Effect of a neural relay on liver regeneration in mice: activation of serotonin release from the gastrointestinal tract

Ryosuke Inoue[1], Kenya Kamimura[1*], Takuro Nagoya[1], Norihiro Sakai[1], Takeshi Yokoo[1], Ryo Goto[1], Kohei Ogawa[1], Yoko Shinagawa-Kobayashi[1], Yukari Watanabe-Mori[1], Akira Sakamaki[1], Satoshi Abe[1], Hiroteru Kamimura[1], Norio Miyamura[2], Hiroshi Nishina[2], and Shuji Terai[1]

[1]Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Niigata, Japan

[2]Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan.

***Correspondence should be addressed to: Kenya Kamimura and Shuji Terai,**

Division of Gastroenterology and Hepatology

Graduate School of Medical and Dental Sciences

Niigata University

1–757 Asahimachi–dori

Chuo–ku, Niigata, Niigata, 9518510

Tel: +81 (25) 227–2207

Fax: +81 (25) 227–0776

E–mail: kenya–k@med.niigata–u.ac.jp

Short running title: **Neural Relay and Liver Regeneration**

Word count: 3,382 words

Abstract

The development of therapeutic options to promote hepatic regeneration following severe liver injury is essential. While humoral factors have been reported as mechanisms of liver regeneration, the contributions of inter-organ communication to liver regeneration have not been reported. In this study, we examined the effect of a neural relay on liver regeneration *via* activation of serotonin release from the gastrointestinal tract. Our results demonstrated that the afferent visceral nerve from the liver activates the efferent vagus nerve from the brain, leading to activation of serotonin release from the gastrointestinal tract and contributing to liver regeneration. While it is difficult to apply these results directly to human health, we believe that this study may represent a step towards developing essential therapeutics to promote liver regeneration.

Keywords: neural relay, liver regeneration, gastrointestinal tract, hormone, serotonin

List of Abbreviations: GI, gastrointestinal; TPH1, tryptophan hydroxylase 1; BrdU, 5-Bromo-2'-deoxyuridine; PH, partial hepatectomy; Cap, capsaicin; HV, hepatic vagotomy; PCNA, proliferating cell nuclear antigen; N.S., not significant; HGF, hepatocyte growth factor; IL-6, interleukin-6

Introduction

The development of therapeutic options to promote hepatic regeneration following severe liver injury is important. It has been reported that in addition to humoral factors [1-15], the autonomic nervous system is also involved in hepatic regeneration [16-18]. Many studies have examined the direct feedback between the brain and liver, starting from the liver via the afferent sympathetic nervous system to the brain, and then to the liver via the hepatic branch of the efferent vagus nerve [16, 17]. Kiba et al. reported that efferent vagal nerve in subdiaphragmatic vagus nerve, but not hepatic vagal nerve containing more than 90% of afferent nerve from the liver, is involved in and contributes to hepatic regeneration in a direct feedback system [16, 18]. Interestingly, this phenomenon occurs following destruction of the ventromedial hypothalamus, which is the center of the sympathetic nervous system, and leads to activation of the vagus nerve [19, 20]. These results suggest the importance of vagal nerve activity for hepatic regeneration [16].

On the other hand, Kiba et al. also showed proliferation of pancreatic beta cells, extrapancreatic secretory cells, and epithelial cells in the gastrointestinal (GI) tract were activated in vagus nerve-activated mice [21, 22]. These results suggest that vagus nerve activation after liver injury activates cell proliferation in multiple organs. Furthermore,

it is suggested that various hormones produced in the GI tract cells are also activated via the vagus nerve. Lai et al. showed that the combined administration of glucagon and insulin was effective for hepatic regeneration in rats that had undergone partial hepatectomy [23]. These results suggest that during liver damage, not only direct liver–brain feedback, but also feedback between other organs activated by the vagus nerve may contribute to hepatic regeneration through the organ network from liver to brain and intestine, and to the liver via a neural relay. While the effector in this neural relay has not yet been determined, GI hormones are known to play an important role in hepatic regeneration.

In this study, we focused on serotonin released from chromaffin cells in the intestine, as this is known to be an important factor to encourage proliferation of liver cells [24-28]. Interestingly, it was reported that mice deficient in tryptophan hydroxylase 1 (TPH1), an enzyme that synthesizes serotonin in chromaffin cells in the intestines, exhibit poor regeneration after hepatectomy [26]. However, no reports have clarified the mechanisms by which serotonin secretion is activated and functions after liver injury through a neural relay.

Therefore, this study aimed to examine whether a neural relay plays a pivotal role in liver regeneration after liver injury, and whether serotonin contributes to liver

regeneration after partial hepatectomy via this neural relay. Our study demonstrated that serotonin activation in the GI tract contributes to hepatic regeneration through a neural relay starting from liver, brain, GI tract, and to the liver.

MATERIALS and METHODS

Animals

All animal experiments were approved by and conducted in full compliance with the regulations of the Institutional Animal Care and Use Committee at Niigata University, Niigata, Japan. Male C57BL/6JJcl mice (n = 120, 8 weeks of age, 25–30 g) were purchased from CLEA Japan, Inc. (Meguro-ku, Tokyo, Japan) were housed under standard conditions in temperature of 20-23℃, humidity of 45-55%, in specific pathogen free facilities, and fed a standard diet.

Development of animal models

The mice were divided into four groups (**Fig. 1**): control, sham operated mice; PH, partial hepatectomy was performed; Cap+PH, direct topical application of capsaicin was utilized to deafferentate the afferent visceral nerve; and HV+PH, transection of the hepatic branch of vagal nerve was performed. Partial hepatectomy was performed by

removing two-thirds of normal liver tissue as previously described [29] at 10 weeks of age. Each of the five mice for each group was analyzed at the appropriate time points shown in **Fig. 1**. For PH, briefly, a midline skin incision was made on the mice under general anesthesia using intraperitoneal injection of pentobarbital sodium (Kyoritsu Seiyaku Corporation, Chiyoda-ku, Tokyo, Japan) at a dosage of 40–50 mg/kg. For the selective afferent visceral nerve blockade, direct topical application of capsaicin (Wako Pure Chemical Industries, Osaka, Japan) dissolved in olive oil (50 mg/mL) was utilized to deafferentate the visceral nerve which contains afferent sympathetic fibers from the hepatobiliary system [30]. This method was reported to show no effect on the other nerves, including vagal nerves [31]. For selective afferent vagus nerve blockade, transection of the hepatic branch of vagal nerve, including more than 90% of afferent vagal nerve from the liver [16, 32], was performed.

Histological analysis

Tissue samples for immunohistochemical staining were collected at appropriate time points after the procedures from each group and fixed in 10% formalin upon tissue collection before embedding in paraffin. A total of five different sections (10 μm) were cut from each of the five mice and standard immunohistochemistry was performed

using mouse anti-insulin monoclonal antibody (I2018; Sigma, St Louis, USA), Vecstain Elite ABC mouse IgG kit (PK-6102; Vector Laboratories, Burlingame, USA), and DAB chromogen tablets (Muto Pure Chemicals, Tokyo, Japan) for pancreatic beta cells. Images were captured from each tissue section randomly and number and size of the islet was measured by ImageJ software (version 1.6.0_20, National Institutes of Health). For hepatocytes, anti-PCNA antibody (2586; Cell Signaling Technology Japan, Tokyo, Japan), Vecstain Elite ABC mouse IgG kit (PK-6102; Vector Laboratories, Burlingame, USA), and DAB chromogen tablets (Muto Pure Chemicals, Tokyo, Japan) were used. For small intestinal tissues, anti-serotonin monoclonal antibody (M0758; DAKO, Santa Clara, USA), Vecstain Elite ABC mouse IgG kit (PK-6102; Vector Laboratories, Burlingame, USA), and DAB chromogen tablets (Muto Pure Chemicals, Tokyo, Japan) were used. For visceral nerves, anti-CGRP monoclonal antibody (4901; AbCam, Cambridge, UK), Vecstain Elite ABC rabbit IgG kit (PK-6101; Vector Laboratories, Burlingame, USA), and DAB chromogen tablets (Muto Pure Chemicals, Tokyo, Japan) were used. Images were captured from each tissue section randomly and aquantitative analysis was performed using ImageJ software (version 1.6.0_20, National Institutes of Health) [33].

BrdU in situ detection

Mice were injected intraperitoneally with 1 mg of BrdU (550891; BD Biosciences, Franklin Lakes, USA), 24 h before liver collection. Livers from five mice in each group were then collected and fixed in 10% formalin before embedding in paraffin. The labeled cells were immunostained with anti-BrdU antibody and BrdU In Situ Detection Kit (551321; BD Biosciences, Franklin Lakes, USA) was used for detection. BrdU-positive cells in the liver were counted in each section and images were captured from each tissue section randomly and a quantitative analysis was performed using ImageJ software (version 1.6.0_20, National Institutes of Health, Bethesda, USA) [33].

Serum cytokines

Blood samples were collected at appropriate time points and serum was used to analyze levels of IL-6 and HGF by enzyme-linked immunosorbent assay using a mouse IL-6 ELISA Kit (KMC0061; Thermo Fisher Scientific, Waltham, USA) and Quantikibe HGF ELISA Kit (MHG00; R&D Systems, Minneapolis, USA).

Statistics

The data of histological analyses and liver body weight were statistically evaluated by two-way factor repeated-measures analysis of variance followed by Bonferroni's multiple comparison test by Graphpad Prism7 software (version 7.03, MDF, Tokyo, Japan).

Results

Development of animal models

Details of the animal groups are shown in **Fig. 1**. Partial hepatectomy (PH) was performed as previously described [29]. For selective afferent visceral nerve blockade, direct topical application of capsaicin (Cap) was utilized to deafferentate the afferent visceral nerve (Cap+PH) [30], and for selective afferent vagus nerve blockade, transection of the hepatic branch of vagal nerve (hepatic vagotomy, HV), which includes more than 90% of afferent nerve from the liver, was performed [16, 32] (HV+PH).

Activation of neural relay following partial hepatectomy

To confirm the activation of the neural relay after PH, the increase of beta cells in the pancreas and its inhibition in the Cap+PH group were examined. The number of islets

was recorded and analyzed for each size category (small, <10,000 μm²; medium, 10,000–30,000 μm²; large, >30,000 μm²) 0, 1, and 3 days after partial hepatectomy in each type of mouse using ImageJ software (**Fig. 2a**).

Three days after partial hepatectomy (PH), modifications to islet size showed a significant increase in the proportion of small-sized islets (1 day after; 58.0±3.0% vs 67.0±2.4%, $p < 0.05$, 3 days after; 58.1±4.4% vs 71.7±3.6%, $p < 0.001$), while medium-sized islets showed a mild decrease (1 day after; 27.0±3.7% vs 19.6±1.4%, N.S., 3 days after; 27.6±2.8% vs 15.7±2.5%, N.S.), although this was not significant. Large-sized islets showed no significant difference between the two groups (1 day after, 14.9 ± 1.2% vs 13.3±3.1%, N.S.; 3 days after, 13.4±2.6% vs 12.3±1.1%, N.S.) (**Fig. 2b, 2c**). Time-dependent changes of small-sized islets in the control, PH, Cap+PH, and HV+PH groups showed a significant increase in the PH group and inhibition by visceral nerve blockade in the Cap+PH group, with relatively weaker inhibition in the HV+PH group (**Fig. 2d**).

These results provide evidence that the efferent vagus nerve was activated after partial hepatectomy and that the afferent visceral nerve is important to send a signal to the central nervous system.

Effect of neural relay on DNA synthesis in hepatocytes after partial hepatectomy

To examine the effects of the neural relay on liver regeneration, DNA synthesis in hepatocytes was analyzed by immunostaining with anti-BrdU antibody (**Fig. 3a, 3b**). Quantitative analysis showed that 37.3±4.7% and 22.3±2.5% of hepatocytes were positively stained for BrdU in a high power field 2 and 3 days after partial hepatectomy, respectively, which was significantly higher than that of the control group (2.7±0.6%, $p < 0.001$, 1.3±0.6%, $p < 0.001$). To examine the role of the afferent neural relay, the same set of analyses were performed in the Cap+PH and HV+PH groups. A significant inhibition in the increase of BrdU positive cells was observed in the Cap+PH group 2 and 3 days after the procedure (9.3±2.1% and 6.7±1.5%, respectively, both N.S. compared with the control), while a similar level of increase in BrdU-positive cells was seen in the HV+PH group compared with the PH group 2 and 3 days after the procedure (31.7±3.5% and 21.0 ± 2.0%, respectively, both $p < 0.001$ compared with control) (**Fig. 3b**). No differences were seen between the groups later than 5 days after the procedure. These results suggest that the neural relay is significantly related to DNA synthesis after partial hepatectomy and that the afferent visceral nerve contributed to relay the signal. The hepatic vagal nerve showed a lower effect on DNA synthesis in hepatocytes.

Effect of neural relay on hepatocyte proliferation after partial hepatectomy

To examine whether the neural relay contributes to hepatocyte proliferation after partial hepatectomy, the number of proliferating cell nuclear antigen (PCNA)-positive cells was assessed by staining. Quantitative analysis showed that 14.3±3.1%, 44.3±7.6%, and 38.3±3.8% of hepatocytes were positively stained for PCNA in a high power field 1, 2, and 3 days after partial hepatectomy, respectively (**Fig. 4a, b**), which was significantly higher than that of the control group (6.3±1.5%, $p < 0.001$; 5.0±3.5%, $p < 0.001$; and 7.3±1.5%, $p < 0.001$, respectively). To examine the role of the afferent neural relay, the same set of analyses was performed in the Cap+PH and HV+PH groups. A significant inhibition in the increase of PCNA-positive cells after partial hepatectomy was observed in the Cap+PH group 2 and 3 days after the procedure (25.3±3.2% and 25.7±6.8%, respectively), although this was higher than that of the control group. The HV+PH group showed a similar level of activation of hepatocyte proliferation compared with the control group 2 and 3 days after the procedure (42.7±5.7%, $p < 0.01$ and 36.7±3.1%, $p < 0.01$ compared with the control, respectively) (**Fig. 4b**). No significant differences between the groups were seen later than 5 days after the procedure. These results suggest that the neural relay is significantly related to hepatocyte proliferation after partial hepatectomy and that the afferent visceral nerve contributes to relay the signal. The hepatic vagal nerve showed a lower effect.

Effect of neural relay on liver volume recovery after partial hepatectomy

To examine the effect of the neural relay on the recovery of liver volume, time-dependent liver weight after partial hepatectomy was assessed. As expected, the PH group showed recovery of liver weight-to-body weight ratio 3 days (4.2±0.2%) and 5 days (4.8±0.4%) after partial hepatectomy to the same level as that of the control group (N.S.). The HV+PH group showed similar results 3 days (4.7±0.1%) and 5 days (4.7±0.03%) after partial hepatectomy, while the Cap+PH group showed a significant delay in recovery 3 days (3.1±0.2%, $p < 0.001$) and 5 days (3.8±0.1%, $p < 0.001$) after partial hepatectomy (**Fig. 5**). The Cap+PH group showed a relatively slower recovery even after 5 days and humoral growth factors such as hepatocyte growth factor (HGF) remained significantly higher in this group compared with the other three groups 7 days after the procedure (**Fig. S1**).

These results suggest that the neural relay is significantly associated with recovery of liver volume after partial hepatectomy via the afferent visceral nerve and not via the hepatic vagus nerve plexus.

Activation of serotonin release from intestinal cells following partial hepatectomy

To determine the factors contributing to liver regeneration after partial hepatectomy in this neural relay, various GI hormones activated by the efferent vagal nerve were tested. Activation of serotonin expression in the enterochromaffin cells in the small intestine was monitored in a time-dependent manner after PH in the groups.

Immunohistochemical staining was performed using an anti-serotonin antibody and the amount of serotonin in the enterochromaffin cells was assessed (**Fig. 6a, 6b**). Quantitative analysis showed 6.1±0.3%, 6.1±0.2%, and 5.6±0.2% of small intestinal mucosal epithelium cells stained positively for serotonin 1, 2, and 3 days after partial hepatectomy, which was statistically higher than that of the control group (3.9±0.6%, $p < 0.001$; 4.2±0.3%, $p < 0.001$; 4.2±0.5%, $p < 0.01$) (**Fig. 6b**). These results suggest that serotonin was induced by partial hepatectomy.

To examine the role of the afferent neural relay, the same analyses were performed in the Cap+PH and HV+PH groups. In the visceral nerve block group (Cap+PH), an increase in serotonin level in the small intestine was not seen compared with the control group (1 day after, 4.7±0.3%; 2 days after, 4.8±0.3%; 3 days after, 4.6±0.3%), indicating suppression of serotonin in the enterochromaffin cells after partial hepatectomy when the afferent visceral nerve was blocked. In addition, vagus nerve

block (HV+PH) showed an increase in serotonin levels to the same level as the PH group (1 day after, 5.3±0.6%; 2 days after, 5.5±0.1%; 3 days after, 5.5±0.5%) indicating no significant association of the afferent vagal nerve in the neural relay from the liver towards the central nervous system to stimulate serotonin (**Fig. 6b**). These results suggest that partial hepatectomy induced activation of the neural relay through afferent visceral nerves through to the efferent vagus nerve which contributes to increased serotonin expression in the enterochromaffin cells. This leads to an increase in DNA synthesis in the hepatocytes, hepatocyte proliferation, and recovery of liver volume.

Discussion

Development of therapeutic options to promote hepatic regeneration following severe liver injury is needed. To date, many studies on the mechanisms of hepatic regeneration have investigated various growth factors [5-14], cytokines [1-4, 15], signal transducers [34, 35], GI hormones [23-28], and direct feedback of autonomic nerves [16-18], which are considered to play an important mechanistic role in homeostasis in various organs. These nerves are distributed in various internal organs including blood vessels, heart, lungs, GI tract, liver, and reproductive organs, and are controlled by a feedback system situated predominantly in the brain, playing important organ-related roles as a network for maintaining homeostasis [36].

The importance of the efferent vagus nerve in hepatic regeneration was reported by Kiba et al. They created a mouse model of vagus nerve activation by destroying the center of the sympathetic nervous system with no direct liver damage [19], and showed the same level of hepatocyte proliferation to that seen after partial hepatectomy [16-18]. In addition, vagus nerve activation following partial hepatectomy increased pancreatic beta cells [37-39]. We also confirmed the same effect in our study by activating the vagus nerve using gene transfer and sympathetic nerve destruction [21, 31, 40] (**Fig. 1**). These results suggest that the neural signal relay from the liver to the efferent vagal nerve is important for hepatocyte proliferation and maintenance of blood glucose level following liver injury. However, no study has focused on the network of these organs through the neural relay, the afferent signal from the injured liver, or the effector for liver regeneration in this neural relay.

On the other hand, inter-organ communication functions in a coordinated manner as a biological mechanism to maintain homeostasis in living organisms and is very important in various pathologies. For example, studies into the relationship between the GI tract and the brain have shown intestinal bacterial flora to be strongly involved in Parkinson's disease [41] and autism [42].

Therefore, our study aimed to investigate whether a neural relay contributes to liver regeneration depending on communication with other organs, such as the GI tract. To confirm the contribution of a neural relay, we utilized a neural blockade procedure (**Fig. 1**), and to examine the association of the GI tract, efferent vagal nerve activation was confirmed upon liver injury (**Fig. 2**) followed by analysis of serotonin levels and liver regeneration. Serotonin is a monoamine neurotransmitter that is mostly found in chromaffin cells in the intestine [24, 25]. Release of serotonin from the GI tract increases when the parasympathetic nervous system is activated [25]. Serotonin is contained in platelet granules and is released when platelets come into contact with liver cells. It mediates 5–HT_2 receptors in the liver to function as a growth factor for liver cells [26-28]. Lesurtel et al. analyzed mice lacking TPH1, which is the rate-limiting enzyme for the synthesis of peripheral serotonin, and reported that platelet-derived serotonin mediates liver regeneration after hepatectomy. In addition, they found that failure to regenerate was rescued by reloading serotonin-free platelets with a serotonin precursor molecule [26]. Similar results have been reported by others: Murata, et al. and Papadimas, et al. found the effects of serotonin on DNA synthesis were arrested by 5-HT2 receptor blockade at the G_1/S transition [13, 43]; Balasubramanian, et al. reported induction of DNA synthesis in primary cultures of rat hepatocyte by serotonin

[44]; and Nocito, et al. used a normothermic hepatic ischemia model mouse [27]. Matondo, et al. reported that a small amount of serotonin in the liver is sufficient for liver regeneration although the biological homeostasis was disturbed by serotonin transporter depletion [45]. In addition, serotonin protected mouse liver from cholestatic injury by stabilizing the bile salt pool after bile duct ligation through adaptation of renal transporters in cholestasis [46]. Reduced serotonin reuptake transporter function caused insulin resistance and hepatic steatosis independent of food intake [47]. These reports support that serotonin functions to activate the proliferation and growth of the hepatocyte *in vitro* and *in vivo*. However, there have been no reports showing how serotonin release is activated after liver injury.

Our results demonstrated that the afferent visceral nerve, not the afferent vagal nerve, significantly contributes to the activation of the efferent vagal nerve upon liver injury and that the GI tract activates the release of serotonin by this signal relay and contributes to liver regeneration evidenced by BrdU (**Fig. 3**), PCNA (**Fig. 4**), and liver weight-to-body weight ratio (**Fig. 5**). A summary of our studies and the reported effect of autonomic nervous system are shown in **Fig. 7**. Other factors such as serum HGF (**Fig. S1**) and IL-6 (**Fig. S2**) are also involved in liver regeneration and, interestingly,

HGF remained at high levels after partial hepatectomy when the visceral nerve was blocked (**Fig. S1**). This result indicates that the afferent visceral nerve contributes to activation of hepatocytes proliferation through signaling, including serotonin, and HGF compensates for the delay in recovery of cellular growth (**Fig. 6 and Fig. S1**).

Further analyses are necessary to fully elucidate the mechanisms of liver regeneration after liver injury. However, our study demonstrates that the autonomic nervous system plays a pivotal role for liver regeneration to maintain homeostasis and inter-organ communication between the liver and the GI tract upon liver injury (**Fig.7**).

In conclusion, we report that a liver damage-mediated neural relay causes the release of serotonin by the GI tract and this directly promotes hepatic regeneration. While it is difficult to directly apply these results to the human health, we believe that this study represents a step towards developing essential therapeutics to promote liver regeneration.

Acknowledgments

The authors would like to thank Takao Tsuchida in the Division of Gastroenterology and Hepatology at the Niigata University for his excellent assistance in histological analyses. The authors would also like to thank Nobuyoshi Fujisawa, Yoshitaka Maeda, Toshikuni Sasaoka, and all staff members at the Division of Laboratory Animal Resources in Niigata University. They also thank Enago for the English language editing. The research in the authors' laboratories has been supported in part by Grant from brain research institute, Niigata University.

Author Contributions

RI, TN, NS, TY, RG, KO, and YSK performed experiments; KK, YWM, AS, SA, HK, and NM analyzed data; KK, HN, and ST conceived and supervised study; KK and ST wrote the manuscript.

References

1. Iwai M, Cui TX, Kitamura H, et al. Increased secretion of tumour necrosis factor and interleukin 6 from isolated, perfused liver of rats after partial hepatectomy. *Cytokine* **13**: 60-64, 2001.

2. Yang L, Magness ST, Bataller R, et al. NF-kappaB activation in Kupffer cells after partial hepatectomy. *Am J Physiol Gastrointest Liver Physiol* **289**: G530-8, 2005.

3. Nakamura K, Nonaka H, Saito H, et al. Hepatocyte proliferation and tissue remodeling is impaired after liver injury in oncostatin M receptor knockout mice. *Hepatology* **39**: 635-644, 2004.

4. Moh A, Iwamoto Y, Chai GX, et al. Role of STAT3 in liver regeneration: survival, DNA synthesis, inflammatory reaction and liver mass recovery. *Lab Invest* **87**: 1018-1028, 2007.

5. Nakamura T, Nishizawa T, Hagiya M, et al. Molecular cloning and expression of human hepatocyte growth factor. *Nature* **342**: 440-443, 1989.

6. Nakamura T, Sakai K, Nakamura T, et al. Hepatocyte growth factor twenty years on: Much more than a growth factor. *J Gastroenterol Hepatol* **Suppl 1**: 188-202, 2011.

7. Fujiwara K, Nagoshi S, Ohno A, et al. Stimulation of liver growth by exogenous human hepatocyte growth factor in normal and partially hepatectomized rats. *Hepatology* **18**: 1443-1449, 1993.

8. Block GD, Locker J, Bowen WC, et al. Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium. *J Cell Biol* **132**: 1133-1149, 1996.

9. Patijn GA, Lieber A, Schowalter DB, et al. Hepatocyte growth factor induces hepatocyte proliferation in vivo and allows for efficient retroviral-mediated gene transfer in mice. *Hepatology* 28: 707-716, 1998.

10. Alroy I, Yarden Y. The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett* **410**: 83-86, 1997.

11. Linggi B, Carpenter G. ErbB receptors: new insights on mechanisms and biology. *Trends Cell Biol* **16**: 649-656, 2006.

12. Mead JE, Fausto N. Transforming growth factor alpha may be a physiological regulator of liver regeneration by means of an autocrine mechanism. *Proc Natl Acad Sci U S A*. **86**: 1558-1562, 1989.

13. Murata S, Ohkohchi N, Matsuo R, et al. Platelets promote liver regeneration in early period after hepatectomy in mice. *World J Surg*. **31**: 808-816, 2007.

14. Matsuo R, Ohkohchi N, Murata S, et al. Platelets Strongly Induce Hepatocyte Proliferation with IGF-1 and HGF In Vitro. *J Surg Res* **145**: 279-286, 2008.

15. Anders RA, Subudhi SK, Wang J, et al. Contribution of the lymphotoxin beta receptor to liver regeneration. *J Immunol* **175**: 1295-1300, 2005.

16. Kiba T. The role of the autonomic nervous system in liver regeneration and apoptosis--recent developments. *Digestion* **66**: 79-88, 2002.

17. Hendrickse MT, Thuluvath PJ, Triger DR. Natural history of autonomic neuropathy in chronic liver disease. *Lancet* **339**: 1462-1464, 1992.

18. Kiba T, Tanaka K, Numata K, et al. Facilitation of liver regeneration after partial hepatectomy by ventromedial hypothalamic lesions in rats. *Pflugers Arch* **428**: 26-29, 1994.

19. Kiba T, Tanaka K, Endo O, et al. Role of vagus nerve in increased DNA synthesis after hypothalamic ventromedial lesions in rat liver. *Am J Physiol* **262**: G483-487, 1992.

20. Kiba T, Tanaka K, Numata K, et al. Hepatocyte proliferation in rats after ventromedial hypothalamic lesions: immunoreactivity patterns of proliferating cell nuclear antigen (PCNA). *J Gastroenterol* **33**: 523-528, 1998.

21. Kiba T, Tanaka K, Numata K, et al. Ventromedial hypothalamic lesion-induced vagal hyperactivity stimulates rat pancreatic cell proliferation. *Gastroenterology* **110**: 885-893, 1996.

22. Kiba T, Tanaka K, Endo O, et al. Ventromedial hypothalamic lesions increase gastrointestinal DNA synthesis through vagus nerve in rats. *Gastroenterology* **104**: 475-484, 1993.

23. Lai HS, Chung YC, Chen WJ, et al. Rat liver regeneration after partial hepatectomy: effects of insulin, glucagon and epidermal growth factor. *J Formos Med Assoc* **91**: 685-690, 1992.

24. Gershon MD. 5-Hydroxytryptamine (serotonin) in the gastrointestinal tract. *Curr Opin Endocrinol Diabetes Obes* **20**: 14-21, 2013.

25. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. *Annu Rev Med* **60**: 355-366, 2009.

26. Lesurtel M, Graf R, Aleil B, et al. Platelet-derived serotonin mediates liver regeneration. *Science* **312**: 104-107, 2006.

27. Nocito A, Georgiev P, Dahm F, et al. Platelets and platelet-derived serotonin promote tissue repair after normothermic hepatic ischemia in mice. *Hepatology* **45**: 369-376, 2007.

28. Ruddell RG, Mann DA, Ramm GA. The function of serotonin within the liver. *J Hepatol* **48**: 666-675, 2008.

29. Higgins GM, Anderson RM. Experimental pathology of liver. I. Restoration of liver of white rat following partial surgical removal. *Arch Pathol* **12**: 186-202, 1931.

30. Berthoud HR. Anatomy and function of sensory hepatic nerves. *Anat Rec A Discov Mol Cell Evol Biol*. **280**: 827-835, 2004.

31. Imai J, Katagiri H, Yamada T, et al. Regulation of pancreatic beta cell mass by neuronal signals from the liver. *Science* **322**: 1250-1254, 2008.

32. Sawchenko PE, Friedman MI. Sensory functions of the liver--a review. *Am J Physiol*. **236**: R5-20, 1979.

33. Vrekoussis T, Chaniotis V, Navrozoglou I, et al. Image analysis of breast cancer immunohistochemistry-stained sections using ImageJ: an RGB-based model. *Anticancer Res* **29**: 4995-4998, 2009.

34. Tan X, Behari J, Cieply B, et al. Conditional deletion of beta-catenin reveals its role in liver growth and regeneration. *Gastroenterology* **131**: 1561-1572, 2006.

35. Sekine S, Gutierrez PJ, Lan BY, et al. Liver-specific loss of beta-catenin results in delayed hepatocyte proliferation after partial hepatectomy. *Hepatology* **45**: 361-368, 2007.

36. Langley JN. Sketch of the progress of discovery in the eighteenth century as regards the autonomic nervous system. *J Physiol* **50**: 225-258, 1916.

37. Moreau F, Seyfritz E, Toti F, et al. Early effects of liver regeneration on endocrine pancreas: in vivo change in islet morphology and in vitro assessment of systemic effects on beta-cell function and viability in the rat model of two-thirds hepatectomy. *Horm Metab Res* **46**: 921-926, 2014.

38. Araujo TG, Oliveira AG, Saad MJ. Partial-hepatectomized (70%) model shows a correlation between hepatocyte growth factor levels and beta-cell mass. *Front Endocrinol (Lausanne)*. **6**: 20, 2015.

39. Araujo TG, Oliveira AG, Carvalho BM, et al. Hepatocyte growth factor plays a key role in insulin resistance-associated compensatory mechanisms. *Endocrinology* **153**: 5760-9, 2012.

40. Imai J, Oka Y, Katagiri H. Identification of a novel mechanism regulating beta-cell mass: neuronal relay from the liver to pancreatic beta-cells. *Islets* **1**: 75-77, 2009.

41. Sampson TR, Debelius JW, Thron T, et al. Gut microbiota regulate motor deficits and neuroinflammation in a model of parkinson's disease. *Cell* **167**: 1469-1480, 2016.

42. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. *N Engl J Med* **375**: 2369-2379, 2016.

43. Papadimas GK, Tzirogiannis KN, Panoutsopoulos GI, et al. Effect of serotonin receptor 2 blockage on liver regeneration after partial hepatectomy in the rat liver. *Liver Int* **26**: 352-361, 2006.

44. Balasubramanian S, Paulose CS. Induction of DNA synthesis in primary cultures of rat hepatocytes by serotonin: possible involvement of serotonin S2 receptor. *Hepatology* **27**: 62-66, 1998.

45. Matondo RB, Punt C, Homberg J, et al. Deletion of the serotonin transporter in rats disturbs serotonin homeostasis without impairing liver regeneration. *Am J Physiol Gastrointest Liver Physiol* **296**: G963-968, 2009.

46. Jang JH, Rickenbacher A, Humar B, et al. Serotonin protects mouse liver from cholestatic injury by decreasing bile salt pool after bile duct ligation. *Hepatology* **56**: 209-218, 2012.

47. Chen X, Margolis KJ, Gershon MD, et al. Reduced serotonin reuptake transporter (SERT) function causes insulin resistance and hepatic steatosis independent of food intake. *PLoS One* **7**: e32511, 2012.

Figure Legends

Figure 1. Animal models and protocol

(a) Four groups of animal model were established: control group (Cnt), peritoneum incision was performed as a sham operation; partial hepatectomy group (PH), partial hepatectomy was performed; partial hepatectomy following visceral nerve block group (Cap+PH); and partial hepatectomy following vagus nerve block group (HV+PH). **(b)** For each group, tissues were collected and expression of serotonin in the small intestine, BrdU uptake, PCNA expression in the liver, and liver weight-to-body weight ratio were assessed at appropriate time points. **(c)** Visceral nerve block was performed by applying capsaicin (Cap) to the sympathetic nervous plexus surrounding the celiac artery. Blockade was confirmed by immunostaining of the nerves with anti-CGRP antibody.

Figure 2. Vagal nerve activation after partial hepatectomy

(a) Representative images from a section of pancreatic islet immunohistochemically stained with anti-insulin antibody 3 days after partial hepatectomy. The number of pancreatic islets was classified with the area and was recorded for each size category (<10,000 μm², 10,000–30,000 μm², and >30,000 μm²) 0, 1, and 3 days after partial hepatectomy in the Cnt **(b)** and PH **(c)** groups. **(d)** Time-dependent change of the

number of small islets in the pancreas in Cnt, PH, Cap+PH, and HV+PH groups. Five different pancreatic sections from each of the five mice in all groups were immunohistochemically stained with anti-insulin antibody and a quantitative analysis was performed using ImageJ software. The values represent mean ± SD (n = 25 for each group). * $p < 0.05$, *** $p < 0.001$, and N.S., no statistical significance compared with Cnt group. Two-way factor repeated-measures analysis of variance followed by Bonferroni's multiple comparison test.

Figure 3. Effect of neural relay on DNA synthesis in hepatocytes after partial hepatectomy

(a) Representative images of BrdU-positive cells in the liver. **(b)** Time-dependent change of the number of BrdU positively stained cells in Cnt, PH, Cap+PH, and HV+PH groups. Five different sections from each of the five mice in all groups were immunohistochemically stained with anti-BrdU antibody and a quantitative analysis was performed using ImageJ software. The values represent mean ± SD (n = 25 for each group). ** $p < 0.01$, *** $p < 0.001$, and N.S., no statistical significance compared with Cnt group. Two-way factor repeated-measures analysis of variance followed by Bonferroni's multiple comparison test.

Figure 4. Effect of neural relay on hepatocyte proliferation after partial hepatectomy

(a) Representative images of PCNA-positive cells in the liver. **(b)** Time-dependent change of the number of PCNA positively stained cells in Cnt, PH, Cap+PH, and HV+PH groups. Five different sections from each of the five mice in all groups were immunohistochemically stained with anti-BrdU antibody and a quantitative analysis was performed using ImageJ software. The values represent mean ± SD (n = 25 for each group). ** $p < 0.01$, *** $p < 0.001$, and N.S., no statistical significance compared with Cnt group. Two-way factor repeated-measures analysis of variance followed by Bonferroni's multiple comparison test.

Figure 5. Effect of neural relay on liver weight/body weight ratio after partial hepatectomy

Time-dependent changes of liver weight-to-body weight ratio after partial hepatectomy in Cnt, PH, Cap+PH, and HV+PH groups. Liver weight (LW) and body weight (BW) ratio were measured in each of the five mice from the four groups at appropriate time points. The values represent mean ± SD (n = 5 for each value). * $p < 0.05$, ** $p < 0.01$,

*** $p < 0.001$, and NS, no statistical significance compared with the PH group. Two-way factor repeated-measures analysis of variance followed by Bonferroni's multiple comparison test.

Figure 6. Activation of the serotonin release from the enterochromaffin cells after partial hepatectomy

(a) Representative images of serotonin-secreting enterochromaffin cells in the small intestine of each group. Expression of the serotonin was confirmed by immunostaining of the cells with anti-serotonin antibody. **(b)** Time-dependent change of the number of serotonin positively stained cells in Cnt, PH, Cap+PH, and HV+PH groups. Five different sections from each of the five mice in all groups were immunohistochemically stained with anti-insulin antibody and a quantitative analysis was performed using ImageJ software. The values represent mean ± SD ($n = 25$ for each group). ** $p < 0.01$, *** $p < 0.001$, and N.S., no statistical significance compared with Cnt group. Two-way factor repeated-measures analysis of variance followed by Bonferroni's multiple comparison test.

Figure 7. Mechanism of hepatic regeneration through neural signal

(a) The direct feedback between the liver and the brain. **(b)** The involvement of GI tract in liver regeneration via the neural relay.

Supplementary Figure S1

Time-dependent changes of serum HGF after partial hepatectomy in Cnt, PH, Cap+PH, and HV+PH groups. Serum HGF was measured in each of the five animal from the four groups. The values represent mean ± SD (n = 3 for each value). * $p < 0.05$, *** $p < 0.001$, and NS, no statistical significance compared with the PH group. Two-way factor repeated-measures analysis of variance followed by Bonferroni's multiple comparison test.

Supplementary Figure S2

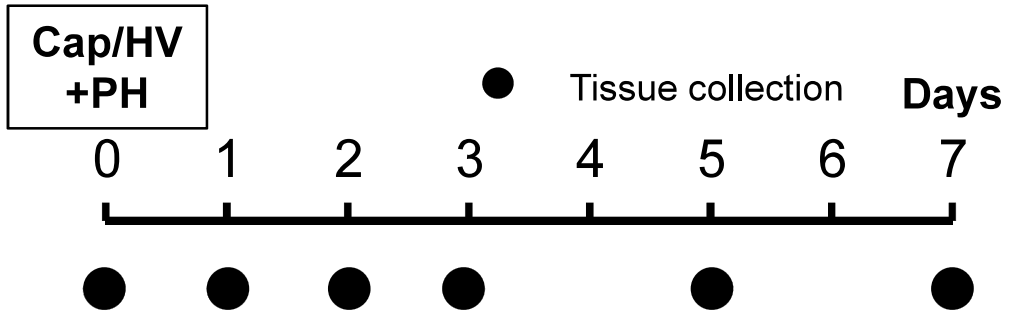
Time-dependent changes of serum IL-6 after partial hepatectomy in Cnt, PH, Cap+PH, and HV+PH groups. Serum IL-6 was measured in each of the five mice from the four groups. The values represent mean ± SD (n = 3 for each value). *** $p < 0.001$, and NS, no statistical significance compared with the PH group. Two-way factor repeated-measures analysis of variance followed by Bonferroni's multiple comparison test.

Figure 1

a

Group	Peritoneotomy	Partial hepatectomy	Capsaicin treatment	Hepatic vagotomy
Cnt	+	-	-	-
PH	+	+	-	-
Cap+PH	+	+	+	-
HV+PH	+	+	-	+

b



C

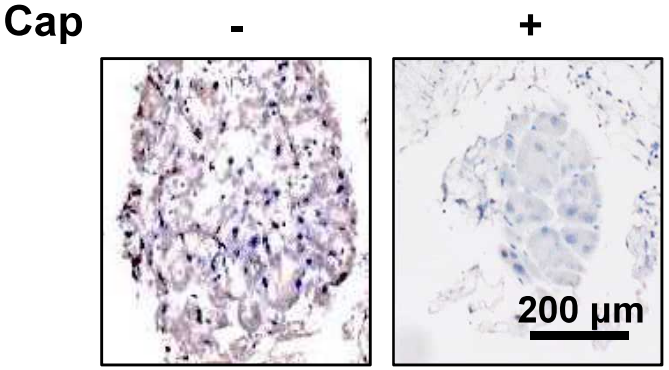


Figure 2

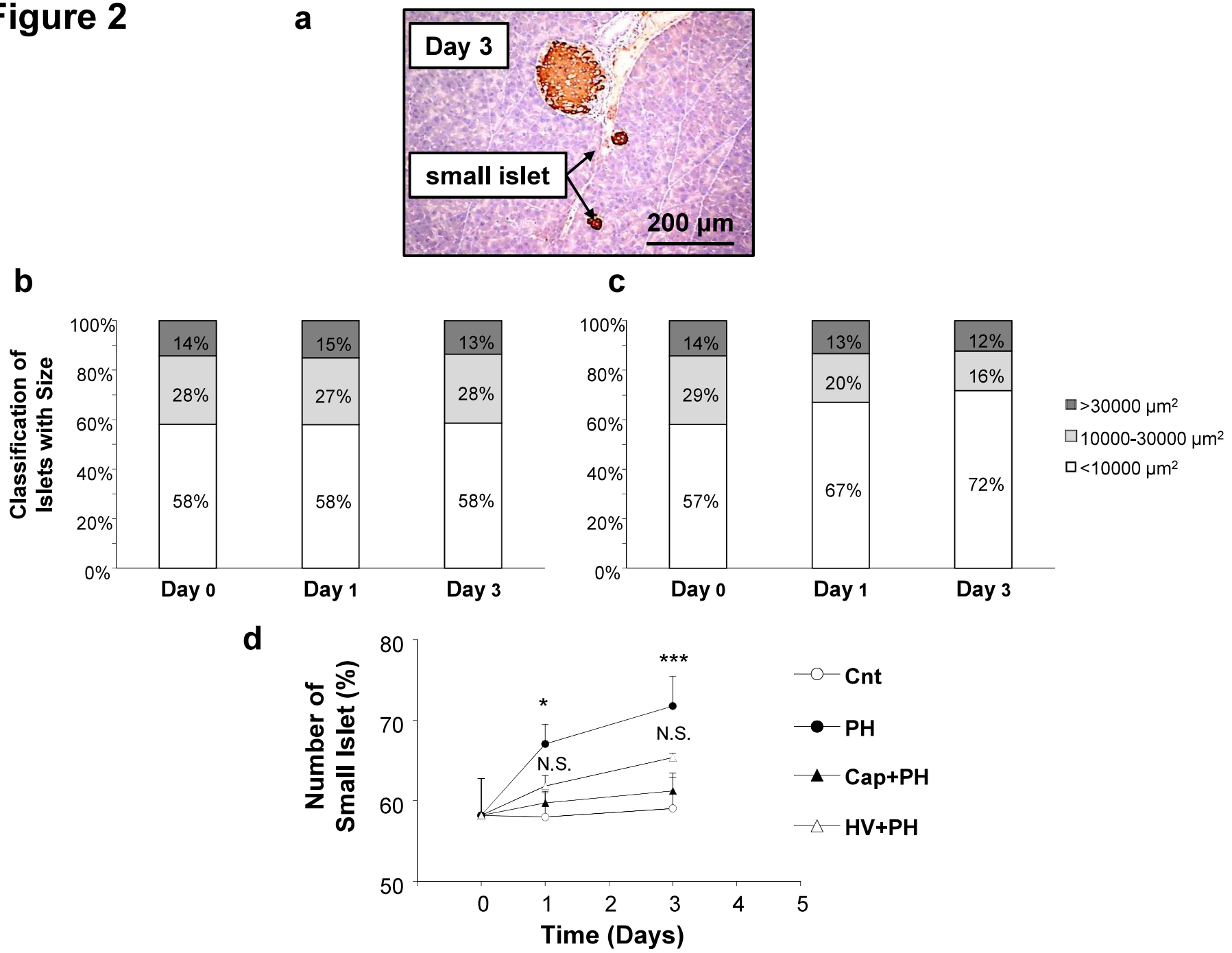

a
Day 3
small islet
200 μm
b
c
Classification of Islets with Size
100%
80%
60%
40%
20%
0%
14%
28%
58%
15%
27%
58%
13%
28%
58%
14%
29%
57%
13%
20%
67%
12%
16%
72%
Day 0
Day 1
Day 3
>30000 μm²
10000-30000 μm²
<10000 μm²
d
Number of Small Islet (%)
80
70
60
50
0
1
2
3
4
5
Time (Days)
*
***
N.S.
N.S.
Cnt
PH
Cap+PH
HV+PH

Figure 5

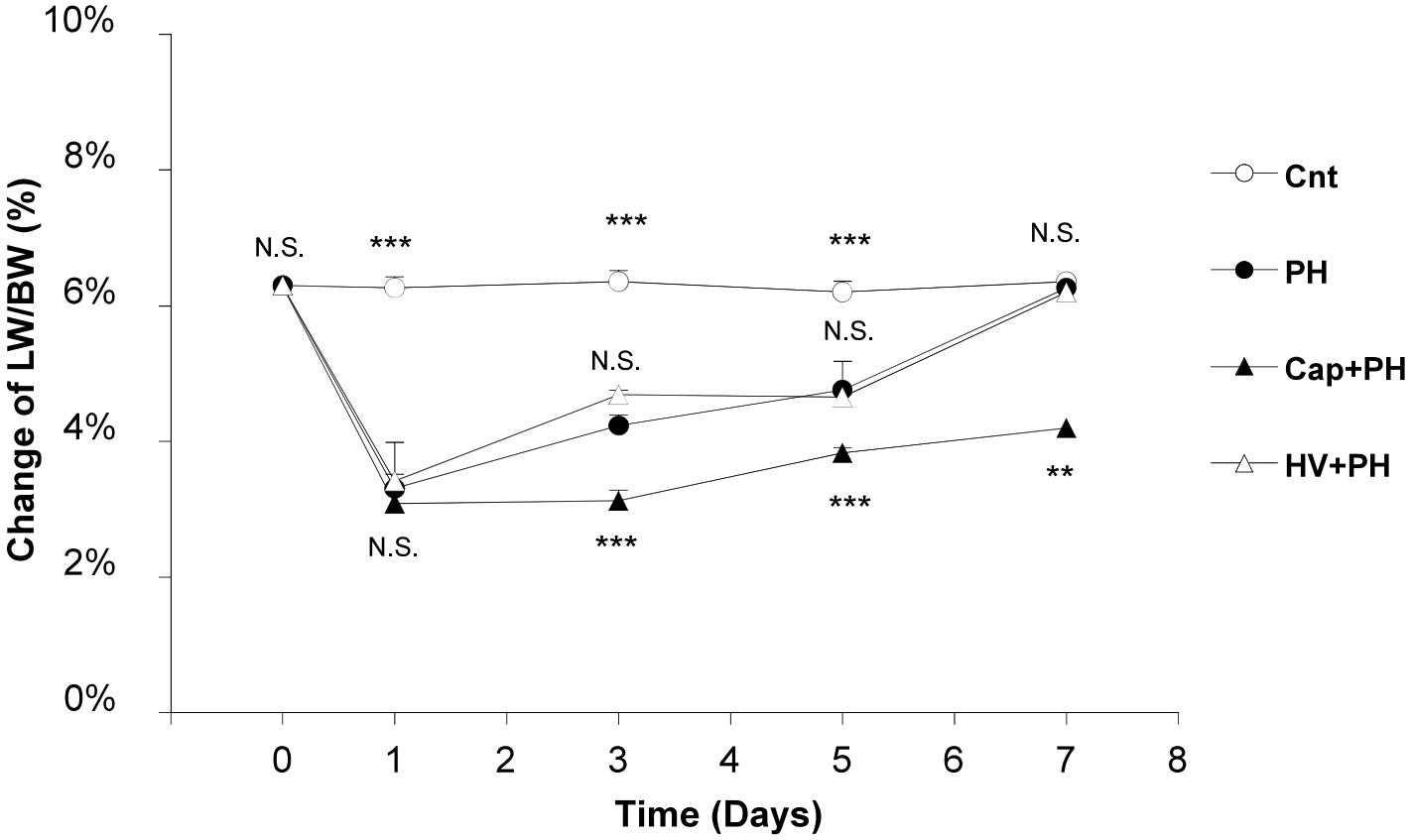
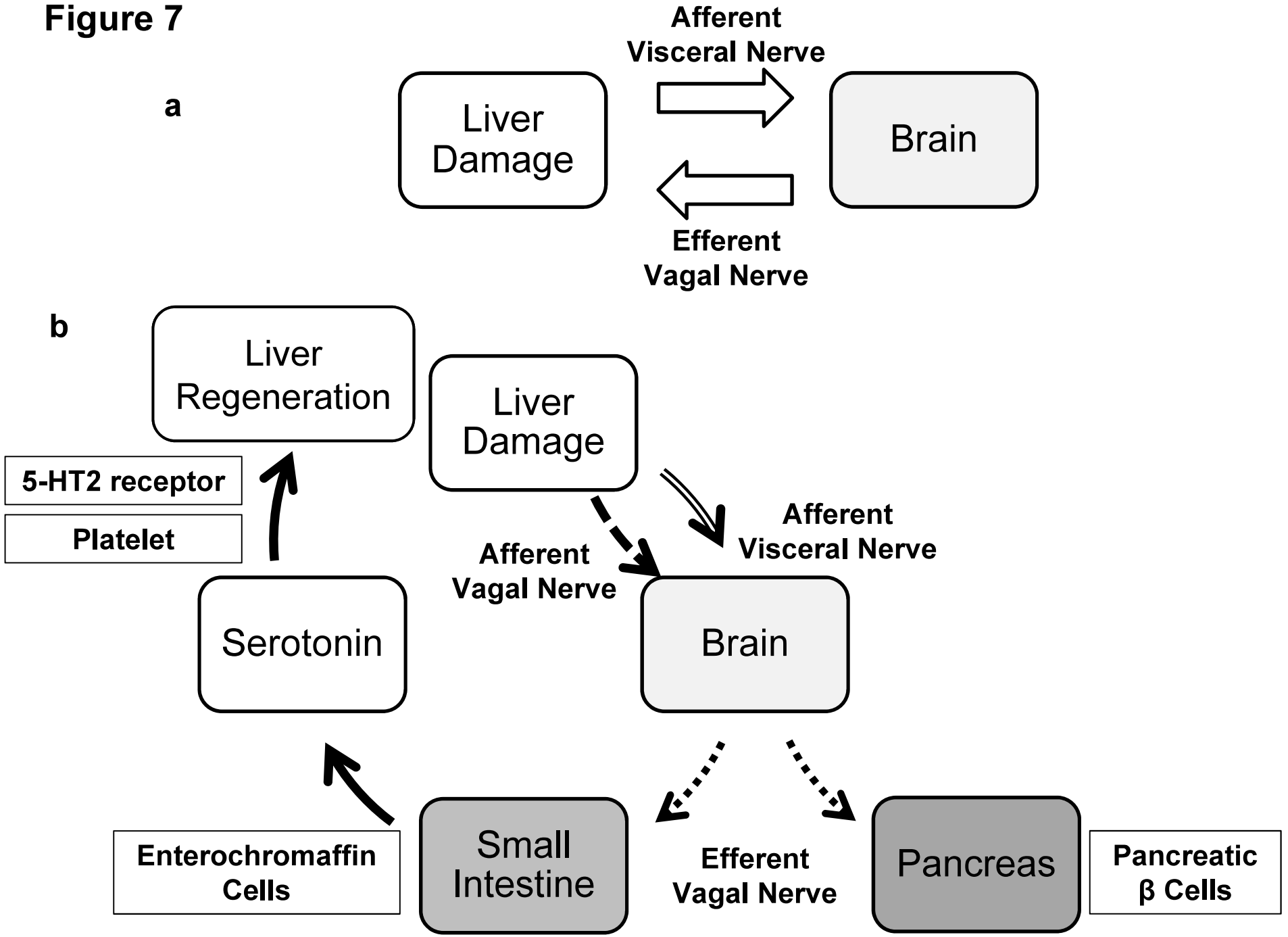
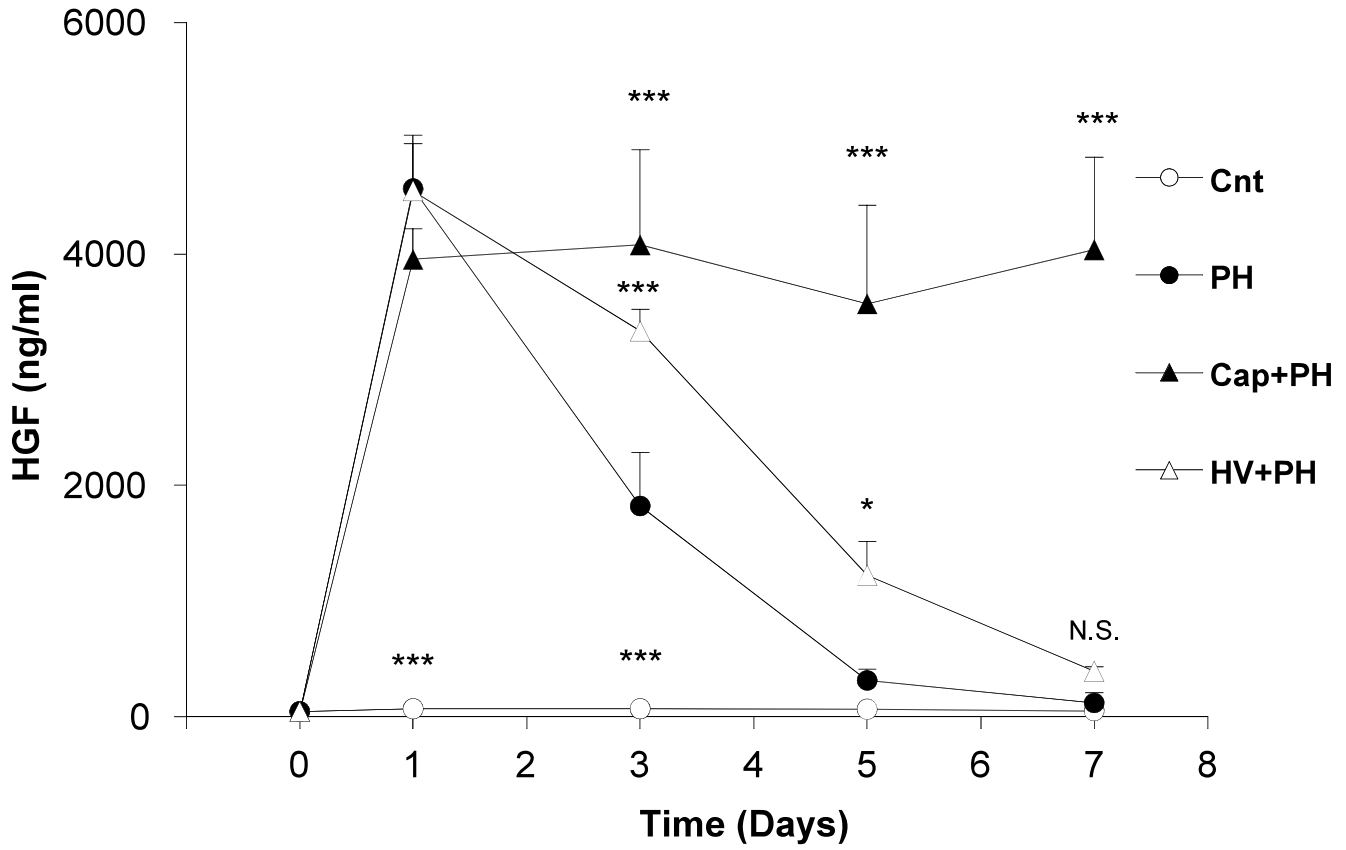

10%
8%
6%
4%
2%
0%
Change of LW/BW (%)
0
1
2
3
4
5
6
7
8
Time (Days)
N.S.
***
***
***
N.S.
N.S.
N.S.
N.S.
***
***
**
Cnt
PH
Cap+PH
HV+PH

Figure 7


a
Afferent
Visceral Nerve
Liver
Damage
Brain
Efferent
Vagal Nerve
b
Liver
Regeneration
Liver
Damage
5-HT2 receptor
Platelet
Afferent
Visceral Nerve
Afferent
Vagal Nerve
Serotonin
Brain
Enterochromaffin
Cells
Small
Intestine
Efferent
Vagal Nerve
Pancreas
Pancreatic
β Cells

Supplementary Figure S1



Supplementary Figure S2

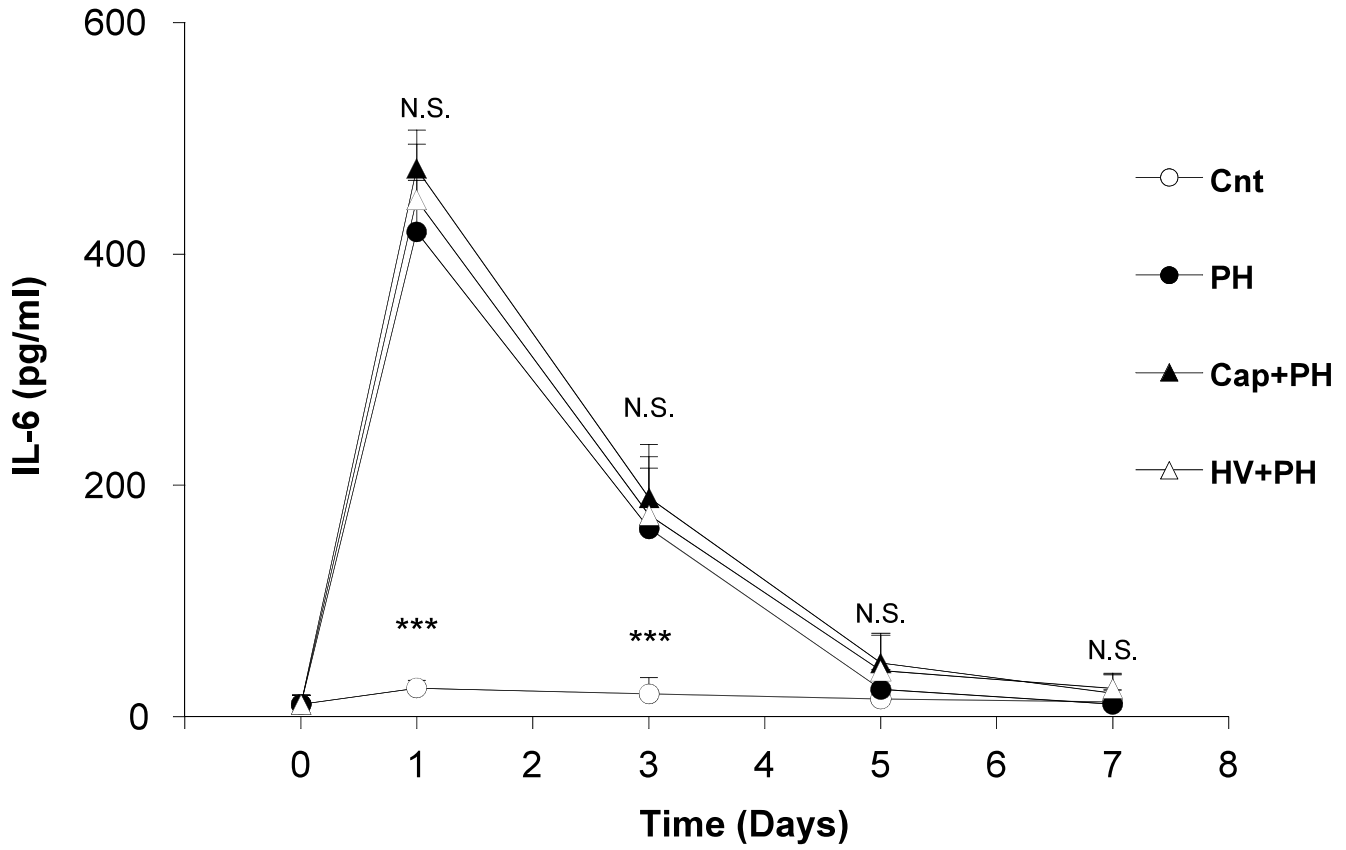
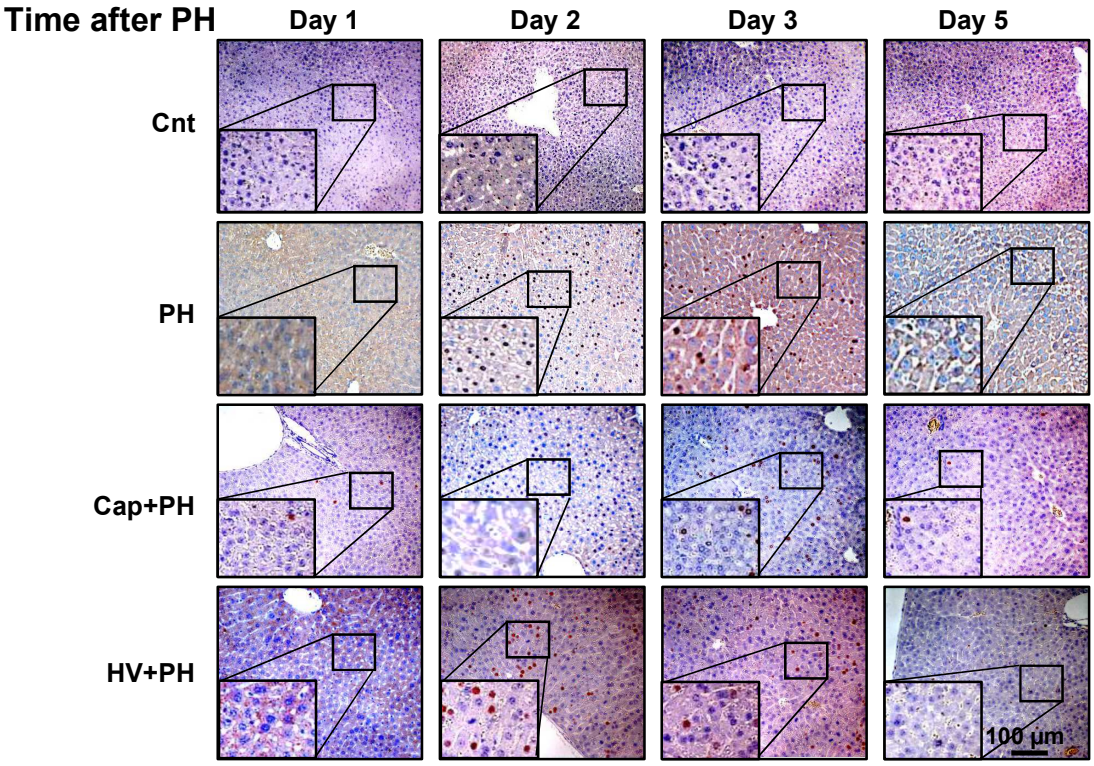


Figure 3

a


Time after PH
Day 1
Day 2
Day 3
Day 5
Cnt
PH
Cap+PH
HV+PH
100 μm

b

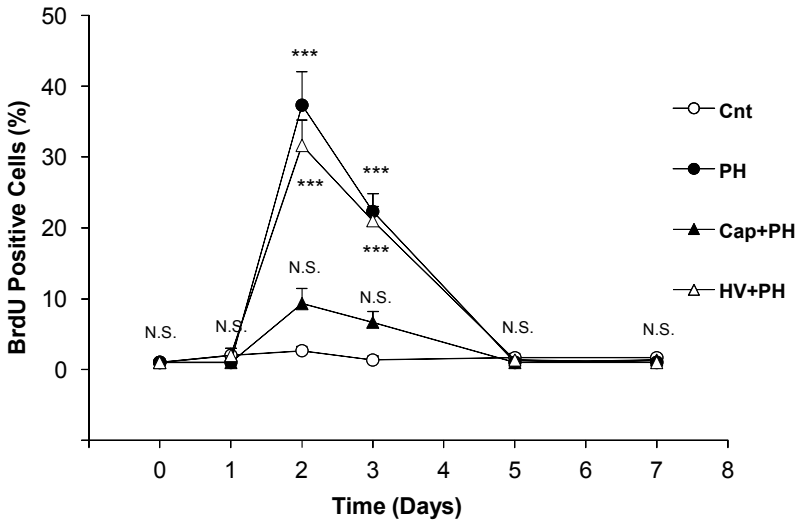

BrdU Positive Cells (%)
Time (Days)
Cnt
PH
Cap+PH
HV+PH
***
N.S.

Figure 4

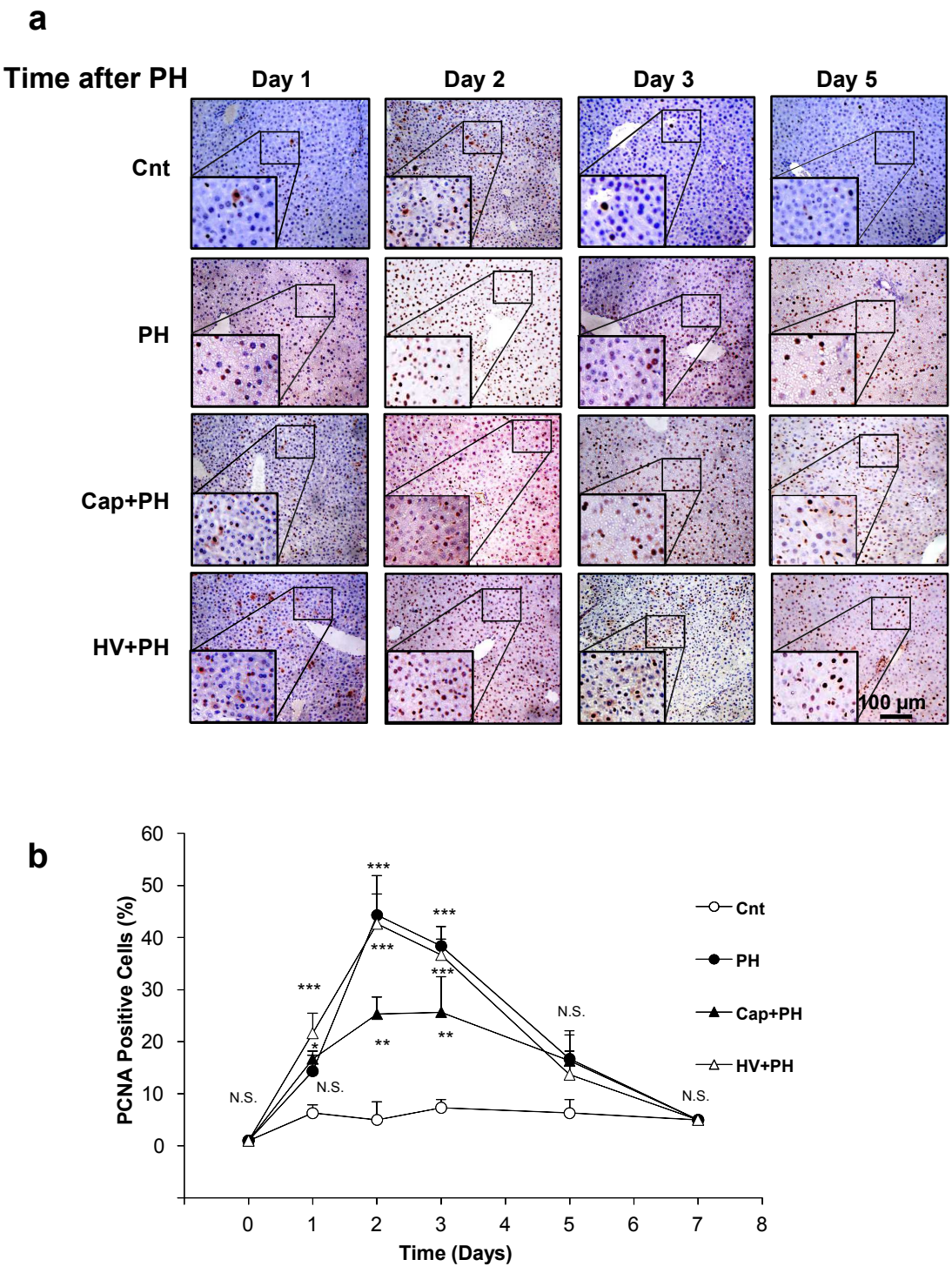
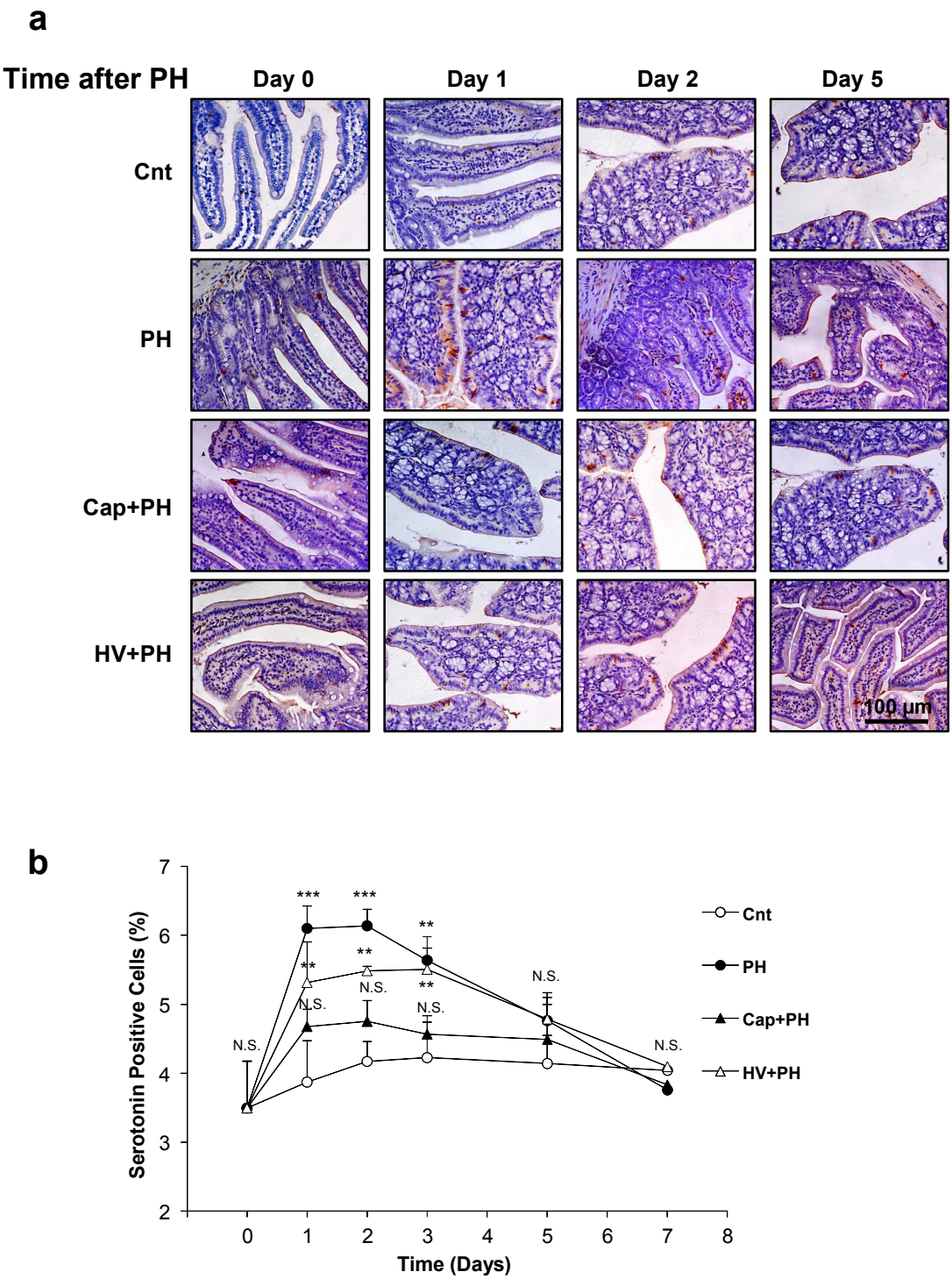

a
Time after PH
Day 1
Day 2
Day 3
Day 5
Cnt
PH
Cap+PH
HV+PH
100 μm
b
PCNA Positive Cells (%)
Time (Days)
Cnt
PH
Cap+PH
HV+PH
N.S.
***
**
*

Figure 6


a
Time after PH
Day 0
Day 1
Day 2
Day 5
Cnt
PH
Cap+PH
HV+PH
100 μm
b
Serotonin Positive Cells (%)
Time (Days)
Cnt
PH
Cap+PH
HV+PH
***
**
N.S.

Graphical Abstract Figure