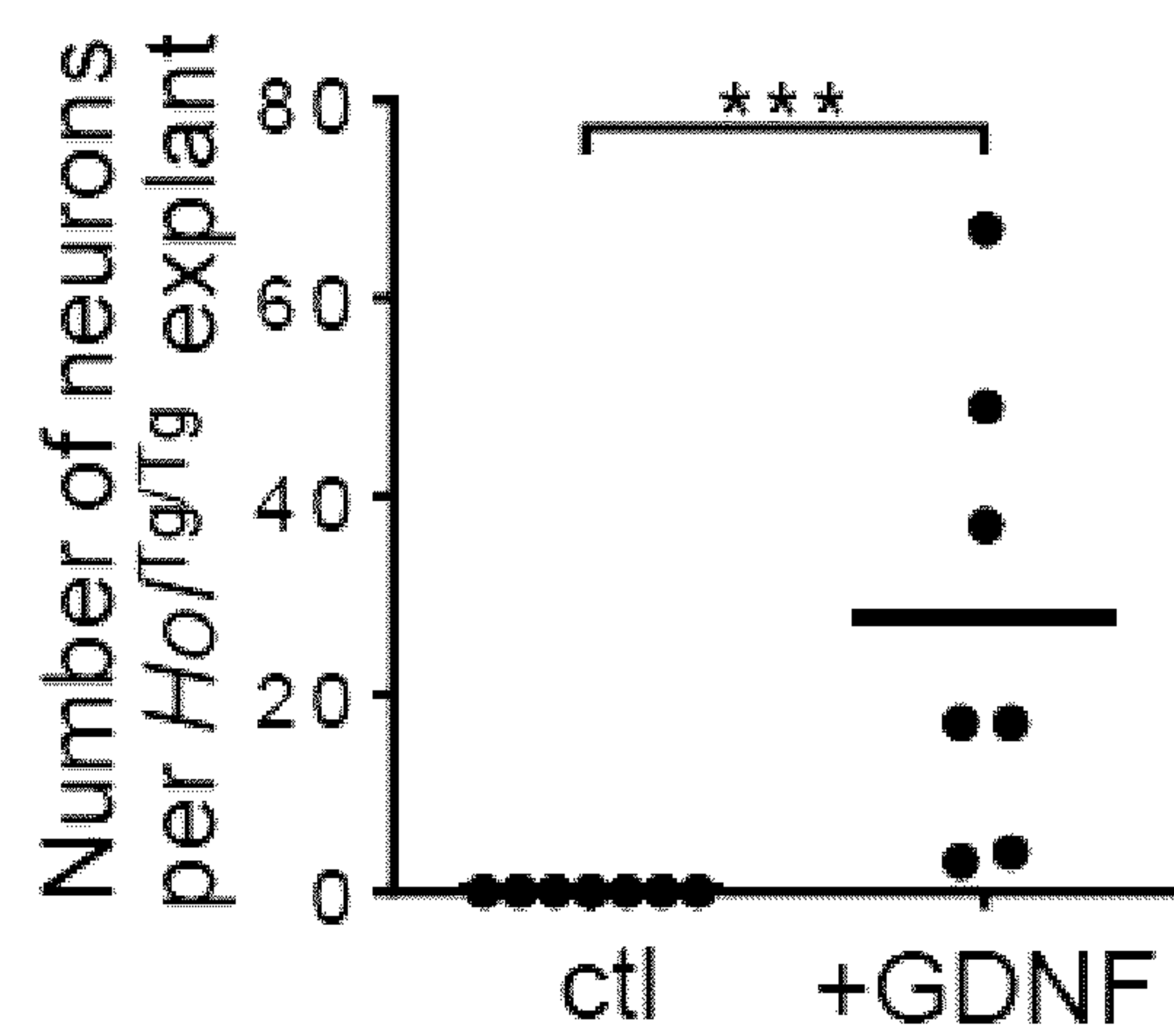


FIG. 14A

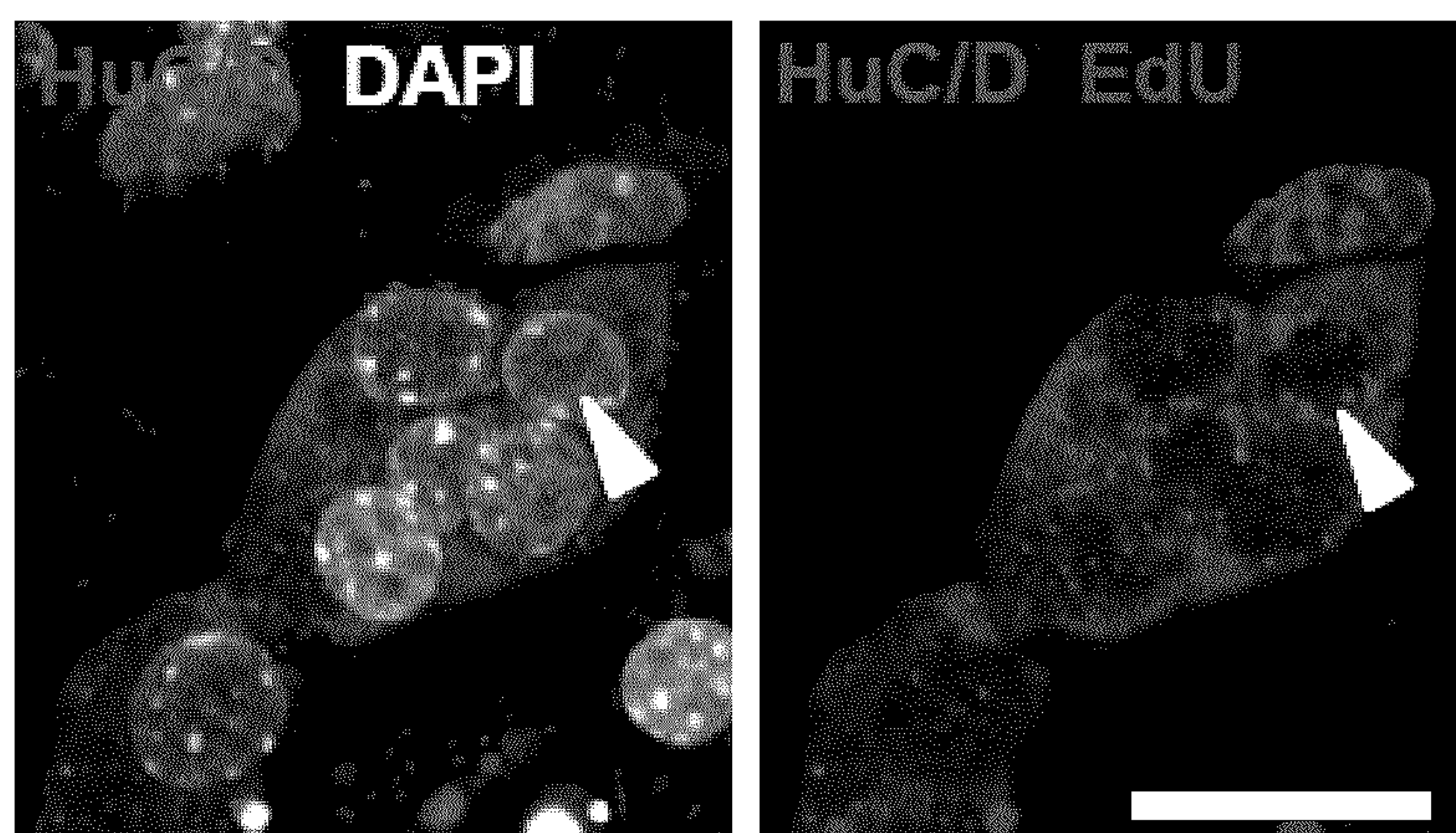


FIG. 14B

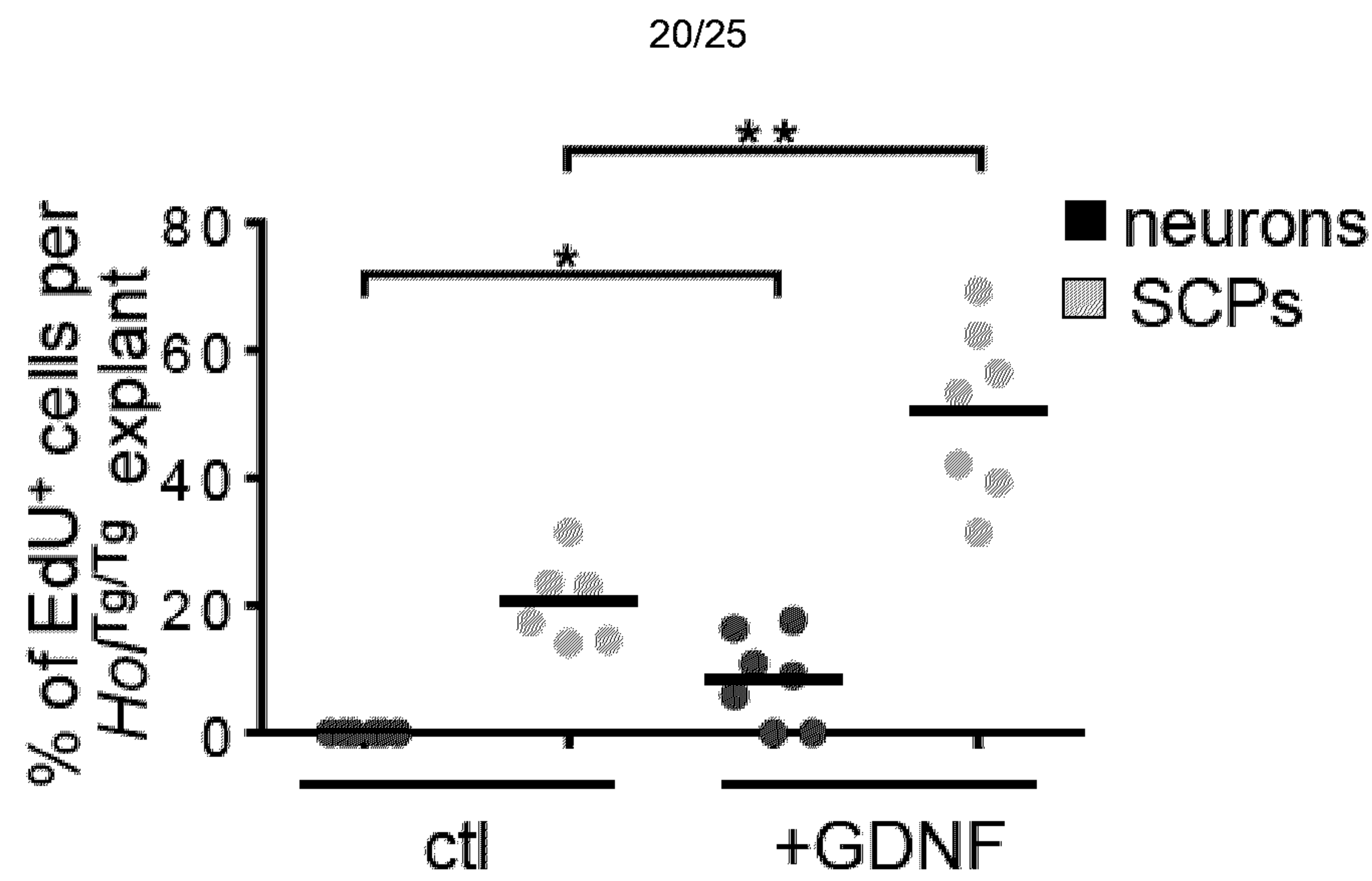


FIG. 14C

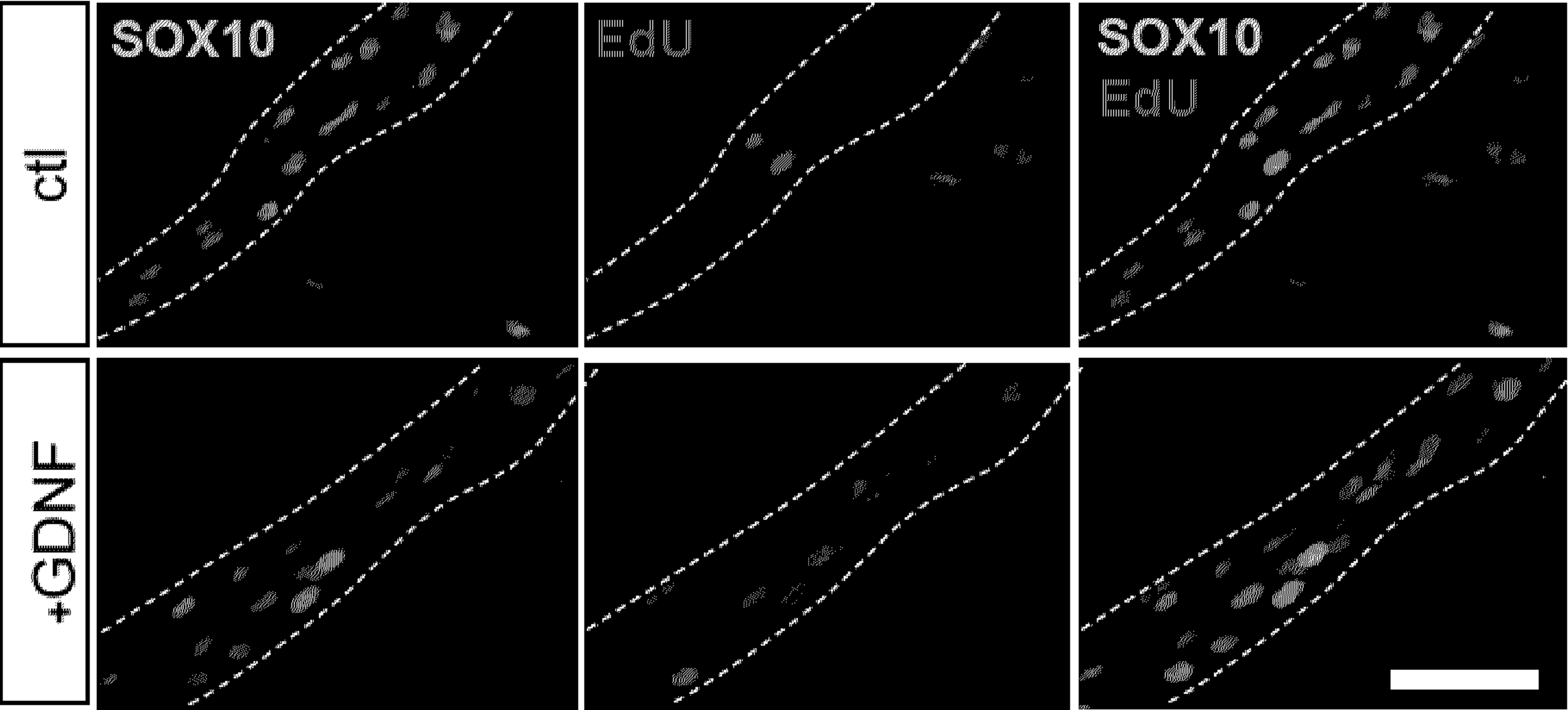


FIG. 14D

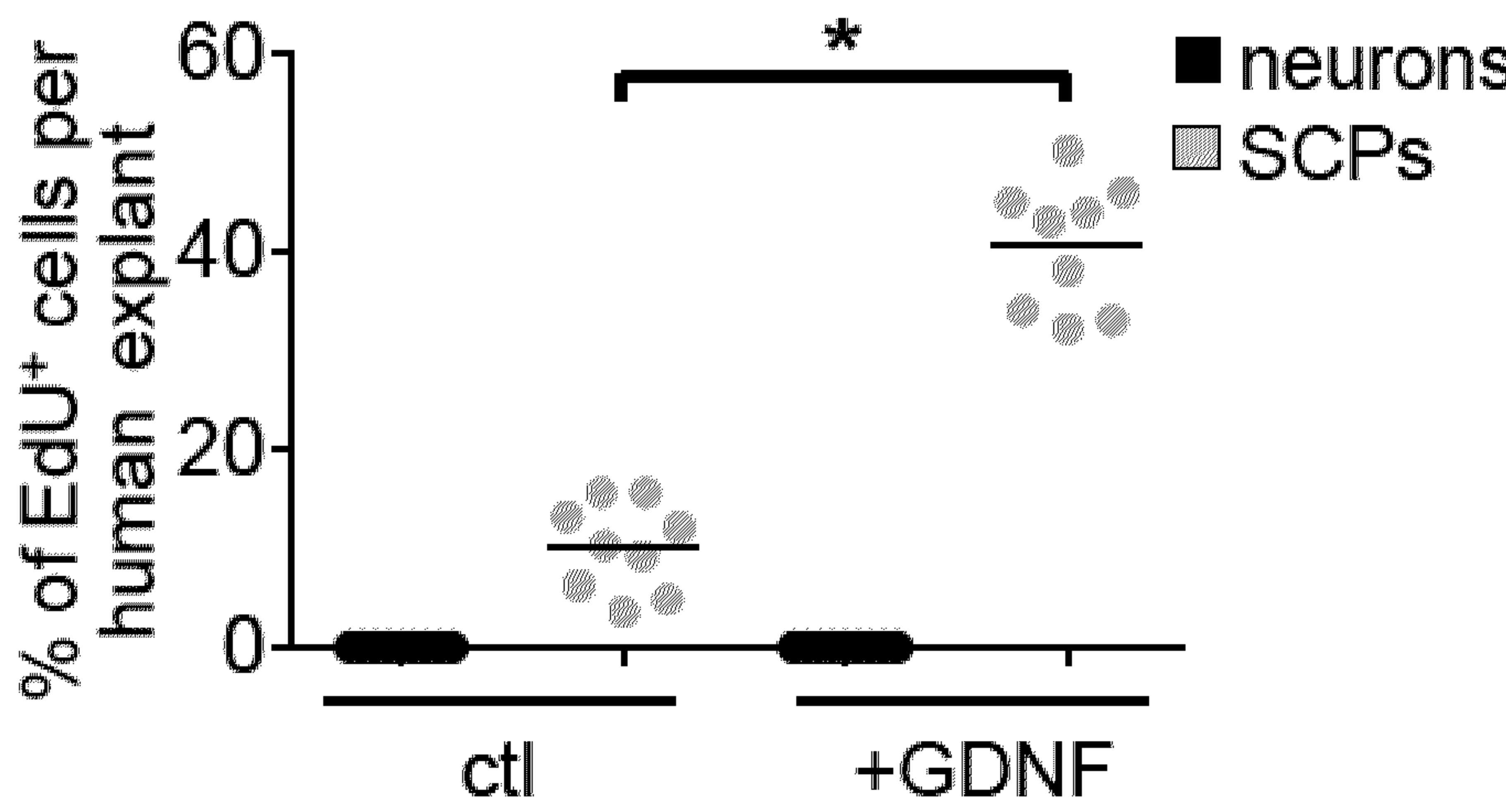


FIG. 14E

21/25

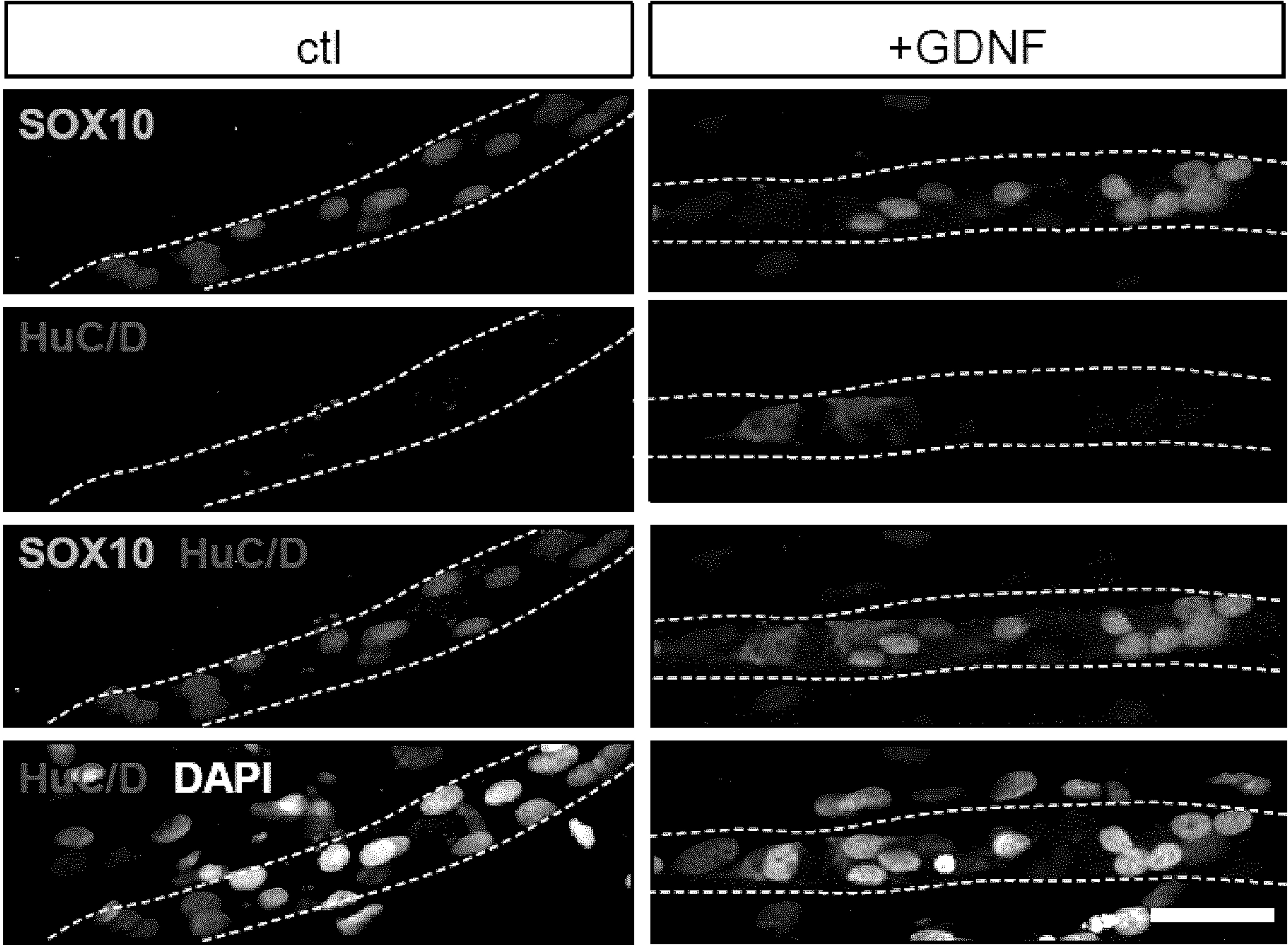


FIG. 14F

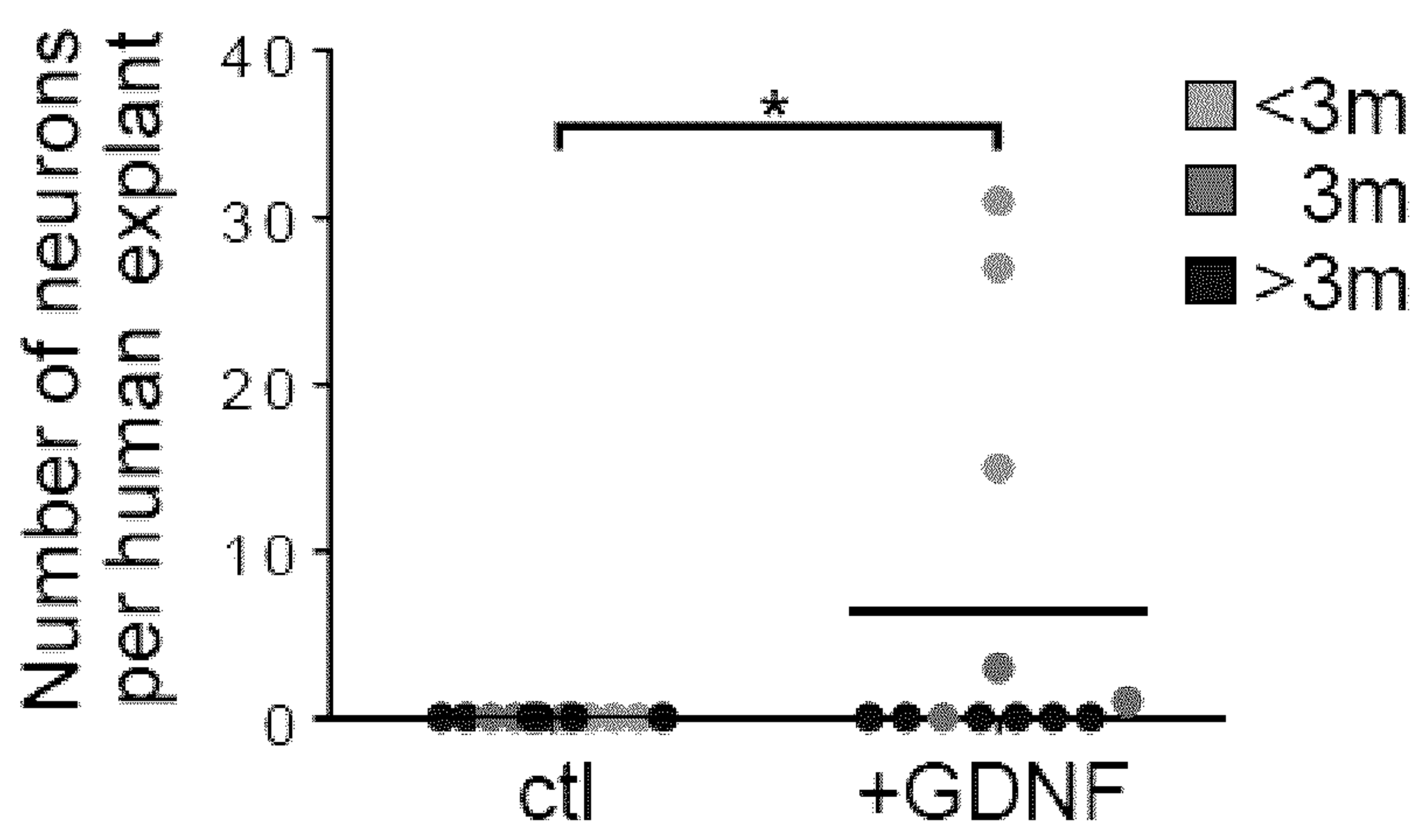


FIG. 14G

22/25

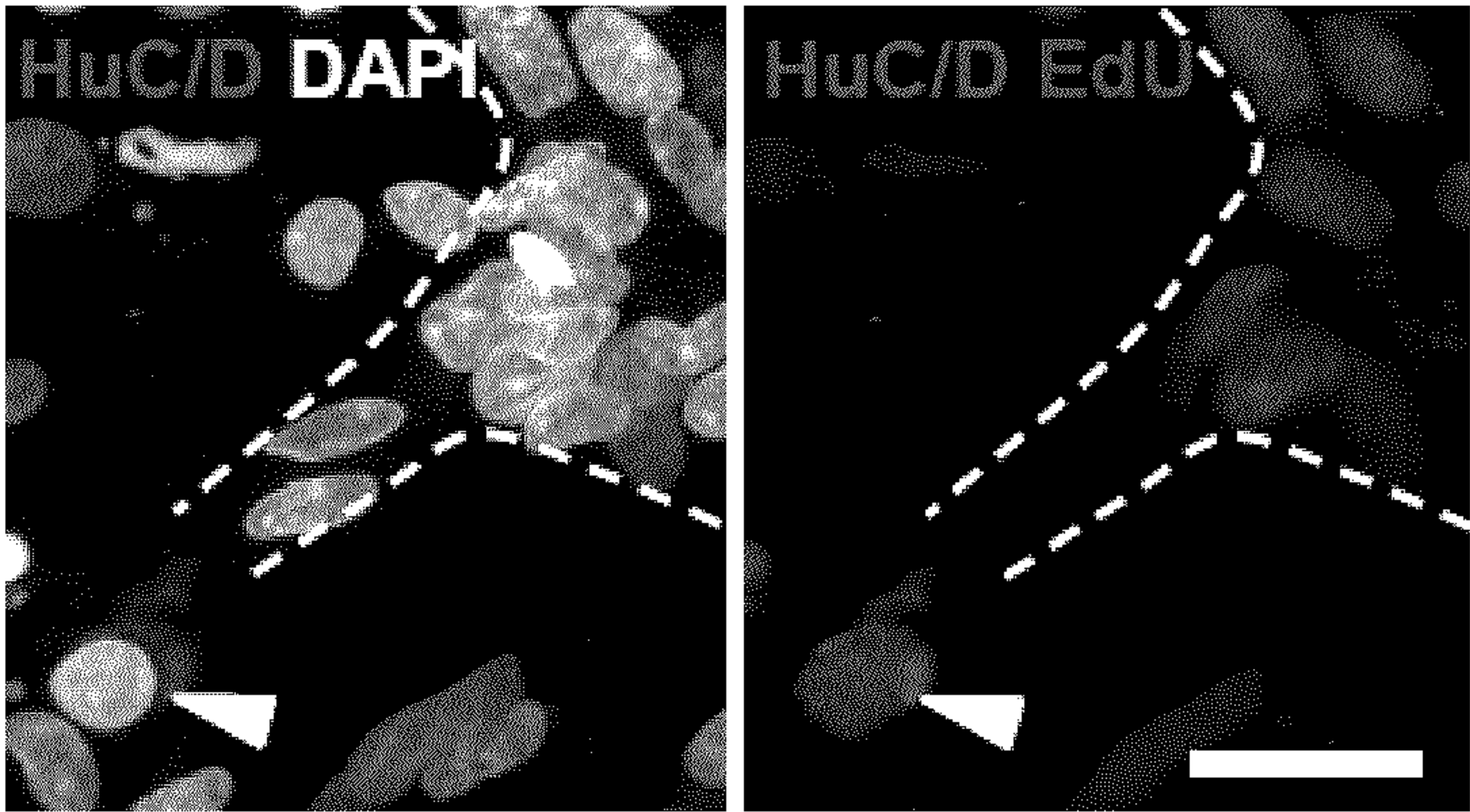


FIG. 14H

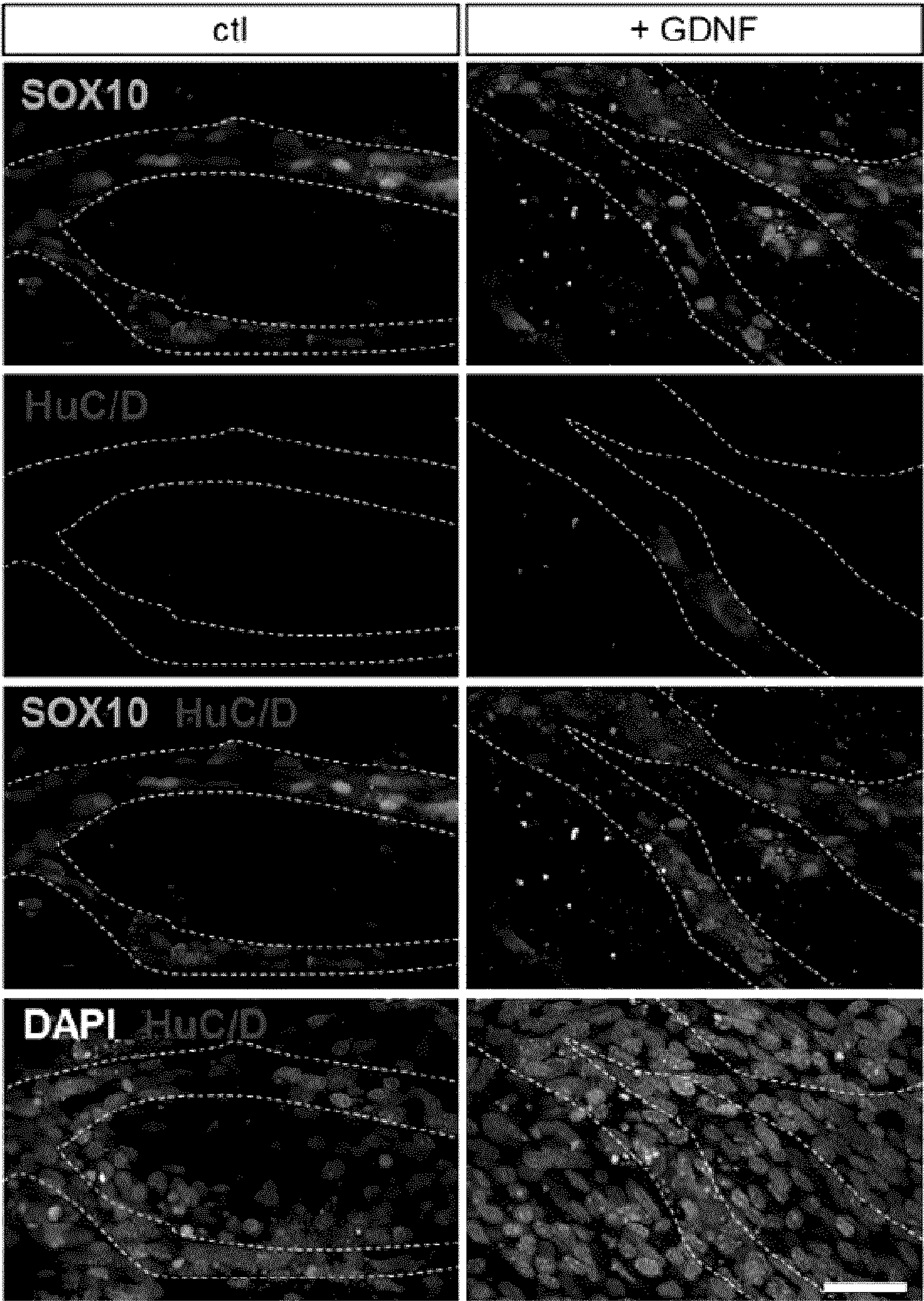


FIG. 15A

23/25

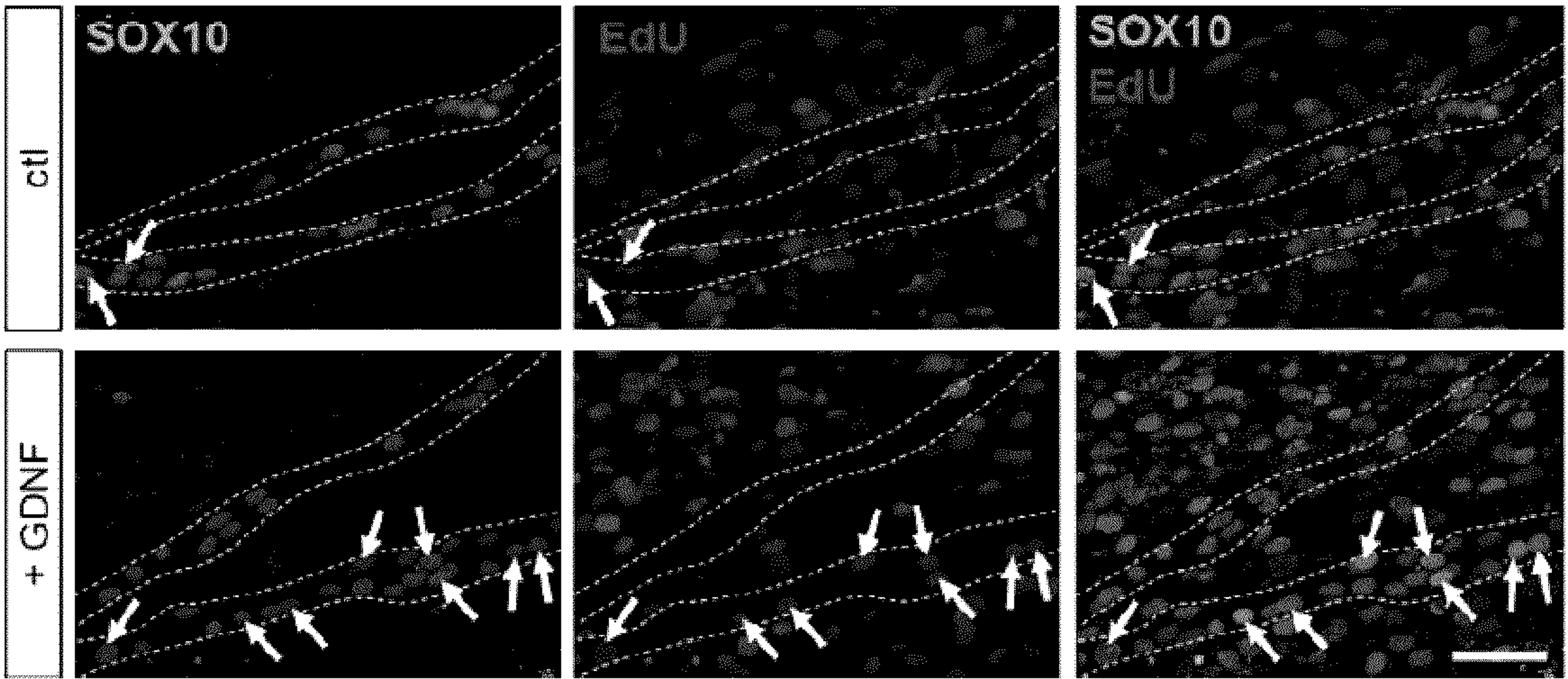


FIG. 15B

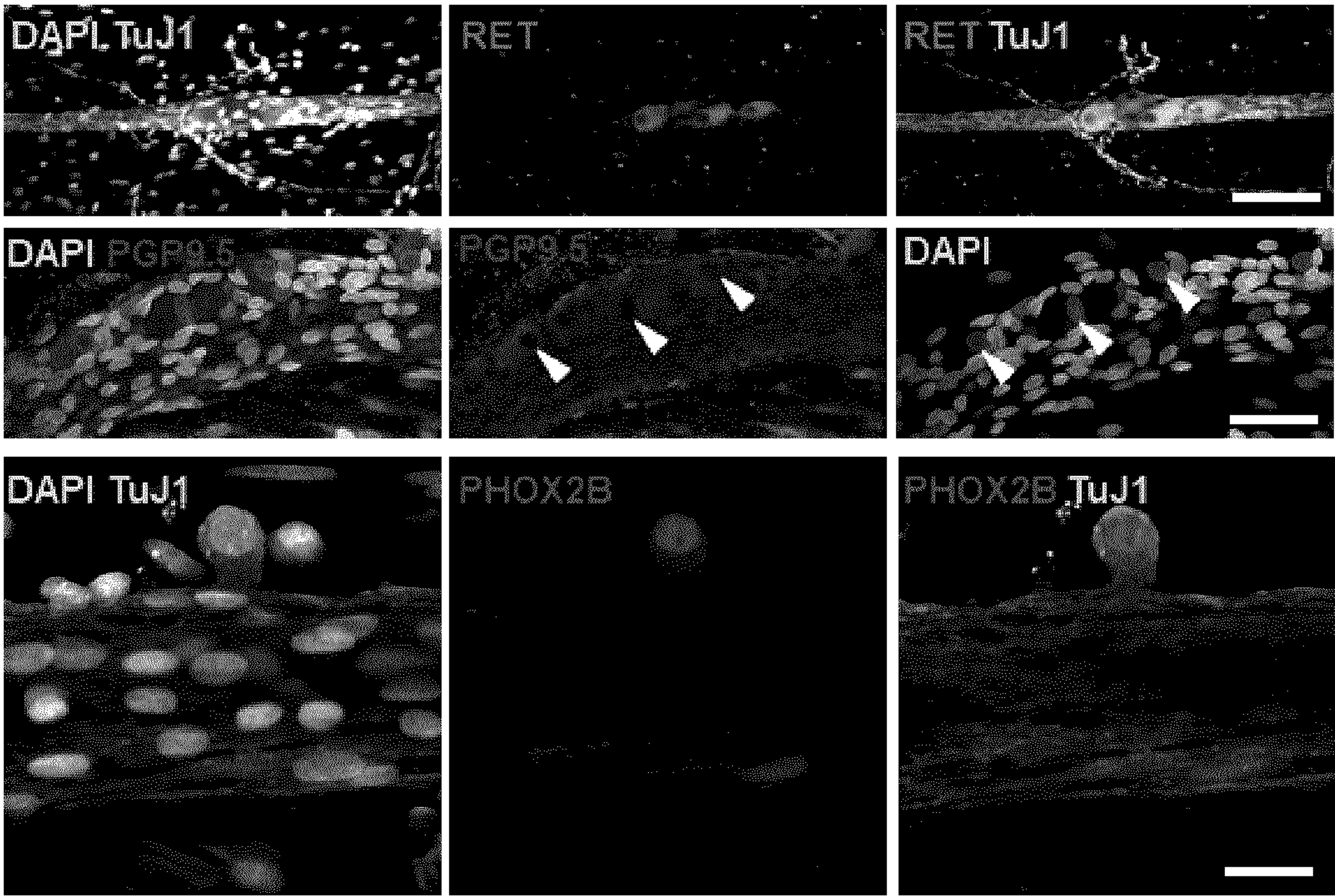


FIG. 16

MKLWDVVAVCLVLLHTASAFPLPAGKRPPEAPAEDRSLGRRRAPFALSSDSNM
PEDYPDQFDDVMDFIQATIKRLKR**SPDKQMAVLPRRERNRQAAAANPENSRGK**
GRRGQRGKNRGCVLTAIHLNVTDLGLGYETKEELIFRYCSGSCDAAETTYDKI
LKNLSRNRRLVSDKVGQACCRPIAFDDDLNFLVYHILRKHS AKRCGCI

FIG. 17A

24/25

```

1  agcctgcgct cctggcgccc tcattgtcttc acgggactcc ccgcgcccgt tgacgtgggtg
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901 aaaaaccggg gttgtgtctt aactgcaata catttaaatg tcactgactt gggtctgggc
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1141 aacctgggtt accatattct aagaaagcat tccgctaaaa ggtgtggatg tatctgactc
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3361 gcagccaaca catgtatgac acactaggcc ctgtattaca gcttgttacg catttcataa

```

FIG. 17B

25/25

3421 aagggatttt cattaaggag ataatctatt actacctacc ttagtggcta ctagtataaa
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3541 agaagcagca gttaccagac atctgatccc cttcccccaa aatacccaaa ttcttcatgg
3601 ttctgccctt ctctgtcctt tctgctcccc ttgctcgcct gggaaatgga ggaaaggcct
3661 tccctctcac actgtcttgg gatcttgctg agaattcaga ctgctcgaaa cagtgacaaa
3721 ccccagccat ccagtcattc gtggagcaca atttggatgt ggcccaggg gcactgttcc
3781 cattcagaga accttggcag tgcgatggcc actgttccca ggcttcaacc tcagtgaccc
3841 cccccaacaa ctccccatgg agagtccctg cccaaaaaag ctgtaggatc caaggggtgt
3901 caatagctcg ttcccggcat cacctacaca ccacaagcag gttttaatgg aagcaagttg
3961 ctccaccaaaa tccacaaaag ggtaaagttt gtgatttttc tttatcattg cgatcaccat
4021 ctgataccgt aaggagtgc cttgtttgga agttctgact tctctgatct gtcttggtcg
4081 tttgtgttat aaaaccaaag ttctctacag actttatattt tgtacaatat cattttgtaa
4141 cttttttacaa ataaaaactc atttctattg c

FIG. 17C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2020/051746

A. CLASSIFICATION OF SUBJECT MATTER
IPC: **A61K 38/17** (2006.01) , **A61K 9/10** (2006.01) , **A61P 25/02** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Key words searched across all IPC. (GDNF) AND ((colon) OR (aganglionic) OR (enteric neuropathy) OR (Hirschprung disease) OR (HSCR))

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Questel-Orbit, PubMed, GooglePatent: (GDNF) AND ((colon) OR (aganglionic) OR (enteric neuropathy) OR (Hirschprung disease) OR (HSCR))
Genomequest: SEQ ID NO:1 aa 78-211

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	SORET, R et al., " <i>Glial Cell-Derived Neurotrophic Factor Induces Enteric Neurogenesis and Improves Colon Structure and Function in Mouse Models of Hirschsprung Disease</i> ". Gastroenterology, November 2020 (11-2020), Vol. 159(5), pp. 1824-1838, ISSN 0016-5085 (whole document)	1-90
Y	BONDURAND, N and SOUTHARD-SMITH, E.M. " <i>Mouse models of Hirschsprung disease and other developmental disorders of the enteric nervous system: Old and new players</i> ". Developmental Biology, 15 September 2016 (15-092016), Vol. 417, pp. 139–157, ISSN 0012-1606 (whole document, particularly Table 1 and Page 140, column 2)	1-90
Y	MCKEOWN, S.J. et al., " <i>Exposure to GDNF Enhances the Ability of Enteric Neural Progenitors to Generate an Enteric Nervous System</i> ". Stem Cell Reports, 14 February 2017 (14-02-2017), Vol. 8, pp. 476-488, ISSN 2213-6711 (whole document, particularly p 485, column 1)	1-90

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* "A" "D" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance document cited by the applicant in the international application earlier application or patent but published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search
15 March 2021 (15-03-2021)

Date of mailing of the international search report
24 March 2021 (24-03-2021)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 819-953-2476

Authorized officer

Marcin Kaminski (819) 639-7692

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2020/051746

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97/11964 A1 (HU, S.F.) 3 April 1997 (03-04-1997) (particularly SEQ ID NO:1)	1-90
Y	WO 2012/141936 A1 (LEUNG, D.D.M. et al) 18 October 2012 (18-10-2012) (particularly SEQ ID NO:3)	1-90
X	TOMAC, A. et AL, " <i>Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo</i> ". Nature, 26 January 1995 (26-01-1995), Vol. 373(6512), pp. 335-339, ISSN 0028-0836 (whole document)	31-60

Box No. I	Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)
1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing: a. ☒ forming part of the international application as filed: ☒ in the form of an Annex C/ST.25 text file. ☐ on paper or in the form of an image file. b. ☐ furnished together with the international application under PCT Rule 13^{ter}.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file. c. ☐ furnished subsequent to the international filing date for the purposes of international search only: ☐ in the form of an Annex C/ST.25 text file (Rule 13^{ter}.1(a)). ☐ on paper or in the form of an image file (Rule 13^{ter}.1(b) and Administrative Instructions, Section 713). 2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished. 3. Additional comments:	

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2020/051746

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2012141936A1	18 October 2012 (18-10-2012)	AU2012243178A1	05 September 2013 (05-09-2013)
		AU2012243178B2	29 September 2016 (29-09-2016)
		BR112013026004A2	29 November 2016 (29-11-2016)
		BR112013026004B1	29 September 2020 (29-09-2020)
		CA2833158A1	18 October 2012 (18-10-2012)
		CA2833158C	17 July 2018 (17-07-2018)
		CN103635201A	12 March 2014 (12-03-2014)
		CN103635201B	09 November 2016 (09-11-2016)
		CY1119750T1	27 June 2018 (27-06-2018)
		DK2696889T3	22 January 2018 (22-01-2018)
		EA201370204A1	28 February 2014 (28-02-2014)
		EA025129B1	30 November 2016 (30-11-2016)
		EP2696889A1	19 February 2014 (19-02-2014)
		EP2696889B1	15 November 2017 (15-11-2017)
		ES2656020T3	22 February 2018 (22-02-2018)
		HRP20171993T1	09 February 2018 (09-02-2018)
		HUE036239T2	28 June 2018 (28-06-2018)
		IL228102D0	30 September 2013 (30-09-2013)
		IL228102A	30 April 2017 (30-04-2017)
		JP2014512369A	22 May 2014 (22-05-2014)
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