2016

Interleukin 10 Restores Gastric Emptying, Electrical Activity, and Interstitial Cells of Cajal Networks in Diabetic Mice

Anthony J. Bauer, *Liberty University*

HHS Public Access

Author manuscript

Cell Mol Gastroenterol Hepatol. Author manuscript; available in PMC 2016 December 15.

Published in final edited form as:

Cell Mol Gastroenterol Hepatol. 2016 July ; 2(4): 454–467. doi:10.1016/j.jcmgh.2016.04.006.

Interleukin 10 Restores Gastric Emptying, Electrical Activity, and Interstitial Cells of Cajal Networks in Diabetic Mice

Kyoung Moo Choi[#1], **Simon J. Gibbons**[#1], **Lei Sha**[1], **Arthur Beyder**[1], **Pieter-Jan Verhulst**[1], **Gianluca Cipriani**[1], **Jessica E Phillips**[1], **Anthony J Bauer**[2], **Tamas Ordog**[1], **Jon J Camp**[1], **Xin Ge**[1], **Adil E Bharucha**[1], **David R Linden**[1], **Joseph H Szurszewski**[1], **Purna C Kashyap**[1], and **Gianrico Farrugia**[1]

[1]Enteric NeuroScience Program, Mayo Clinic School of Medicine, Rochester, MN, USA

[2]Department of Integrative Physiology and Pharmacology, Liberty University College of Osteopathic Medicine Lynchburg VA, USA

[#] These authors contributed equally to this work.

Abstract

Background and Aims—Gastroparesis is a complication of diabetes, characterized by delayed emptying of stomach contents and accompanied by early satiety, nausea, vomiting, and pain. No safe and reliable treatments are available. Interleukin 10 (IL10) activates the M2 cytoprotective phenotype of macrophages and induces expression of heme oxygenase 1 (HO1) protein. We investigated whether IL10 administration could improve gastric emptying and reverse the associated cellular and electrical abnormalities in diabetic mice.

Correspondence: Gianrico Farrugia MD, Enteric NeuroScience Program, Mayo Clinic, 200 First St SW, Rochester MN 55905, USA, Phone: 507-284-4695, Farrugia.gianrico@mayo.edu.

Disclosures: KMC, SJG, LS, AB, P-J V, GC, JEP, AJB, TO, XG, AEB, DRL, JHS, PCK and GF declare that they have no conflict of interest to disclose. Jon J. Camp and Mayo Clinic has a financial conflict of interest in technology (medical image analysis software) used in this research and Jon J. Camp and Mayo Clinic may stand to gain financially from the successful outcome of the research. This technology is not the subject of the research; instead, it is a software tool in the research.

Author Contributions: KMC - study design, acquisition, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis; **SJG** – study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, administrative, technical, or material support, study supervision; **LS** - acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, statistical analysis; **AB** - analysis and interpretation of data, statistical analysis, critical revision of the manuscript for important intellectual content; **PJV** - acquisition, analysis and interpretation of data, statistical analysis; **GC** - acquisition, analysis and interpretation of data, statistical analysis; **JEP** - acquisition of data; **AJB** – study concept, critical revision of the manuscript for important intellectual content; **TO** - study concept and design, critical revision of the manuscript for important intellectual content; **JJC** - analysis and interpretation of data, statistical analysis, drafting of the manuscript; **XG** - analysis of data; **AEB** - study concept and design, critical revision of the manuscript for important intellectual content; **DRL** - study concept and design, administrative, technical, or material support, critical revision of the manuscript for important intellectual content; **JHS** - study concept and design, critical revision of the manuscript for important intellectual content, obtained funding, administrative, technical, or material support, study supervision. **PCK** - study concept and design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, statistical analysis, obtained funding, administrative, technical, or material support, study supervision; **GF** -study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, obtained funding, administrative, technical, or material support, study supervision.

Methods—Non-obese diabetic mice with delayed gastric emptying were given either IL10 (0.1-1μg, twice/day) or vehicle (controls). Stomach tissues were isolated, and sharp microelectrode recordings were made of the electrical activity in the gastric muscle layers. Changes to interstitial cells of Cajal (ICC), NADPH-diaphorase and levels and distribution of HO1 protein were determined by histochemical and imaging analyses of the same tissues.

Results—Gastric emptying remained delayed in vehicle-treated diabetic mice but returned to normal in mice given IL10 ($n = 10$ mice, $P < 0.05$). In mice given IL10, normalization of gastric emptying was associated with a membrane potential difference between the proximal and distal stomach and lower irregularity and higher frequency of slow-wave activity, particularly in the distal stomach. Levels of HO1 protein were higher in stomach tissues from mice given IL10, and ICC networks were more organized, better connected, and more evenly distributed compared with controls.

Conclusions—IL10 increases gastric emptying in diabetic mice and has therapeutic potential for patients with diabetic gastroparesis. This response is associated with up-regulation of HO1 and repair of connectivity of ICC networks.

Keywords

Alternatively-Activated Macrophages; Heme Oxygenase 1; Electrical Slow Wave

INTRODUCTION

Gastroparesis is a complication of diabetes defined by delayed gastric emptying without obstruction that is accompanied by early satiety, nausea, vomiting and pain.[1-3] No safe and reliable treatments are available.[4] Identification of the cellular changes associated with gastroparesis in humans[5, 6] and in mouse models of gastroparesis[7, 8] have identified possible therapeutic targets. The non-obese diabetic (NOD) mouse in particular, has proved to be a useful model of human diabetic gastroparesis.[7, 9-11]

In previous studies using a non-invasive gastric emptying test, a proportion of diabetic NOD mice developed delayed emptying of solids while the remainder remained resistant to this complication.[11] While most animals demonstrate loss of neuronal nitric oxide synthase (nNOS) upon development of diabetes, development of delayed gastric emptying was concurrent with reduced expression of the receptor tyrosine kinase, Kit, a marker for interstitial cells of Cajal (ICC).[7] This is consistent with studies showing impairment to ICC networks and loss of nNOS in mice with long standing diabetes.[9, 10] Subsequent studies determined that animals resistant to development of delayed emptying maintained high expression of heme oxygenase-1 (HO1) in alternatively activated M2-macrophages in stomach muscle layers.[7, 8] In studies on human gastric body, a correlation between numbers of ICC and of CD206-positive M2-macrophages was found.[12]

These studies suggest that targeting HO1 may ameliorate diabetic gastroparesis. Indeed, treatment of diabetic mice with delayed gastric emptying by inducing HO1 normalized gastric emptying and Kit expression levels.[7, 8] These therapies were accompanied by increased numbers of HO1-positive, M2-macrophages in gastric muscularis propria.[8] Hemin

(as Panhematin™) also up-regulates HO1 expression in humans, although administration of hemin must be done under close supervision.[13] These data suggest that HO1 is a promising therapeutic target for diabetic gastroparesis[14] but treatment with hemin has significant disadvantages including the delivery method and side effects.

There consequently exists a need to determine other modalities to target HO1. In the present study we tested interleukin-10 (IL10) as a treatment for delayed gastric emptying in NOD mice. The rationale was that IL10 expression is up-regulated during treatment by CO of post-operative ileus in mice[15] and that IL10 had an obligatory role in post-operative intestinal recovery.[16] IL10 up-regulates HO1 expression[17] and is expressed at high levels in M2-macrophages. IL10 increases numbers of M2-macrophages[18] directly and suppresses expression of M1-macrophages.[19] Also, in diabetic mice with delayed emptying IL10 mRNA levels were decreased.[8]

In previous studies on diabetic mice and rats, ICC networks and the electrical slow wave activity that originates from ICC were absent or abnormal, especially in the distal antrum.[9, 20] In humans, electrical dysrhythmias were also reported in diabetic gastroparesis.[21-23] However electrical properties and immuno-histochemical differences were not comparatively studied in rodents with delayed gastric emptying or after normalization of gastric emptying. Thus, for the current study we made electrical recordings from multiple locations in the stomach of treated mice and subsequently immuno-labeled for HO1 and Kit. Therefore we were able to examine associations between gastric emptying of the mice and physiological and anatomical changes to the stomach and the response of those changes to treatment with IL10.

METHODS

Animals and Experimental Design

Animal procedures were done using protocols approved by Mayo Clinic's Institutional Animal Care and Use Committee. Female NOD/ShiLtJ mice were used as previously described.[7, 8, 11] Blood glucose levels were measured every week until the onset of diabetes after which point they were measured daily. Single drops of blood were collected from the vascular bundle located at the rear of the jaw bone of the mice. The amount of blood collected was carefully monitored to avoid anemia. Mice were considered diabetic when the glucose levels were over 250 mg/dl. The incidence of diabetes was 57% in this study. Sub-therapeutic insulin (Lantus insulin glargine, Sanofi-Aventis U.S. LLC, Bridgewater, NJ) was injected once daily i.p. when the glucose levels were over 500 mg/dl to keep the diabetic mice alive yet also keep blood glucose levels between 400 and 600 mg/dl to allow complications of diabetes to develop. In order to determine the levels of oxidative stress in the diabetic mice, malondialdehyde (MDA) was measured in blood plasma. The concentration of thiobarbituric acid reactive substances was calculated as malondialdehyde equivalents using a commercial kit (Oxi-Tek; Zeptometrix Corp, Buffalo, NY). Five microliters of plasma sample was mixed with an equal volume of sodium dodecyl sulfate solution and 125 μL of 5% thiobarbituric acid/acetic acid reagent. Samples were incubated for 60 minutes at 95°C. After centrifugation at 1600*g*, the absorbances of supernatants from

samples were read at 532 nm using a spectrophotometer (NanoDrop Technology, Wilmington, DE).

Gastric emptying of solids was measured using a ^{13}[C]-octanoic acid breath test.[11] Three baseline values of $T_{1/2}$ for gastric emptying were obtained prior to development of diabetes. After onset of diabetes, gastric emptying was measured weekly. After development of delayed gastric emptying, mice were assigned to treatment with vehicle or IL10 (GenScript, Piscataway, NJ or Insight Genomics, Falls Church, VA, 1 or 0.1μg intraperitoneally, twice a day). We alternated the assignment to treatment based on development of delayed gastric emptying only and without regard to any metabolic parameters such as blood glucose or MDA levels. Treatment began after 2 consecutive measurements of delayed gastric emptying and continued as two doses every day until 2 consecutive normal values for $T_{1/2}$ were obtained or a maximum of 10 weeks after the start of treatment. The duration and frequency of vehicle treatment was matched to the length of IL10 treatment. All mice with delayed gastric emptying also had MDA levels that were significantly greater than the upper limit of the normal range for MDA in diabetic NOD mice (> 73 nmol/mL).[7] At the completion of the study, mice were killed by carbon dioxide exposure followed by cervical dislocation. Whole stomachs were cut along the lesser curvature and the mucosa removed. Electrical recordings and immunohistochemical studies were then done on the tissues. The experimental protocol is outlined in Figure 1A.

Electrophysiological Recordings from the Gastric Smooth Muscle

Smooth muscle membrane potential and electrical slow waves were recorded from the circular muscle of every tissue from the treated mice at 12 defined regions distributed from proximal body to distal antrum (Fig 1B). Sharp glass microelectrodes filled with 3M KCl (input resistances: 40 to 70MΩ) were used to record membrane potential and electrical slow wave. The locations of the recording sites were documented on a digital image after which the tissue was fixed in the recording dish (Fig 1B). Electrical slow wave events were analyzed using Clampfit (Molecular Devices, LLC., Sunnyvale, CA). Analysis was done on recordings longer than 2min in duration in which at least 12 slow wave events were observed as follows. Slow wave events from stable recordings were identified using the “template search” event discriminator function in Clampfit. For each trace a template was derived from a typical event. All the events were then automatically fit using the established template, and each event was confirmed by the user for goodness of fit. Automated fitting allowed quantification of the baseline membrane potential, peak amplitude and inter-event interval. The inter-event interval represents the frequency of the pacemaking depolarization. Averages and variances were obtained for each recording from each area.

Immunohistochemistry

After completion of the electrical recordings, the intact tissues from each mouse were fixed in acetone and doubly labeled for Kit and HO1. Antibodies used were a rabbit polyclonal anti-HO-1 antibody (Stressgen Biotech. Corp., Victoria, BC, Canada) and rat monoclonal anti-Kit antibody (ACK2; eBioscience, San Diego, CA, USA). The tissue was washed 3 times in 0.1 M phosphate buffered saline (PBS) then blocked in 10% normal donkey serum (NDS) in PBS and 0.3% Triton overnight at 4°C. The Kit antibody (1.7 μg/ml final

concentration) was applied by incubation in 5% NDS/PBS/0.3% Triton for 2 days at 4°C. After the tissue was fixed again in 4% paraformaldehyde in 0.1M phosphate buffer and washed 3 times in PBS, anti-HO-1 antibody (Stressgen Biotech Corp., Victoria, Canada, 0.125 μg/ml, final concentration) in 5% NDS/PBS/0.3% Triton was applied overnight at 4°C. After rinsing in PBS, donkey anti-rabbit FITC (3 μg/ml, final concentration , Chemicon, Temecula, CA) and donkey anti-rat Cy3 (3 μg/ml, final concentration, Chemicon, Temecula, CA) were added in 2.5% NDS/ PBS/0.3% Triton and incubated overnight at 4°C in the dark. After rinsing in PBS, nuclei in the tissue were stained by incubating the tissue for 30 min at 4°C with 0.3 μM 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes, Inc., Eugene, OR) in water.

In order to identify the HO1-positive cells that had macrophage-like morphology in the muscularis propria of diabetic mice treated with IL10, we treated two diabetic mice with 1 μg twice daily IL10 for 1 week. These mice were B6;C3Fe wild type mice that were made diabetic by treatment with 160 mg/kg streptozotocin as described previously.[24] Treatment with IL10 was started after 10 weeks of diabetes. The mice had normal gastric emptying. After killing the mice, we immuno-labeled the gastric muscularis propria as a whole mount preparation with the pan-macrophage marker F4/80, the M2 macrophage marker CD206 and HO1 as previously reported [8]. Briefly, tissues were incubated in F4/80 rat anti-mouse antibody (0.4 μg/mL, Thermo Fisher, Waltham MA) in Ca^{2+}-containing, HEPES-buffered physiologic salt solution (CaPSS; 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with Tris) at 37°C for 90 min in dark. The tissue was then fixed in 4% paraformaldehyde in 0.1M phosphate buffer. After blocking in 10% NDS, 0.1M PBS, 0.3% Triton overnight at 4°C, rabbit anti-HO1 (0.125 μg/mL) and goat anti-CD206 (0.5 μg/mL, Santa Cruz Biotech, Santa Cruz, CA) antibodies were applied by incubation in 5% NDS/1X PBS/0.3% Triton overnight at 4°C. After rinsing in PBS, donkey anti-rabbit cy3 (0.5 μg/mL; Jackson ImmunoResearch, West Grove PA) and donkey anti-goat cy5 (0.5 μg/mL; Jackson Immuno), both in 2.5% NDS/1X PBS/0.3% Triton were added overnight at 4°C in the dark. The tissues were then washed in PBS and water and the nuclei in the tissue stained by incubating for 30 minutes at 4°C with 0.3 μM DAPI in water and mounted for viewing.

Assessment of Nitric Oxide Synthase expression in Myenteric Neurons

Nitric oxide synthase expression was determined by NADPH-diaphorase histochemistry as previously reported.[25] Tissues, fixed in acetone and paraformaldehyde were washed 5 times over 30 minutes in 1X PBS then pinned out on Sylgard in a small Petri dish. Tissues were then washed 2 times in 100 mM TrisCl for 5 min and permeabilized in 100 mM TrisCl/ 0.3% Triton X100. The labeling solution contained 0.2 mg/mL nitroblue tetrazolium and 1 mg/mL NADPH dissolved in 100 mM TrisCl/ 0.3% Triton X100 and was incubated with the tissue at 37 °C until the reaction was complete. The reaction was stopped by washing in 100 mM TrisCl/ 0.3% Triton X100. Tissues were then mounted for examination at 20X magnification on an upright microscope and counting of positively labeled cells.

Assessment and Quantification of Kit and HO-1 Immunoreactivity

Tissues were examined with a confocal microscope using a 20X (NA0.95) XLUMPlanFl objective (Olympus Japan) in Fluoview (Olympus) at a resolution of 0.994x0.994x1.13μm (XxYxZ). Stacks of images across the full thickness of the muscles were collected from the documented electrical recording sites. To assess labeling across the whole stomach, tissues were examined with an Olympus IX70 microscope by two investigators blind to the tissue source. For quantification of the labeling specifically at the electrical recording sites, all of the confocal image stacks (120 images for each immunolabel) were flattened into projections using the FV10-ASW Viewer (Olympus) assigned a random number and up-loaded into a digital album in random order. The images were then assessed using the same parameters as used for the whole tissue by two investigators blind to the tissue source. The images were scored on a 10 cm visual analogue scale for network density, connectivity and structural preservation. The scores for each image were then averaged and the code broken to find which image belonged to which field and animal.

Quantification of Connectivity in the confocal images of Kit immunoreactivity

The absolute volumes of Kit-positive immunoreactivity in 4 fields from two areas in each tissue where electrical abnormalities were most evident in the vehicle-treated mice were obtained by volume rendering and 3D reconstruction from confocal image stacks of ICC across the full thickness of the muscle using Analyze™ (Mayo Foundation, Rochester, MN) as previously described.[26] The reconstructed Kit positive 3D structures from each field were subjected to a morphological 3×3×3 opening operation. This deleted any region of the structures that did not contain a 3×3×3 voxel (12.86μm^3) cube, thereby breaking up larger tenuously-connected structures into two or more smaller structures. After opening, remaining connected structures larger than 100 voxels (143μm^3) were counted. A larger number of such structures within similar total volumes indicates reduced connectivity of the total volume.

Statistics were done with Prism (Graphpad, La Jolla CA) using the appropriate tests as described in the results. All authors had access to the study data and have reviewed and approved the final manuscript.

RESULTS

Blood Glucose and Oxidative Stress Levels in the Diabetic Mice

Mice used for this study developed diabetes at 19.6±1.6 weeks (mean±SEM, n=10 mice) and remained diabetic throughout the study. Thirty percent of diabetic mice developed delayed gastric emptying with a mean of 5.4±0.5 weeks (mean±SEM, n=10 mice) after onset of diabetes. This is consistent with previous reports.[7] Animals with delayed gastric emptying were consecutively enrolled until 10 completed the study. Five received vehicle and five IL10. There were no significant differences in blood glucose levels for the mice whether treated with vehicle or IL10 at any point during the progression of the study and diabetic mice with delayed gastric emptying did not have significantly different blood glucose levels compared to diabetic mice with normal gastric emptying (Fig 2A). Vehicle

and IL10-treated mice received similar amounts of insulin during the study (3.4±1.80iu/day vs 2.6±0.98iu/day respectively, mean±SEM, n=5 mice, P=0.67, t test).

Systemic oxidative stress was measured using plasma malondialdehyde (MDA) levels. Non-diabetic mice had low levels of MDA (6.0±0.6 and 6.0±0.5nmol/ml for mice assigned to vehicle and IL10 respectively, mean±SEM, n=5 in each group, Fig 2B). After development of diabetes but while gastric emptying remained normal, MDA levels were not significantly different between the mice assigned to the two treatments (41±16.3 and 30±3.7nmol/ml for mice assigned to vehicle and IL10 respectively, mean±SEM, n=5 in each group, Fig 2B). At onset of delayed gastric emptying, MDA levels were further elevated above the previously established normal range for diabetic NOD mice with normal gastric emptying (normal mean 53.05, range 28-73 nmol/ml, n = 19 mice,[7] in both groups of mice (129±28.9 and 226±16.8nmol/ml, mean±SEM, vehicle and IL10 respectively). These levels at onset of delayed gastric emptying were higher than the 73 nmol/ml threshold found previously to be associated with delayed gastric emptying in diabetic NOD mice.[7] In mice treated with IL10, MDA levels decreased to the levels (56±9.9nmol/ml, mean±SEM) typical for diabetic NOD mice with normal gastric emptying whereas MDA levels in the vehicle-treated mice remained above the upper limit of this range (Fig 2B, 221±37.2nmol/ml, mean±SEM). These values were higher than those prior to development of delayed gastric emptying and higher than the levels in mice treated with IL10 (Fig 2B).

Gastric Emptying in Diabetic Mice after IL10 Treatment

Gastric emptying values are shown in Figure 2C. Prior to development of diabetes, gastric emptying values were in the normal range.[11] $T_{½}$ values were 97±7.1 and 98±6.0min for mice assigned to vehicle and IL10 respectively (mean±SEM, n=5 in each group, Fig 2C). The gastric emptying values in diabetic mice after development of delay were not different between groups ($T_{½}$=174±13.9min for vehicle, 183±19.4min for IL10, mean±SEM, n=5, Fig 2C). In mice that were treated with IL10, gastric emptying returned to normal in all animals after 8.8±1.9weeks ($T_{½}$=106±3.4min, mean±SEM, n=5, Fig 2C) whereas mice treated for the same period with vehicle continued to have delayed gastric emptying ($T_{½}$=159±9.5min, mean±SEM, n=5, Fig 2C).

These data support the conclusion that IL10 can reverse delayed gastric emptying over an 8 week period with no effects on blood glucose levels. We next determined the effect of IL10 on electrical activity.

Electrical Activity after Treatment with IL10

Stable electrical recordings were made from 12 areas in the corpus and antrum of all diabetic mice with delayed gastric emptying that then completed the study of the effects of treatment with vehicle or IL10 on gastric emptying (120 regions in total). In regions where slow waves could be detected (116 areas), there were differences in the properties of the electrical activity between vehicle and IL10-treated mice. Quiescent areas were all in the proximal body (areas 1-3), close to the fundus, an area where slow waves are not usually detected. The quiescent areas were found in 2 tissues from both vehicle- and IL10-treated mice. Peak amplitudes of the slow waves recorded in the distal region (areas 10-12) of the IL10-treated

mice did not vary as much as the peak amplitudes of slow waves in the same regions from vehicle-treated mice. In the more proximal regions of the stomach, irregularities in the slow waves from 3 out of 5 vehicle-treated animals were also observed but this was not observed for any IL10-treated mice. In all tissues from vehicle-treated mice, an irregular slow wave pattern was recorded in the distal antrum (Fig 3A) whereas the slow wave activity for the IL10-treated animals did not exhibit these irregularities in the equivalent areas of the distal antrum (Fig 3B). This reduction in the variability of the slow wave in the antrum following IL10 treatment was confirmed by quantifying the peak amplitude of the slow waves. We averaged the amplitudes of all events in each recording, both low and high amplitude events, but found that there was no difference in the actual amplitude of the slow waves by comparing either individual regions or averages of the observations at all regions but there was significantly more variation in the amplitude for the most distal regions (Fig 4A, $P<0.05$, unpaired t-test, n=5). The events recorded from IL10-treated mice also had significantly higher frequencies (4.05±0.34 cycles per min) with, on average, a 22% shorter peak to peak inter-event interval compared to vehicle-treated mice (3.18±0.4 cycles per min, Fig 4B, $P=0.0052$, unpaired t-test, n=5). A faster frequency was also detected in tissues from IL-10 treated mice by comparing the inter-event intervals at every individual recording location ($P<0.05$ Wilcoxon matched-pairs test). There exists a smooth muscle membrane potential gradient between proximal and distal stomach in dogs.[27] In healthy wild-type mice, the smooth muscle membrane potential in the antrum is reported to be hyperpolarized relative to the fundus [28] and the values are similar to those found in the equivalent areas of the canine stomach.[27] The membrane potential difference between proximal and distal areas of the stomach was not significant in tissues from the diabetic with delayed emptying, vehicle-treated mice (corpus 1-3, E_m= −54.2±1.76 vs antrum 7-9, antrum, −60.6±2.87 mV, NS, n=5), while there was a significant membrane potential difference in tissues from IL10-treated mice, with proximal regions (areas 1-3, corpus, E_m= −51.0±0.96 mV, mean±SEM) significantly more depolarized than distal regions (areas 7-9, antrum, E_m= −64.7±3.00 mV, mean±SEM, $P<0.01$, 2 way ANOVA with Bonferoni post test, n=5, Fig 4C). Also the variation in the membrane potential values across the whole tissue was significantly greater in the IL10-treated mice compared to the vehicle-treated mice (Fig 4D, $P=0.03$, F-test).

Changes in Kit-positive ICC, HO1- positive Cells and Neuronal NOS Activity after IL10 Treatment

We next determined if the IL10-induced changes in gastric emptying and electrical activity were accompanied by cellular changes. All of the tissues used for electrophysiology were processed as whole mounts and immuno-labeled using antibodies to HO1 and to Kit. The whole tissues were examined at low power under an epifluorescence microscope. High resolution confocal images were also collected from the electrical recording sites as identified by careful mapping. Overall, there was a clear difference between the tissues from IL10- and vehicle-treated mice (Fig 5A,B). Blind scoring showed that expression of HO1 protein was significantly higher in tissue from IL10-compared to vehicle-treated mice (Fig 5C) and that tissues from IL10-treated mice had more HO1-positive cells than tissues from vehicle-treated mice. HO1-positive cells were distributed unevenly across the tissue with patches of positively labeled cells in some areas but not others (Fig 5A, B). Most of the labeled cells had the macrophage-like morphology (Fig 5D) that we previously reported for

CD206 positive, F4/80 positive cells in the gastric muscularis propria of diabetic NOD mice with normal gastric emptying and were likely M2 macrophages.[7] HO1 protein expression was also up-regulated in some myenteric ganglia (Fig 5D) from 2 out of 5 IL10-treated mice. To test whether the HO1-positive, macrophage-like cells were M2 macrophages, we triply labeled gastric body muscularis propria from two diabetic mice that had been treated with IL10 for 2 weeks. As shown in Fig 5E, the majority of HO1-positive cells with macrophage-like morphology expressed not only the pan-macrophage marker F4/80 but also the M2 macrophage marker CD206. We examined NOS activity in myenteric plexuses of vehicle and IL10-treated tissues by counting NADPH-diaphorase positive neurons. There was no difference in the number of labelled neurons (Vehicle: 46±11, IL10 42.8±5.9 neurons/field. NS, unpaired t test, Fig 6).

ICC networks, as identified by Kit immunoreactivity were significantly different between IL10 and vehicle-treated groups. Quantification of the differences at the whole tissue level was done by collecting high resolution confocal images at the location of all the electrical recording sites (120 images, Fig 7A,B) and scoring of network density in a blind fashion by two independent investigators on a 10cm scale. The blinded observers reported that tissues that were subsequently identified as vehicle-treated had clear regions where ICC networks were depleted and in other areas, where ICC were present the networks were scored as more disorganized. In tissues subsequently identified as from IL10-treated mice, the density of ICC networks was more even across the tissue and the networks were scored as more organized and more evenly labeled for Kit. These scores found that ICC in IL10-treated mice had significantly higher ICC densities across the whole tissue compared to vehicle-treated mice (Fig 7C). We separately investigated whether local ICC network changes corresponded with abnormalities in electrical slow waves by selecting two recording locations from each tissue, collecting high resolution images and determining Kit-positive ICC volume and connectivity at those recording locations in the proximal and distal antrum from both IL10 and vehicle-treated mice. The investigator doing this work was blind to the source of the images that were reconstructed. The volume of the Kit-positive structures in these images was not different between ICC networks from the two groups of mice in either the distal antrum where the slow wave changes were most prominent or in the proximal antrum where they were less common (Fig 7D, n=5 mice, $P > 0.05$, unpaired t-test). However, analysis of the count of connected structures after morphological opening revealed that ICC networks from IL10-treated mice, were significantly more connected than vehicle-treated networks (Fig 7E). The algorithm used for this analysis examines the impact of eroding the map equally across the volume and examining the size and number of the resulting fragments. In vehicle-treated mice, the network breaks up into more objects with the same processing compared to the samples from IL10-treated mice (Fig 7E, n =75 areas, $P=0.026$, unpaired t-test). These data quantify the greater organization of the ICC networks in IL10-treated mice. To illustrate this difference, we show examples of the largest connected Kit-positive ICC networks in two fields in the distal antrum that were segmented from a vehicle-treated and an IL-10 treated mouse (Fig 7F). These selections show a higher level of connectivity of the ICC networks in the IL-10 treated mice.

Effect on Gastric Emptying of Treatment with a Lower Dose of IL10

We also tested a 10 fold lower dose of IL10 in a separate cohort of diabetic NOD mice with delayed emptying. After onset of delay, mice were treated with 100ng IL10 twice a day. One mouse was removed from the study because glucose levels dropped to pre-diabetic levels. Gastric emptying returned to normal values in all 4 remaining mice (Fig 8, $T_{1/2}$ pre-delay = 87±8, pre IL10 treatment 196±11 and post IL10 treatment 110±8 min, $P<0.05$ pre versus post IL10) in a mean of 4.8 weeks, which was not a significantly different time to respond than found for the higher 1μg dose of IL10.

DISCUSSION

This study demonstrates that treatment with IL10 for delayed gastric emptying in diabetic mice restores gastric emptying to normal. This effect was accompanied by reduced levels of oxidative stress indicated by lower levels of the systemic lipid peroxidation marker, malondialdehyde, up-regulation of HO1 expression in the muscularis propria, higher integrity of Kit-positive ICC networks and by normalization of electrical slow wave activity.

Mice received 0.1 or 1μg IL10 twice a day, which is equivalent to 5-50μg/kg twice a day in humans.[29] Animals receiving the high dose of IL10 were not more sick than vehicle-treated mice, even though the mice under study had elevated blood glucose levels and gastroparesis. This is consistent with the experience with IL10 in humans, where treatment was shown to be safe and well tolerated at doses up to 25μg/kg with a few complications in subjects receiving 50-100μg/kg.[29, 30]

There was no consistently beneficial clinical response when IL10 was tested as a treatment for inflammatory bowel disease, rheumatoid arthritis or psoriasis[31] and in trials studying the effectiveness of IL10 in treatment of Crohn's disease, 20μg/kg resulted in increased expression of blood markers for inflammation.[32] The side effects observed, and lack of response to treatment has resulted in withdrawal of many patients treated with IL10 for Crohn's disease.[33] Our studies indicate that diabetic delayed gastric emptying in mice is not the result of an adaptive immune response but is due to a particular, macrophage-dependent mechanism,[8] which may explain why IL10 was effective in treating delayed emptying. Interestingly, IL10 was very effective in preventing post-operative ileus in mice,[16] also an innate response and supporting the concept that IL10 has more promise in innate immune rather than adaptive immune disorders. This study tested IL10 as a treatment for delayed gastric emptying and our study was not designed to find out if continued treatment is necessary to sustain the benefit. Further studies are necessary to determine the optimal dose and means of delivery of IL10 to most effectively treat gastroparesis. One possibility includes delivering IL10 orally using IL10-releasing lactococci as has been recently demonstrated in another study.[34]

We also showed that IL10 was effective in normalizing disrupted slow wave activity. Disrupted slow wave activity was previously reported in studies that compared the electrical properties of the gastric muscularis propria in NOD mice with long-standing and uncontrolled diabetes to activity in non-diabetic mice.[9] Irregular electrical rhythmicity in human stomach is also reported in diabetic gastroparesis.[21-23] In diabetic rodents, other

studies have reported observations similar to those that we found in vehicle-treated mice with delayed emptying.[9, 20] Both groups observed more severe changes in slow wave activity than we did but the pattern of changes was the same. Ordog et al reported diminished slow wave frequencies and amplitudes in cells that had electrical activity, although a majority of cells in distal stomach (81%) exhibited no slow wave.[9] Others reported absence of slow waves in 13 out of 19 preparations from diabetic rats with irregular and small events in preparations where activity was detected.[20] The disadvantage of these previous studies was that they could not do what we did for this report where we examined the electrical properties and cellular pathology of the tissues from each mouse knowing the history of the gastric emptying status of that mouse. All our mice received low doses of insulin to maintain blood glucose levels at 500-600 mg/dL; so they were not subject to severe ketoacidosis and could be maintained alive for longer than animals that did not receive insulin. This insulin treatment may account for the more modest abnormalities in slow wave properties from our vehicle-treated animals compared to previous reports.[9, 20]

The intrinsic pacing of the electrical slow wave in the stomachs of dogs, pigs and humans has been shown, using multi-electrode recordings, to be driven by a region close to the greater curvature in the proximal corpus[35-37] and tissue dissection studies indicate that this is likely also true for mouse stomach.[38] This pacemaker region corresponds to region 5 (proximal corpus) in our experiments and our observations suggest that IL10 treatment results in better coupling of the pacemaker in the corpus to antral ICC. The immuno-histochemical data showing that the vehicle-treated mice have disrupted ICC networks and IL10-treated mice have more even, and better connected ICC networks across the whole tissue, support inefficient coupling of the pacemaker in the corpus to other parts of the tissue in vehicle-treated mice that continue to have delayed gastric emptying. Alternatively, abnormal slow waves in the distal antrum of vehicle-treated mice may reflect an accumulation of electrical defects as the slow wave propagates from corpus to antrum through the disrupted ICC networks. Our previous studies have reported that Kit protein expression by immunoblotting is low in diabetic mice with delayed gastric emptying, high in diabetic mice with normal gastric emptying and high in mice treated to restore gastric emptying to normal.[7, 8, 39] Since we observed whole tissue changes to ICC networks but no direct correlation between the uneven slow wave activity and loss of ICC at the level of the recording sites, we conclude that the key improvement in ICC networks was network integrity. Differences in Kit protein levels might be partly due to the brighter Kit immuno-reactivity in the tissues from IL10-treated mice.

The variable that was most affected by IL10 treatment was the degree of variability in the electrical slow wave amplitudes. IL10 reduced peak to peak variability by more than 50% (see Fig 4A for quantification). These differences in peak to peak height are unusual and are predicted to disrupt coordination and force of contractility in the distal stomach regions where the abnormalities were most prominent. In humans with severe symptoms of diabetic gastroparesis and delayed gastric emptying, the electrogastrogram recordings show a high degree of variability consistent with disorganized pacemaking activity.[21-23] This variability is normalized following successful treatment.[21] It is difficult to correlate the magnitude of the improvement in electrical activity necessary for treating gastroparesis in humans compared to our studies in mice since the extracellular human electrogastrogram and the

mouse intracellular slow waves are two different readouts of associated phenomena. However the observations are congruent in that disorganization and variability in electrical activity in the signal directly correlates with impaired gastric emptying. Irregular slow wave activity may emerge in the distal stomach as a cumulative effect of ICC defects proximal to the site of the recorded electrical defect. The presence of a difference in the resting membrane potential from proximal to distal regions of the stomach in IL10-treated mice may also reflect improved function of ICC networks in mice with normal emptying. ICC have been shown to regulate membrane potential and by doing so to act as a rheostat setting the local efficiency of excitation-contractility coupling.[28]

Treatment with IL10 resulted in increased HO1 expression in macrophage-like cells and changes to ICC networks. It is likely that these macrophage-like cells are M2, CD206-positive macrophages but we cannot be certain because we did not directly determine the CD206 or F4/80 expression of the HO1 positive cells in the diabetic mice with delayed gastric emptying that were successfully treated with IL10. The likelihood that the macrophage-like cells are M2, CD206-positive macrophages is based on the findings that HO1 expression was identified in CD206-positive, F4/80-positive macrophages but not other cell types in diabetic mice with baseline normal gastric emptying that were treated with IL10. Furthermore, we have not previously seen HO1 expression in CD206-negative, gastric muscularis propria macrophages and HO1 expression is very low in gastric muscularis propria in non-diabetic mice or diabetic mice with delayed gastric emptying.[7, 8] The mechanism for the response to IL10 may be due to intrinsic cyto-protective properties of M2-macrophages[40] or due to suppression of pro-inflammatory M1 macrophages.[19] Up-regulation of CO production due to increased expression of HO1 may also be key as we showed that CO alone is sufficient to normalize gastric emptying[39] and multiple studies have demonstrated HO1 activation in cyto-protective responses to IL10.[17, 41] The impact of changing macrophage numbers and phenotype on gastric emptying may also be mediated by other factors that have not yet been identified. Our results also demonstrate that IL10 induces HO1 in myenteric ganglia in some mice: an effect that has not been previously reported but which might be a specific response in some diabetic NOD mice to IL10. Changes to MDA values may be a consequence or a mediator of the effect of IL10. We did not power our study to determine how IL10 affects MDA values. MDA values in diabetic mice with delayed gastric emptying were elevated and highly variable, as previously observed.[7] We randomly assigned the 10 mice with diabetes and delayed gastric emptying to treatment with IL10 or vehicle. By chance, the mice receiving IL10 had the highest MDA values yet they still responded with normalization of gastric emptying, despite being sicker than the mice receiving vehicle, which did not reverse the delay in gastric emptying and did not achieve normal MDA values. However given the study was not powered to distinguish MDA levels between the groups assigned to vehicle and IL10 we cannot assume a trend because the mice came from a population of animals that was blindly assigned without respect to MDA levels.

We did not observe a restoration of NOS activity as previously reported when we treated delayed gastric emptying in diabetic NOD mice using hemin or CO.[7, 39] This was unexpected and requires further confirmation.

The most parsimonious explanation for the cellular response to IL10 is that IL10 induces several molecular pathways that are beneficial to gastric function, including reduced oxidative stress, suppression of pro-inflammatory cytokines and activation of cellular repair. In these respects, IL10 may have an advantage over inducing HO1 with hemin or administering CO since, for example, suppression of M1-macrophages is a direct effect of IL10[42] and activation of monocytes by IL10 is not dependent on HO1 activity.[43] The mechanisms for repair to ICC networks were also likely to be due to an interaction of several processes including reduced oxidative stress, which lowers the levels of toxic products of lipid peroxidation, as well as activation of proliferative and anti-cell death pathways by CO.[14]

In conclusion, these studies suggest that IL10 is a promising therapy for diabetic gastroparesis in humans because the response to treatment in diabetic NOD mice affects two well-defined characteristics of human diabetic gastroparesis. Those features are irregular electrical rhythmicity[21-23] and a correlation between damage to ICC and delayed emptying.[5] There is also reason to propose that modulating macrophage phenotype using IL10 will be effective in humans because the number of CD206 positive macrophages also correlates with number of ICC in humans with diabetic gastroparesis.[12]

ACKNOWLEDGEMENTS

We thank Mr. G. Charles Forde, Mr. Gary Stoltz, Dr. Ariel Caride, Mrs. Kristy Zodrow, Mrs. Stephanie Hein, Mr. Ron Karwoski and Mr. Robert Allen for their assistance.

Grant Support: Supported by NIH P01DK068055, a Pilot and Feasibility Award from the Mayo Clinic Center for Cell Signaling in Gastroenterology (P30DK084567) and the Mayo Clinic Metabolomics Resource Core (U24DK100469).

Abbreviations

HO1	heme oxygenase 1
ICC	interstitial cells of Cajal
IL10	interleukin 10
MDA	malondialdehyde
NOD	non-obese diabetic
nNOS	neuronal nitric oxide synthase

REFERENCES

1. Farrell FJ, Keeffe EB. Diabetic gastroparesis. Dig Dis. 1995; 13:291–300. [PubMed: 8542664]
2. Vittal H, Farrugia G, Gomez G, et al. Mechanisms of disease: the pathological basis of gastroparesis--a review of experimental and clinical studies. Nat Clin Pract Gastroenterol Hepatol. 2007; 4:336–46. [PubMed: 17541447]
3. Camilleri M, Grover M, Farrugia G. What are the important subsets of gastroparesis? Neurogastroenterol Motil. 2012; 24:597–603. [PubMed: 22626059]
4. Camilleri M. Clinical practice. Diabetic gastroparesis. N Engl J Med. 2007; 356:820–9. [PubMed: 17314341]

5. Grover M, Bernard CE, Pasricha PJ, et al. Clinical-histological associations in gastroparesis: results from the Gastroparesis Clinical Research Consortium. Neurogastroenterol Motil. 2012; 24:531–9, e249. [PubMed: 22339929]
6. Grover M, Farrugia G, Lurken MS, et al. Cellular changes in diabetic and idiopathic gastroparesis. Gastroenterology. 2011; 140:1575–85. e8. [PubMed: 21300066]
7. Choi KM, Gibbons SJ, Nguyen TV, et al. Heme oxygenase-1 protects interstitial cells of Cajal from oxidative stress and reverses diabetic gastroparesis. Gastroenterology. 2008; 135:2055–64. 64, e1–2. [PubMed: 18926825]
8. Choi KM, Kashyap PC, Dutta N, et al. CD206-positive M2 macrophages that express heme oxygenase-1 protect against diabetic gastroparesis in mice. Gastroenterology. 2010; 138:2399–409. 409, e1. [PubMed: 20178793]
9. Ordog T, Takayama I, Cheung WK, et al. Remodeling of networks of interstitial cells of Cajal in a murine model of diabetic gastroparesis. Diabetes. 2000; 49:1731–9. [PubMed: 11016458]
10. Watkins CC, Sawa A, Jaffrey S, et al. Insulin restores neuronal nitric oxide synthase expression and function that is lost in diabetic gastropathy. J Clin Invest. 2000; 106:373–84. [PubMed: 10930440]
11. Choi KM, Zhu J, Stoltz GJ, et al. Determination of gastric emptying in nonobese diabetic mice. Am J Physiol Gastrointest Liver Physiol. 2007; 293:G1039–45. [PubMed: 17884976]
12. Bernard CE, Gibbons SJ, Mann IS, et al. Association of low numbers of CD206-positive cells with loss of ICC in the gastric body of patients with diabetic gastroparesis. Neurogastroenterol Motil. 2014; 26:1275–84. [PubMed: 25041465]
13. Bharucha AE, Kulkarni A, Choi KM, et al. First-in-human study demonstrating pharmacological activation of heme oxygenase-1 in humans. Clin Pharmacol Ther. 2010; 87:187–90. [PubMed: 19956091]
14. Gibbons SJ, Verhulst PJ, Bharucha A, et al. Review article: carbon monoxide in gastrointestinal physiology and its potential in therapeutics. Aliment Pharmacol Ther. 2013; 38:689–702. [PubMed: 23992228]
15. Moore BA, Otterbein LE, Turler A, et al. Inhaled carbon monoxide suppresses the development of postoperative ileus in the murine small intestine. Gastroenterology. 2003; 124:377–91. [PubMed: 12557144]
16. Stoffels B, Schmidt J, Nakao A, et al. Role of interleukin 10 in murine postoperative ileus. Gut. 2009; 58:648–60. [PubMed: 19359433]
17. Lee TS, Chau LY. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med. 2002; 8:240–6. [PubMed: 11875494]
18. Deng B, Wehling-Henricks M, Villalta SA, et al. IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. J Immunol. 2012; 189:3669–80. [PubMed: 22933625]
19. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol. 2010; 11:889–96. [PubMed: 20856220]
20. Xue L, Suzuki H. Electrical responses of gastric smooth muscles in streptozotocin-induced diabetic rats. Am J Physiol. 1997; 272:G77–83. [PubMed: 9038879]
21. Koch KL, Stern RM, Stewart WR, et al. Gastric emptying and gastric myoelectrical activity in patients with diabetic gastroparesis: effect of long-term domperidone treatment. Am J Gastroenterol. 1989; 84:1069–75. [PubMed: 2773901]
22. Chen J, McCallum RW. Gastric slow wave abnormalities in patients with gastroparesis. Am J Gastroenterol. 1992; 87:477–82. [PubMed: 1553934]
23. Chen JD, Lin Z, Pan J, et al. Abnormal gastric myoelectrical activity and delayed gastric emptying in patients with symptoms suggestive of gastroparesis. Dig Dis Sci. 1996; 41:1538–45. [PubMed: 8769276]
24. Cipriani G, Gibbons SJ, Verhulst PJ, et al. Diabetic mice lacking macrophages are protected against the development of delayed gastric emptying. Cell Mol Gastroenterol Hepatol. 2016; 2:40–7. [PubMed: 26771000]
25. Miller SM, Reed D, Sarr MG, et al. Haem oxygenase in enteric nervous system of human stomach and jejunum and co-localization with nitric oxide synthase. Neurogastroenterol Motil. 2001; 13:121–31. [PubMed: 11298990]

26. Miller SM, Farrugia G, Schmalz PF, et al. Heme oxygenase 2 is present in interstitial cell networks of the mouse small intestine. Gastroenterology. 1998; 114:239–44. [PubMed: 9453482]
27. el-Sharkawy TY, Morgan KG, Szurszewski JH. Intracellular electrical activity of canine and human gastric smooth muscle. J Physiol. 1978; 279:291–307. [PubMed: 671352]
28. Farrugia G, Lei S, Lin X, et al. A major role for carbon monoxide as an endogenous hyperpolarizing factor in the gastrointestinal tract. Proc Natl Acad Sci U S A. 2003; 100:8567–70. [PubMed: 12832617]
29. Huhn RD, Radwanski E, Gallo J, et al. Pharmacodynamics of subcutaneous recombinant human interleukin-10 in healthy volunteers. Clin Pharmacol Ther. 1997; 62:171–80. [PubMed: 9284853]
30. Moore KW, de Waal Malefyt R, Coffman RL, et al. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001; 19:683–765. [PubMed: 11244051]
31. Asadullah K, Sterry W, Volk HD. Interleukin-10 therapy--review of a new approach. Pharmacol Rev. 2003; 55:241–69. [PubMed: 12773629]
32. Tilg H, van Montfrans C, van den Ende A, et al. Treatment of Crohn's disease with recombinant human interleukin 10 induces the proinflammatory cytokine interferon gamma. Gut. 2002; 50:191–5. [PubMed: 11788558]
33. Buruiana FE, Sola I, Alonso-Coello P. Recombinant human interleukin 10 for induction of remission in Crohn's disease. Cochrane Database Syst Rev. 2010:CD005109. [PubMed: 21069683]
34. Robert S, Gysemans C, Takiishi T, et al. Oral delivery of glutamic acid decarboxylase (GAD)-65 and IL10 by Lactococcus lactis reverses diabetes in recent-onset NOD mice. Diabetes. 2014; 63:2876–87. [PubMed: 24677716]
35. Egbuji JU, o'grady G, du P, et al. Origin, propagation and regional characteristics of porcine gastric slow wave activity determined by high-resolution mapping. Neurogastroenterol Motil. 2010; 22:e292–300. [PubMed: 20618830]
36. Lammers WJ, Ver Donck L, Stephen B, et al. Origin and propagation of the slow wave in the canine stomach: the outlines of a gastric conduction system. Am J Physiol Gastrointest Liver Physiol. 2009; 296:G1200–10. [PubMed: 19359425]
37. O'Grady G, Du P, Cheng LK, et al. Origin and propagation of human gastric slow-wave activity defined by high-resolution mapping. Am J Physiol Gastrointest Liver Physiol. 2010; 299:G585–92. [PubMed: 20595620]
38. Ordog T, Baldo M, Danko R, et al. Plasticity of electrical pacemaking by interstitial cells of Cajal and gastric dysrhythmias in W/W mutant mice. Gastroenterology. 2002; 123:2028–40. [PubMed: 12454859]
39. Kashyap PC, Choi KM, Dutta N, et al. Carbon monoxide reverses diabetic gastroparesis in NOD mice. Am J Physiol Gastrointest Liver Physiol. 2010; 298:G1013–9. [PubMed: 20378827]
40. Laskin DL, Sunil VR, Gardner CR, et al. Macrophages and tissue injury: agents of defense or destruction? Annu Rev Pharmacol Toxicol. 2011; 51:267–88. [PubMed: 20887196]
41. Chen S, Kapturczak MH, Wasserfall C, et al. Interleukin 10 attenuates neointimal proliferation and inflammation in aortic allografts by a heme oxygenase-dependent pathway. Proc Natl Acad Sci U S A. 2005; 102:7251–6. [PubMed: 15878989]
42. Mantovani A, Sica A, Sozzani S, et al. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 2004; 25:677–86. [PubMed: 15530839]
43. Jung M, Sabat R, Kratzschmar J, et al. Expression profiling of IL-10-regulated genes in human monocytes and peripheral blood mononuclear cells from psoriatic patients during IL-10 therapy. Eur J Immunol. 2004; 34:481–93. [PubMed: 14768053]

Synopsis

Interleukin 10 reversed delayed gastric emptying in diabetic mice. It increased heme oxygenase 1 expression and normalized electrical activity and networks of interstitial cells of Cajal in the stomach. Thus, interleukin 10 is a potential therapy for diabetic gastroparesis.

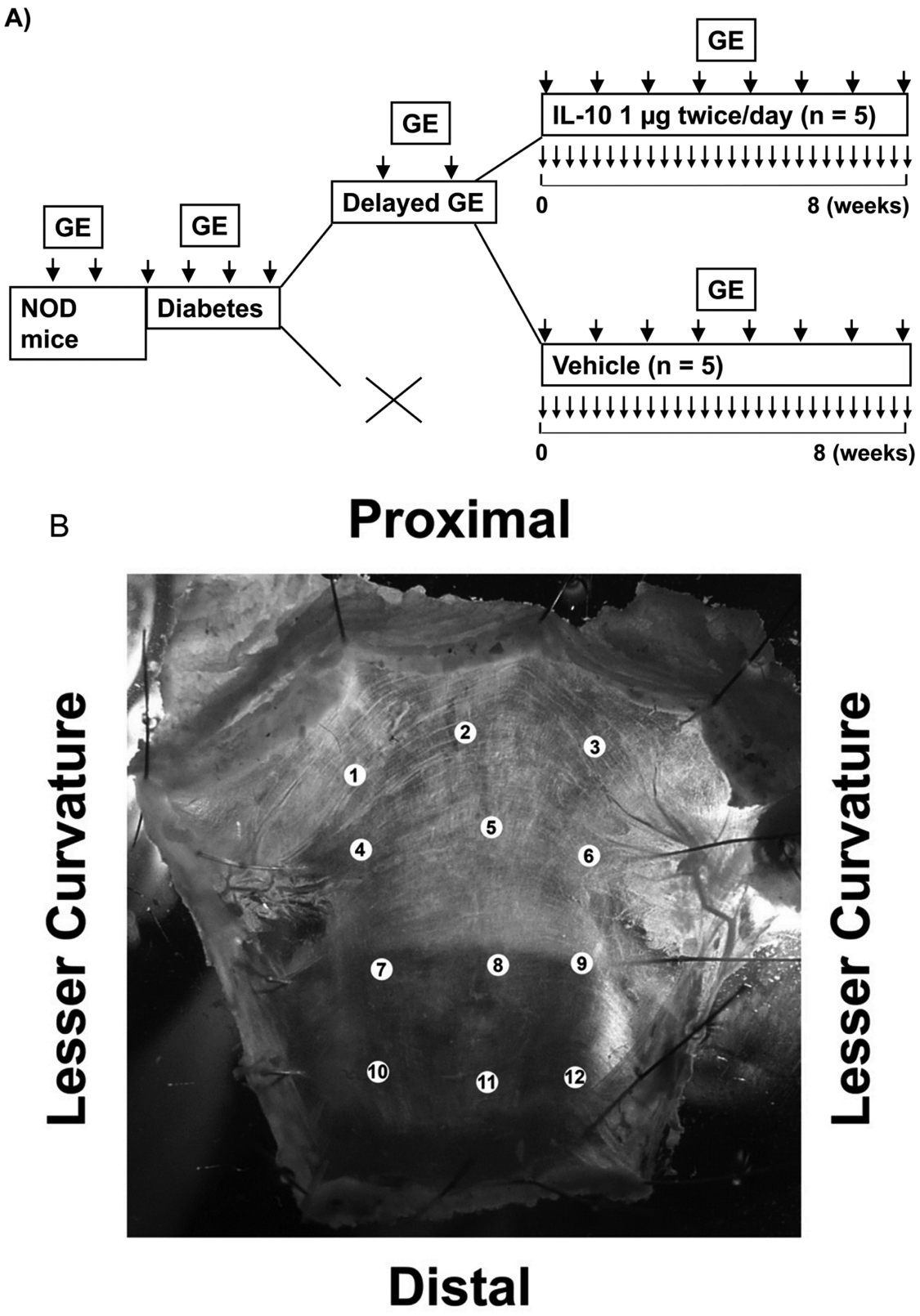
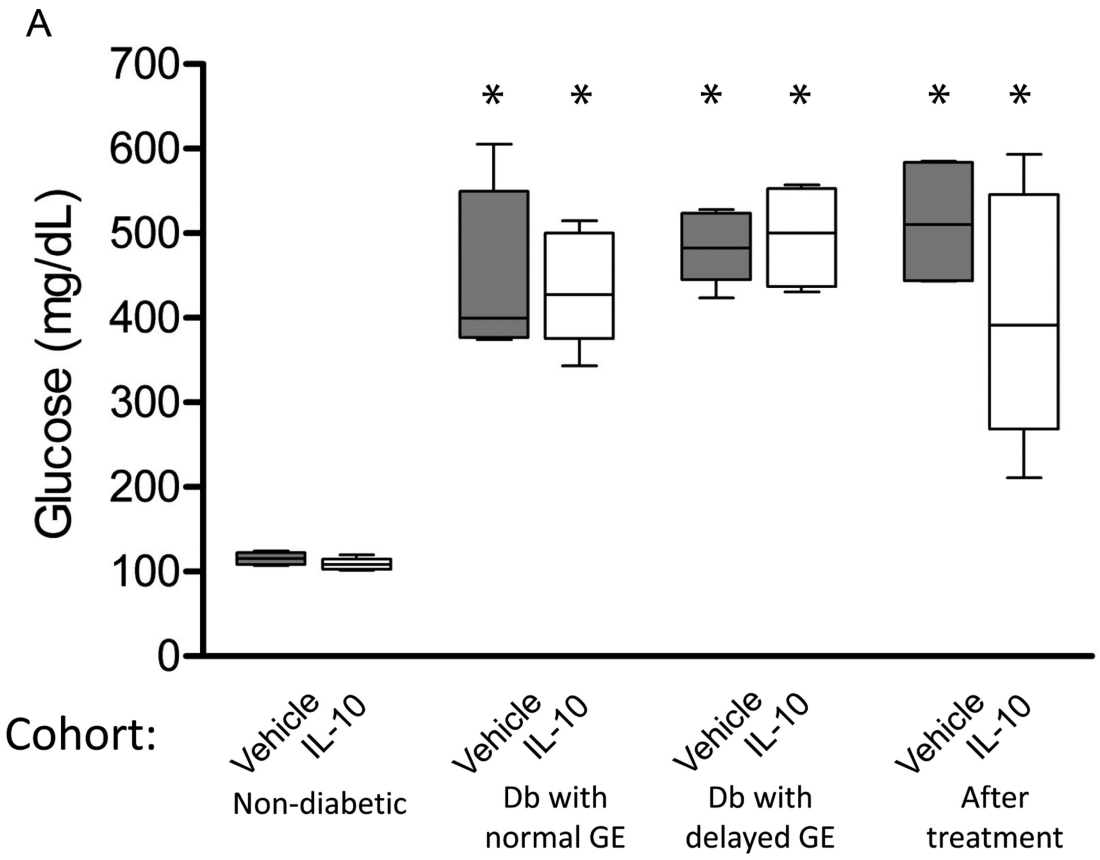
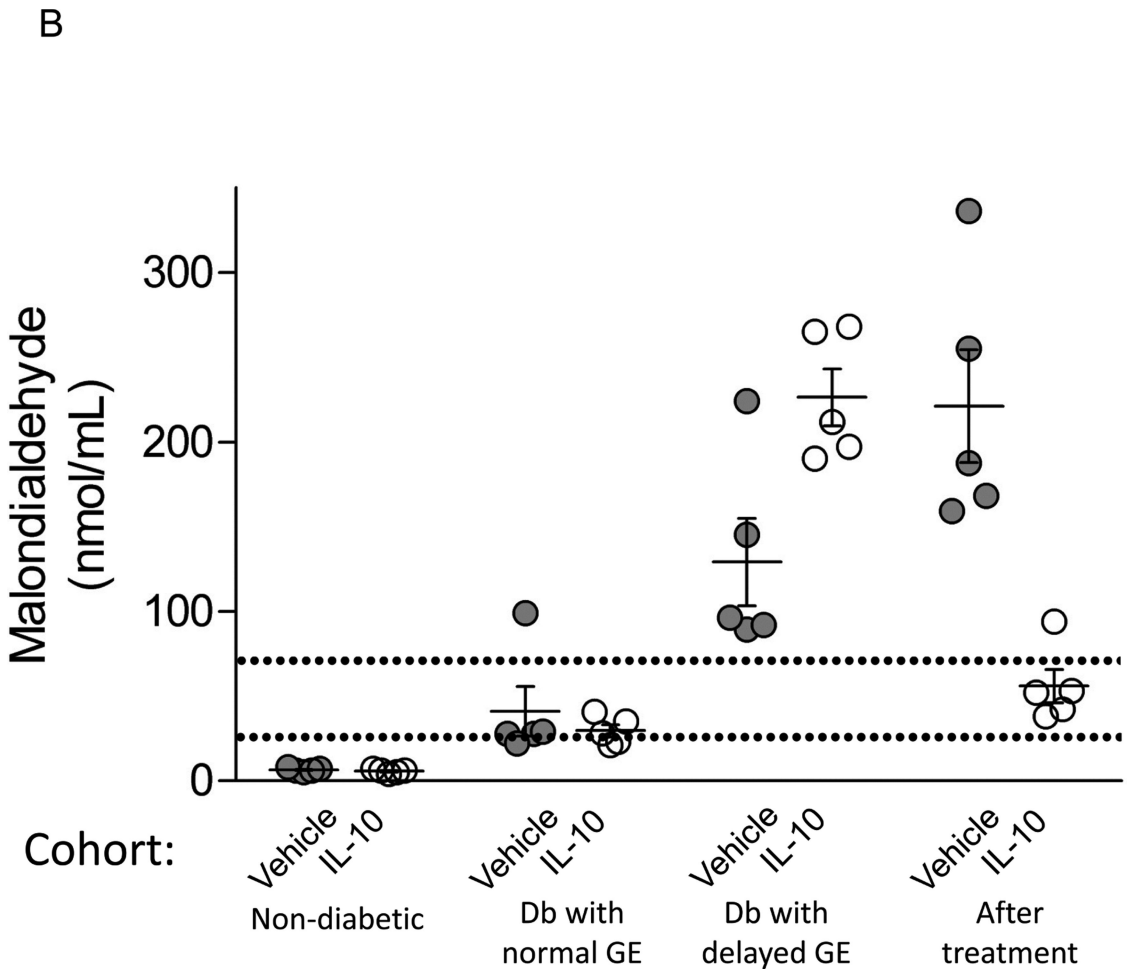


Figure 1.

A) Experimental design: Gastric emptying is indicated by larger arrows, treatment by small arrows. B) Location of electrical recording and image collection sites.





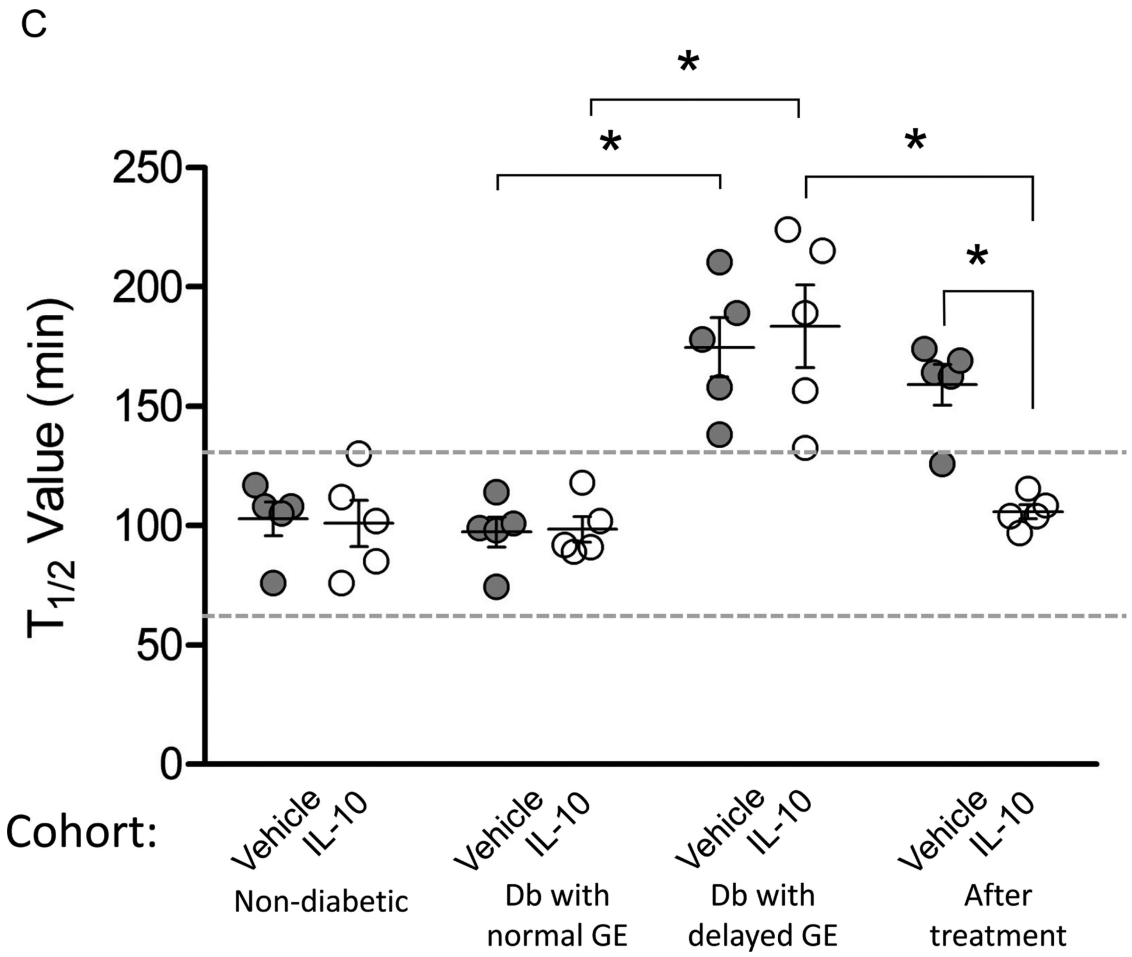
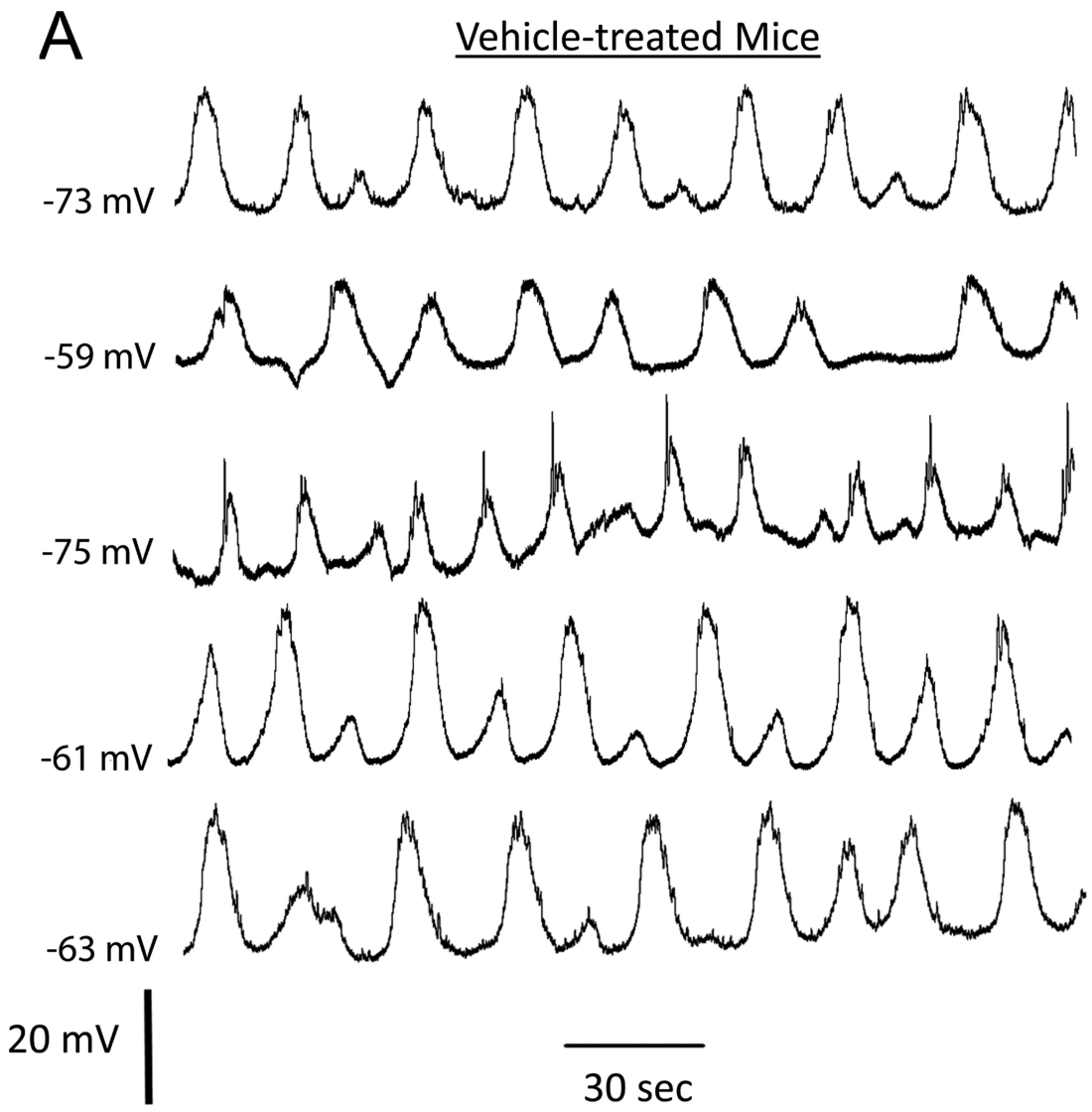


Figure 2.

Effects of IL10 treatment. A) Glucose levels (medians with IQR: bars, $5^{th}/95^{th}$ percentiles: whiskers). Higher glucose for diabetic vs non-diabetic-*. (n=5, 2-way ANOVA with Bonferroni post-test). B) Malondialdehyde levels were significantly associated with delayed gastric emptying and response to IL10 treatment but not diabetes. Circles: values for each mouse. Whiskers: mean±SE. Dotted lines: $5^{th}/95^{th}$ percentile of MDA levels in diabetic NOD mice with normal gastric emptying. C) Gastric emptying: Data are circles: each mouse, means±SE: whiskers, dotted lines: $5^{th}/95^{th}$ percentile of T½ values in non-diabetic mice. $P<0.05$ indicated by * (n=5, 1-way ANOVA with Tukey's post-test)



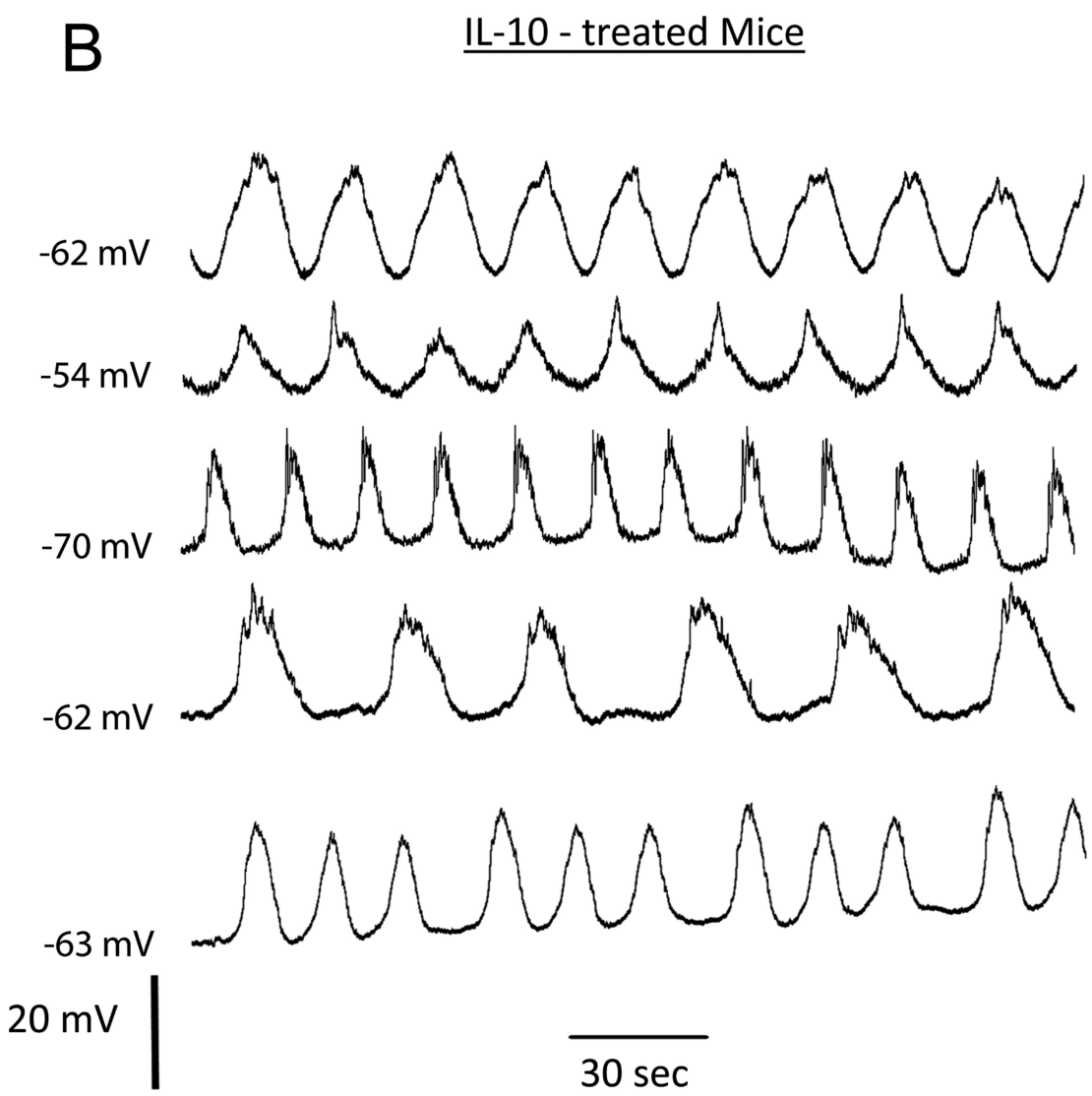
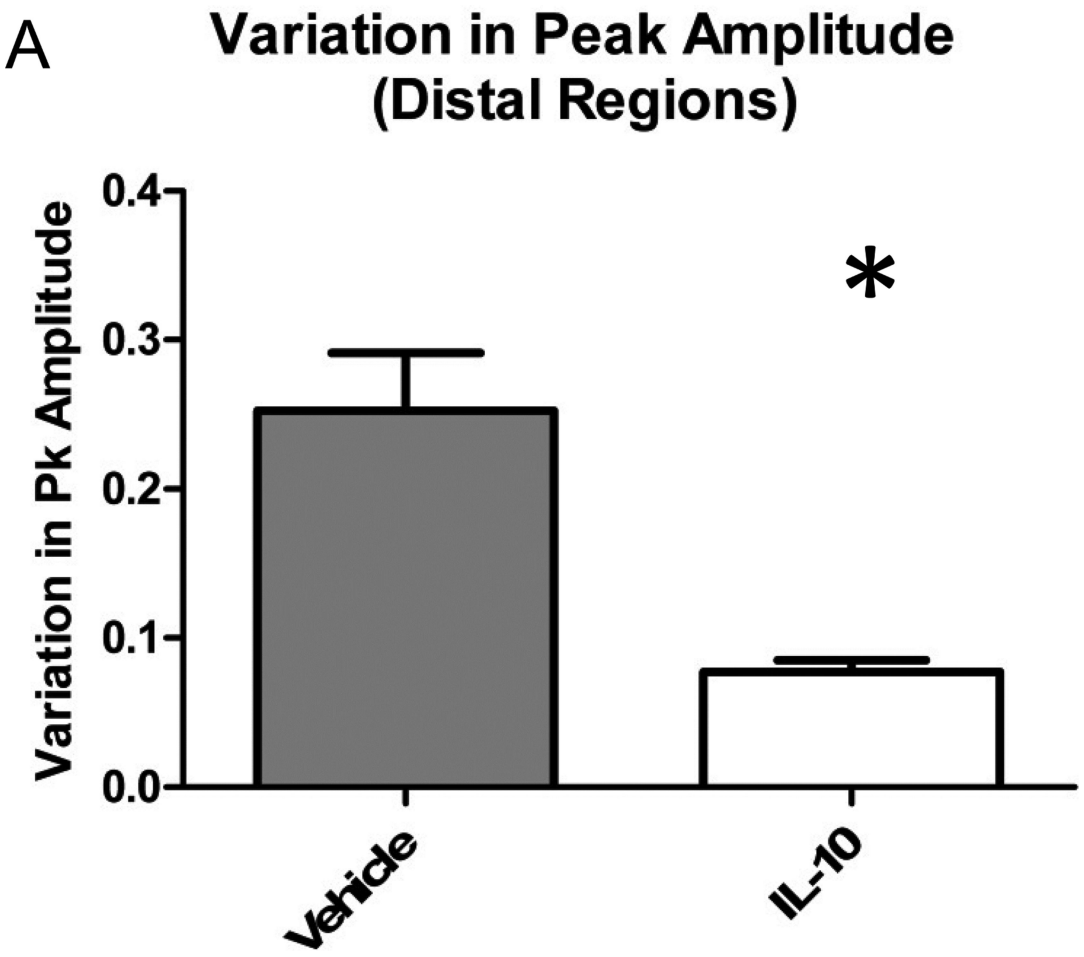
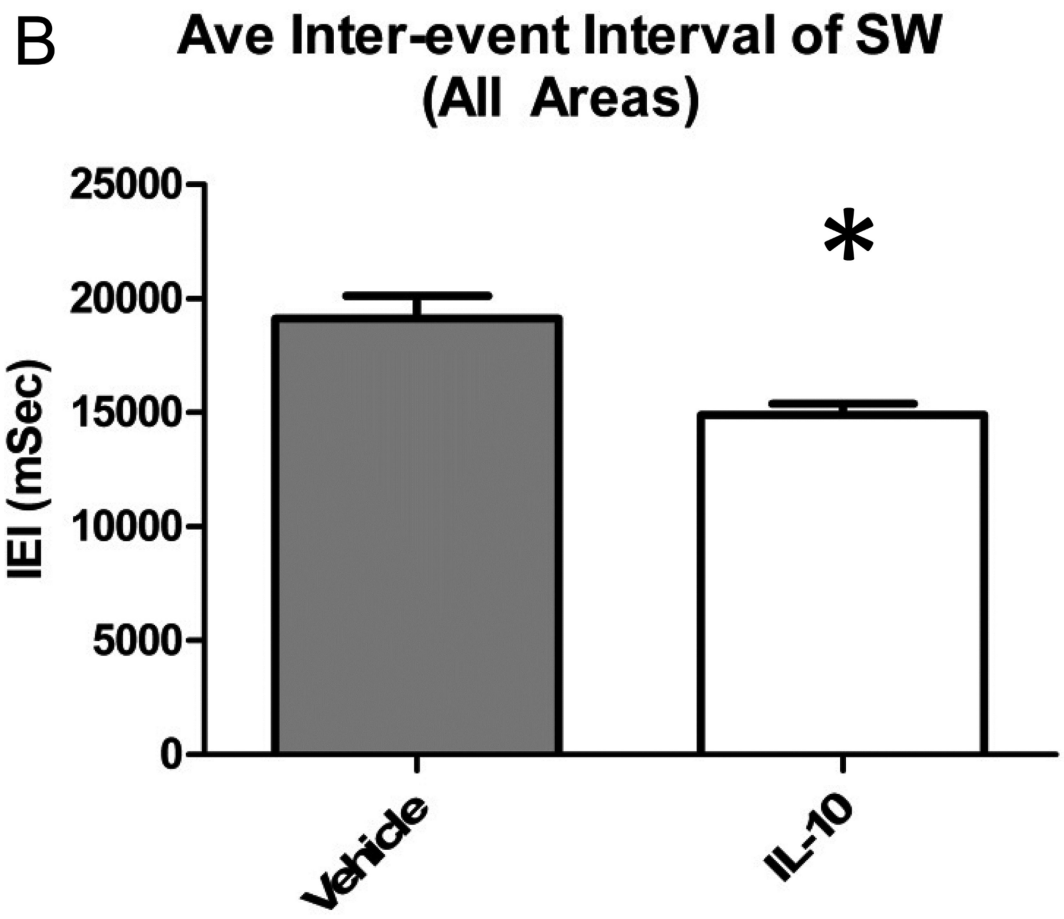
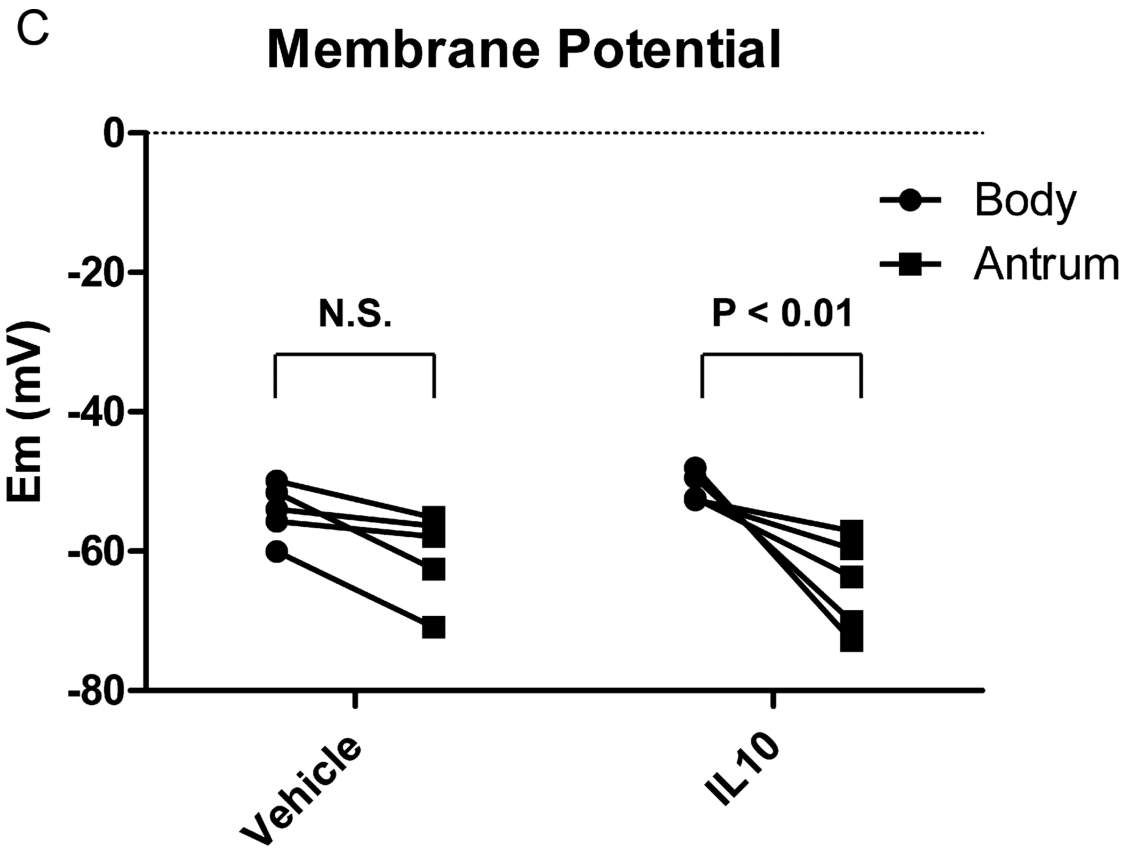


Figure 3.

Electrical Activity. Slow wave activity in the distal antrum of all vehicle-treated (A) and IL10-treated mice (B). Note the variations in peak amplitude of the slow waves in the vehicle-treated mice that are absent in the IL10 treated mice.







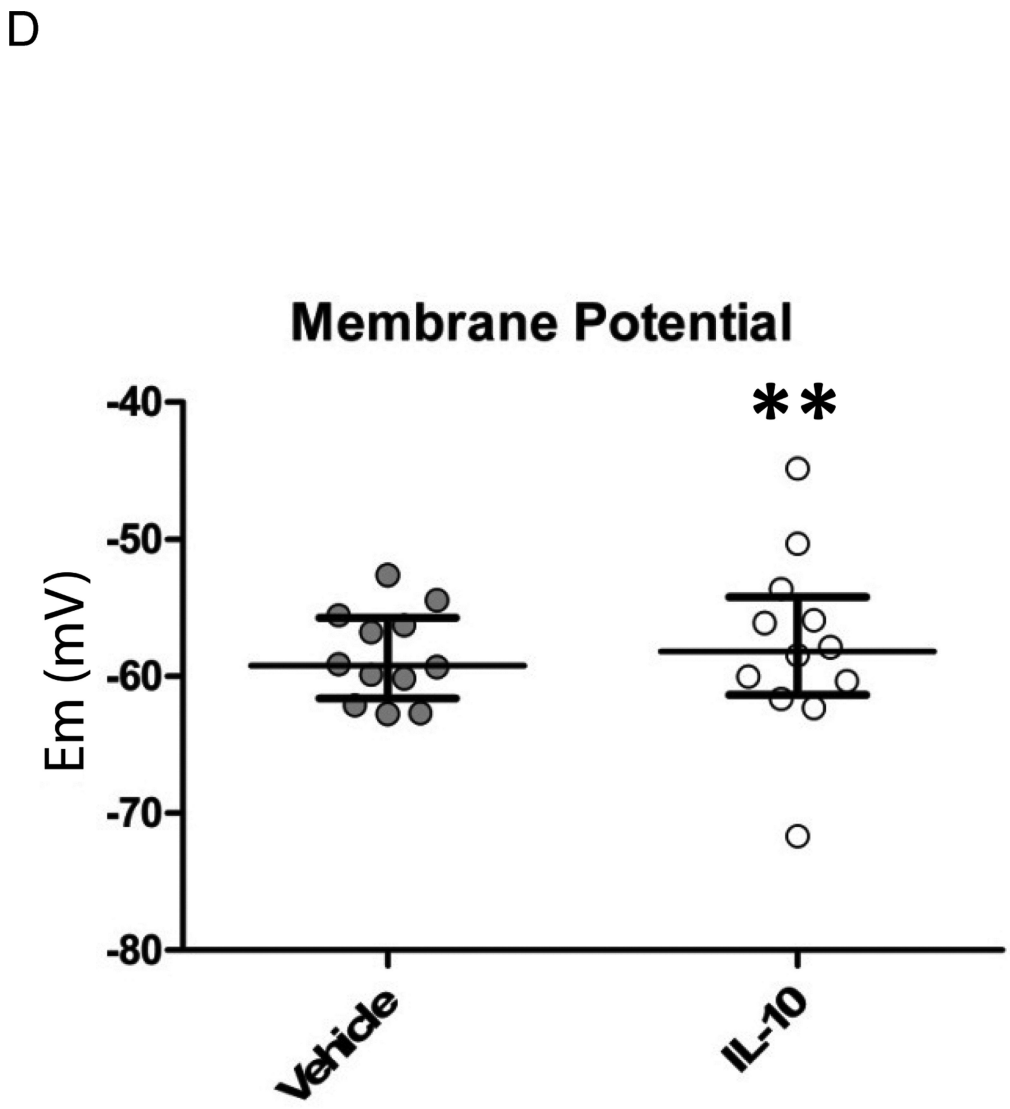
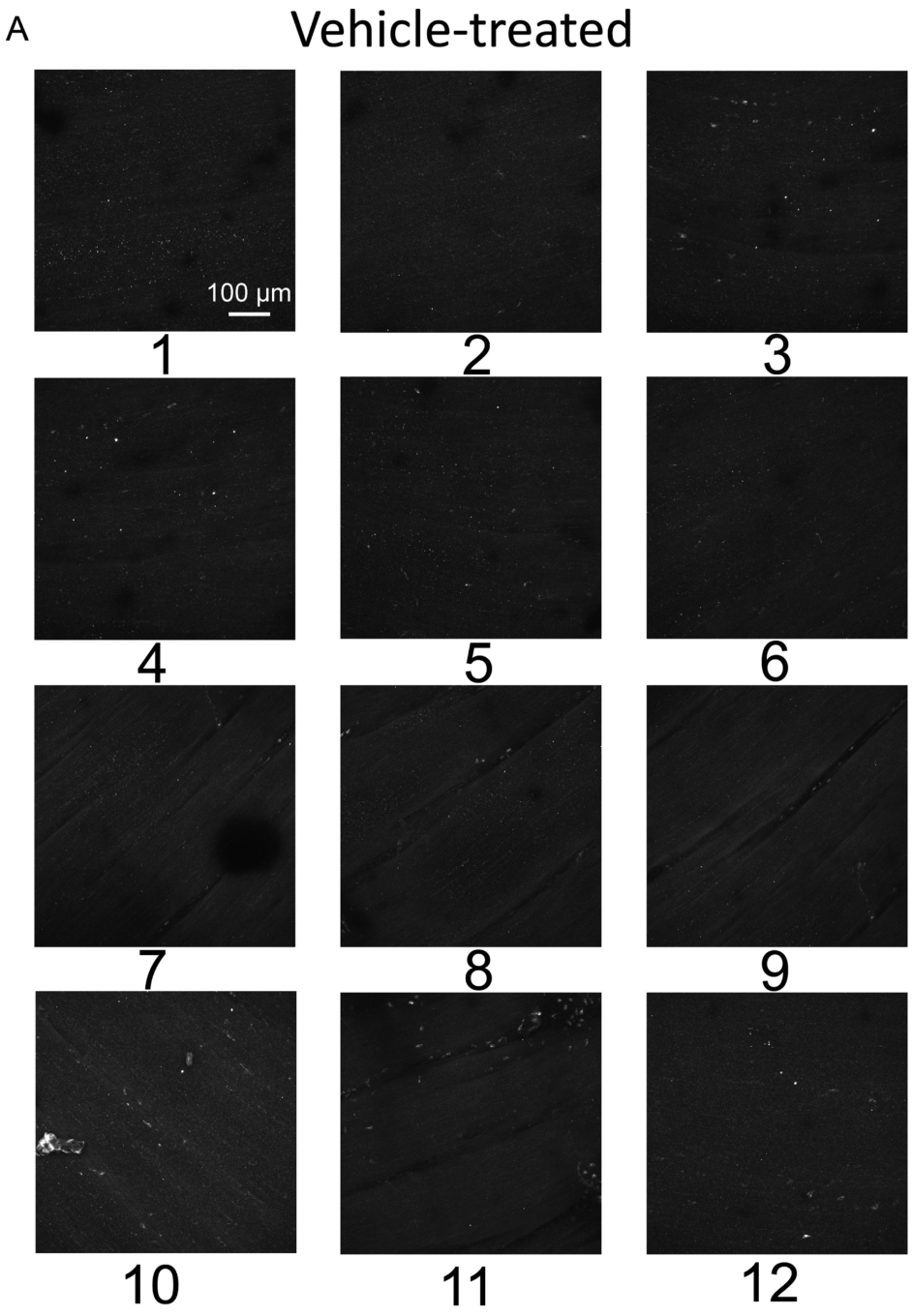
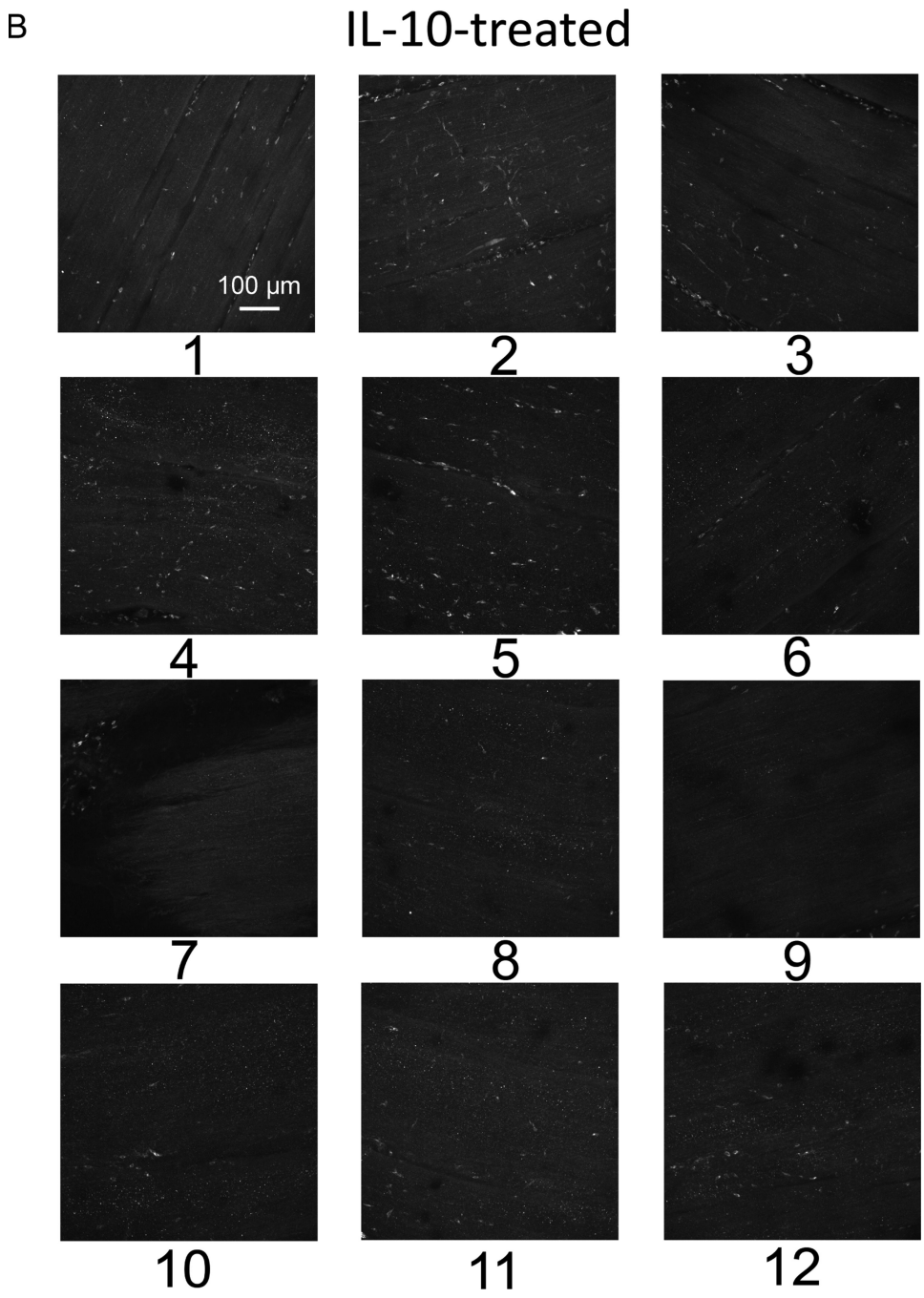
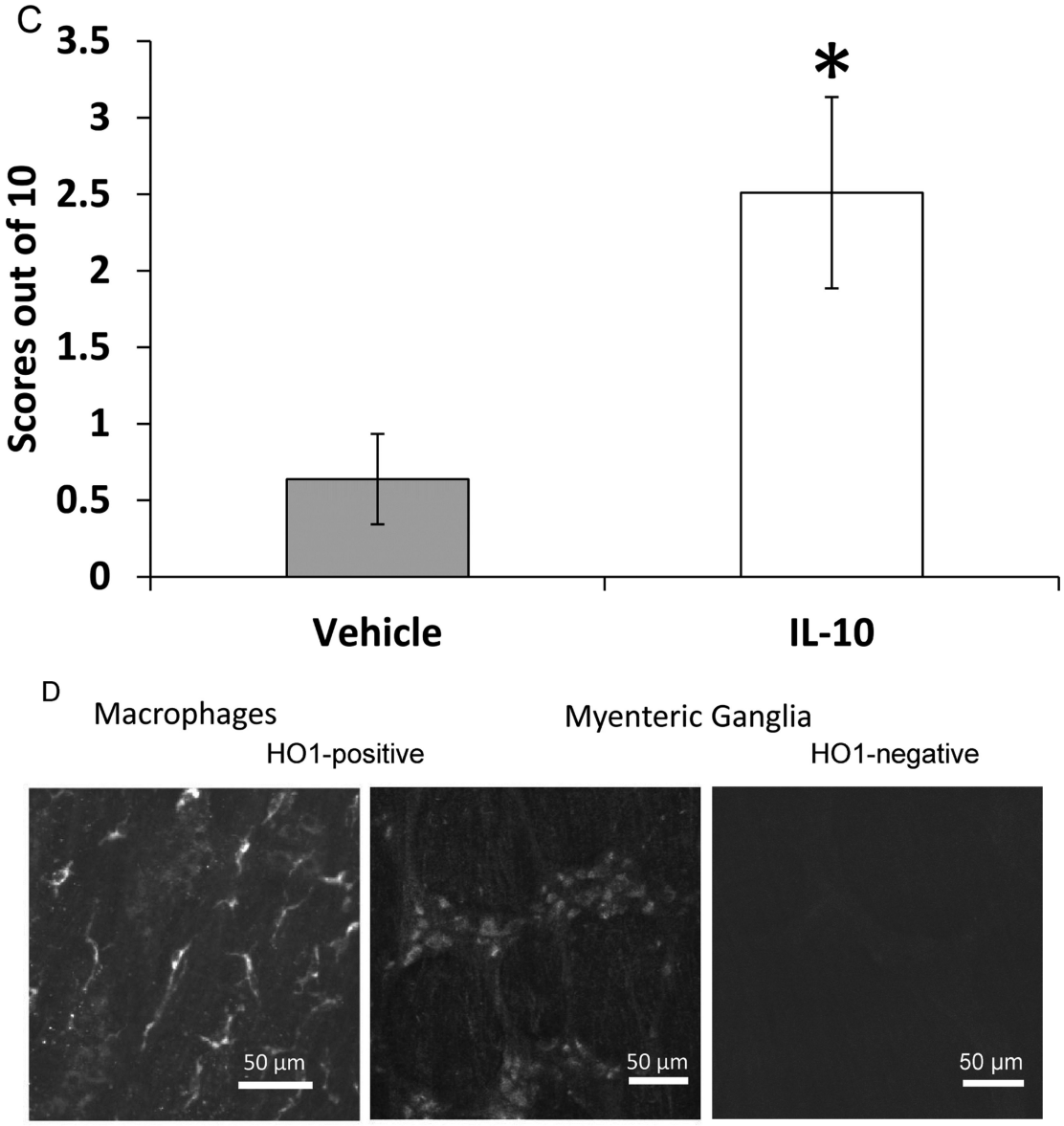


Figure 4.

Electrical Activity: Response to IL10 treatment. A) Variability of peak amplitudes in distal (regions 7-12) of the stomach. Means±SEM, *-P<0.05, one way ANOVA with Bonferroni correction, n=5. B) Quantification of the inter-event intervals for slow waves from all areas of all mice. Means±SEM, n=5. *=P<0.05, unpaired t-test. C) Resting membrane potential in the corpus (regions 1-3) and antrum (regions 7-9) of vehicle and IL10 treated diabetic mice. Each point represents the membrane potential for each mouse. Statistical differences were determined by repeated measures 2-way ANOVA with a Bonferoni post-test. N = 5 mice. D) Resting membrane potential. Note variance in membrane potential across tissue was different **-P=0.03, F-test, Means for 12 recording sites, n=5 mice. Whiskers: medians-IQR.







E

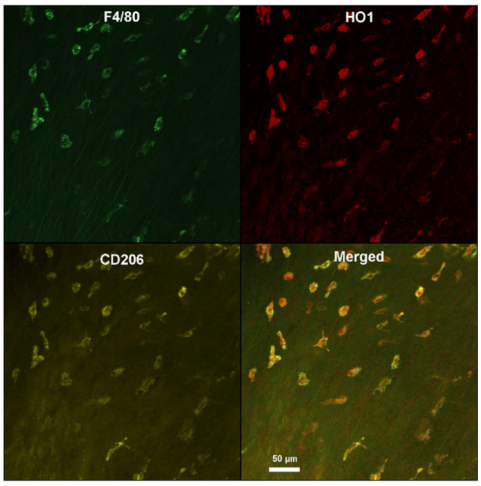


Figure 5.

HO1 expression. Image stacks of HO1 immunoreactivity in vehicle (A) and IL10 mice (B). Numbers indicate regions. C) Scores for HO1 immunoreactivity. Means±SEM, n=5, *-P<0.01, t-test). D) HO1 positive macrophagess (left panel) and neurons in a myenteric ganglion (middle panel) from the gastric body of IL10-treated mouse. The right panel shows a ganglion from an IL10-treated mouse where HO1 was not detected in the neurons. E) Image stacks of F4/80, HO1 and CD206 immunoreactivity in the muscularis propria of a diabetic mouse treated with IL10. Data are representative of immunolabeling in two different mice.

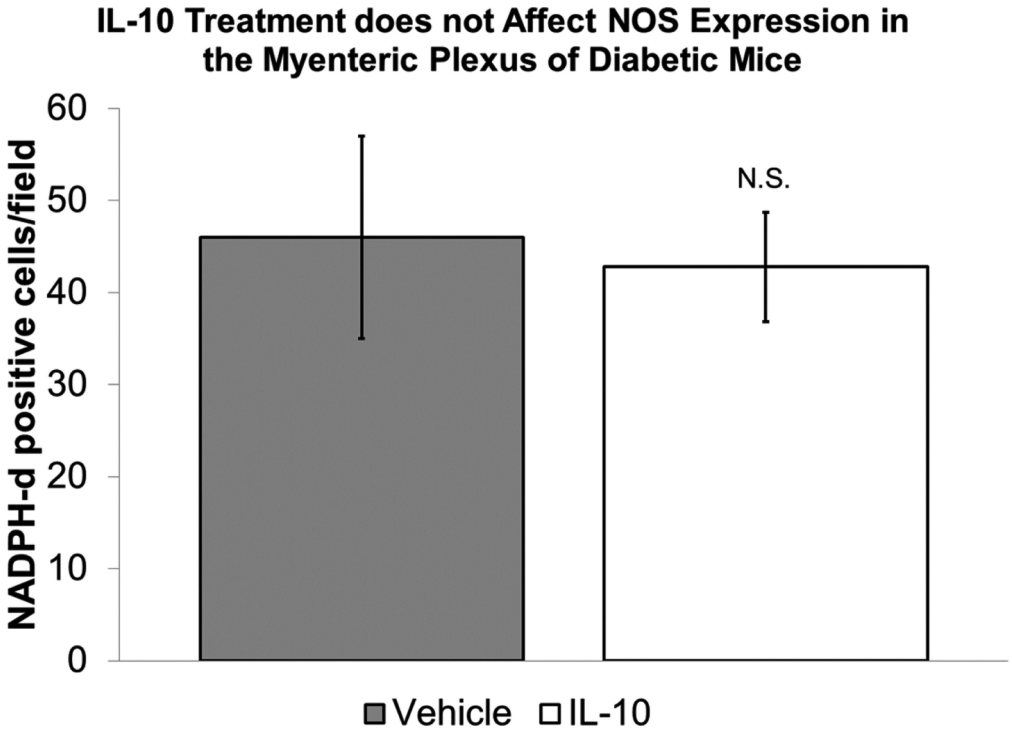
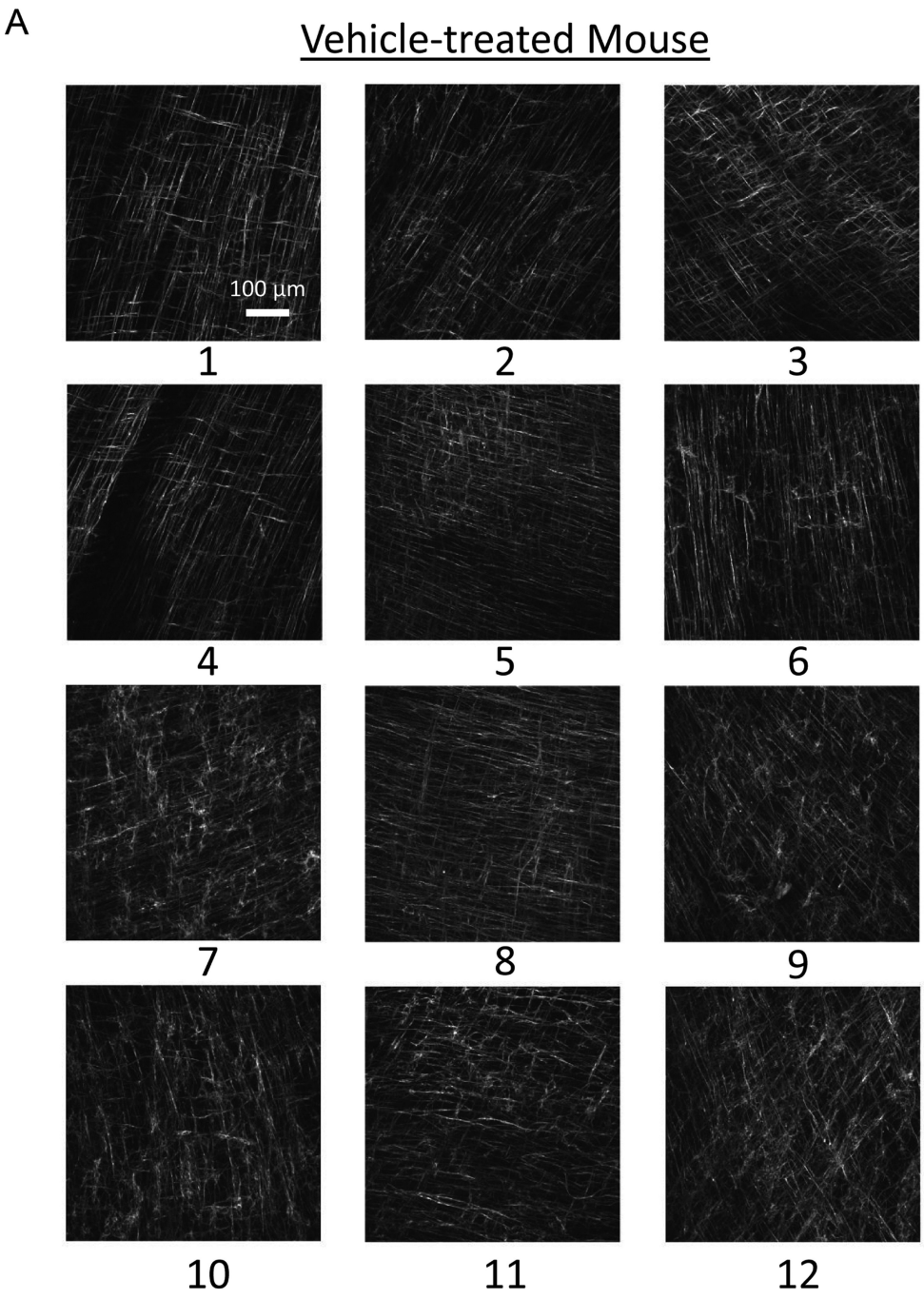
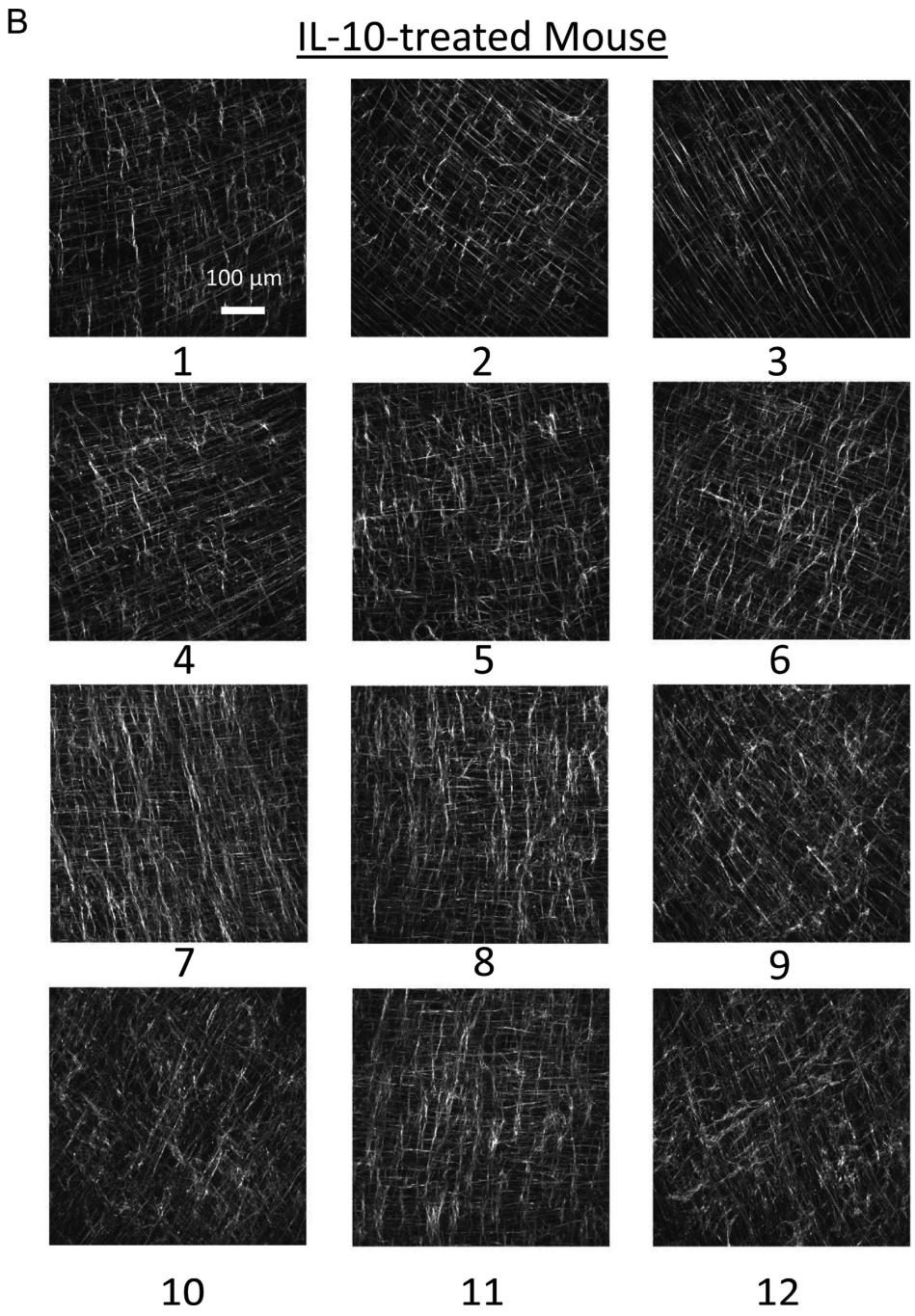


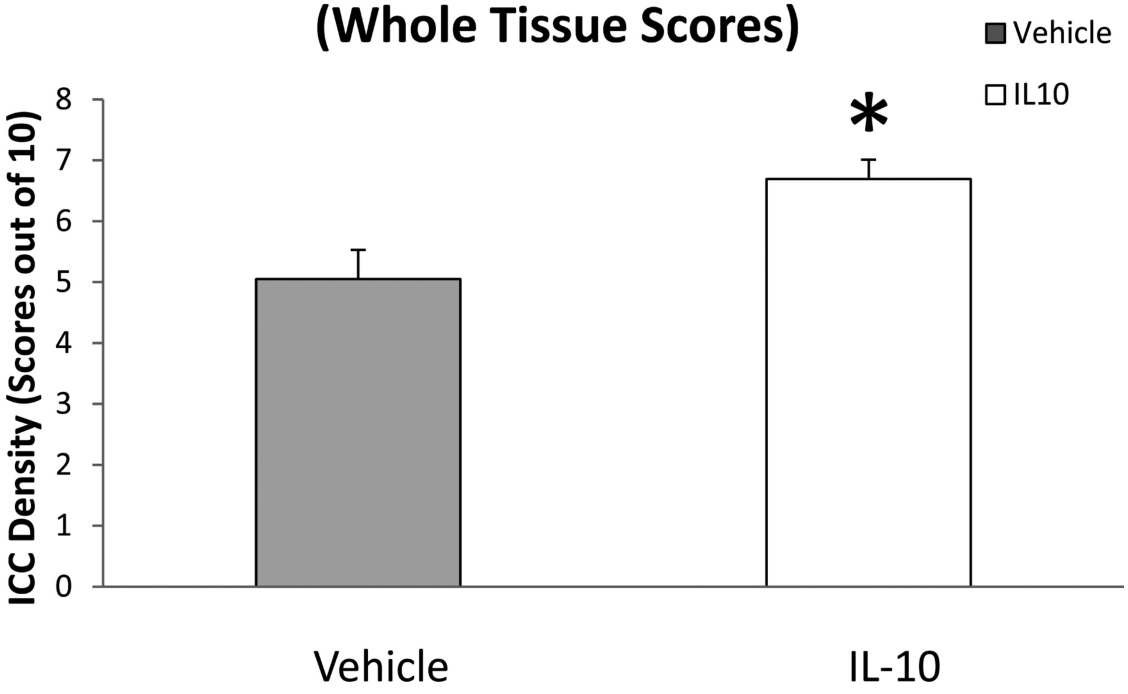
Figure 6.

NADPH-diaphorase activity. Quantification of neurons in the myenteric plexus from diabetic NOD mice with delayed gastric emptying treated with vehicle or 1 μg IL-10 twice a day. Positively labeled neurons were counted from 7 high power fields using a 20X objective from each tissue. A minimum of 174 neurons were counted from each tissue. There was no significant difference in the number of positively labeled neurons between the two groups (Mean ± SEM, t test).

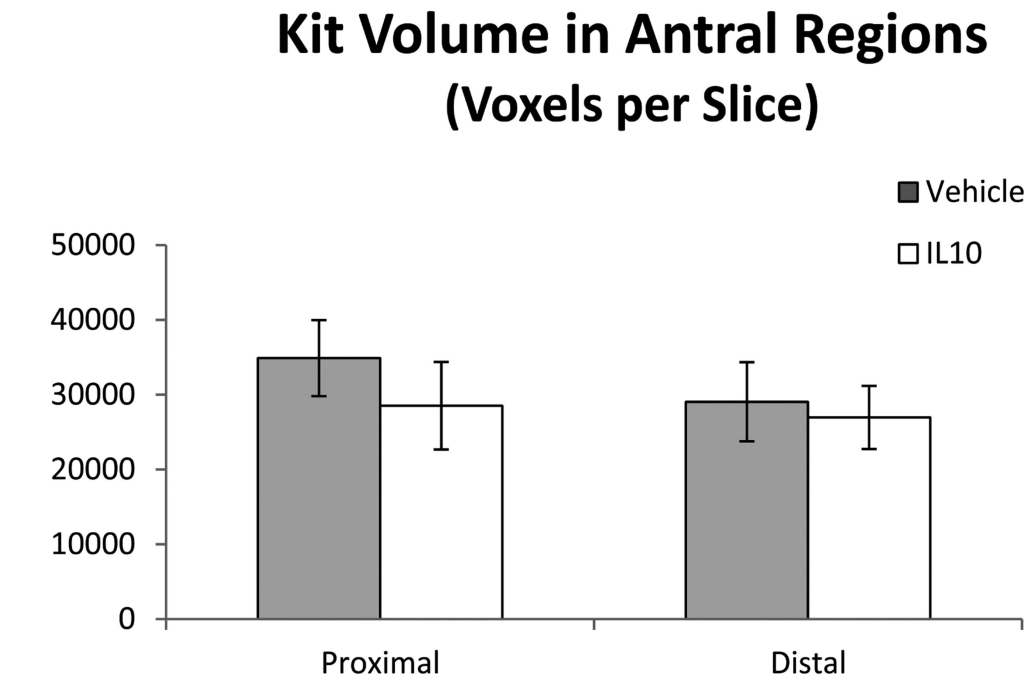




C



D



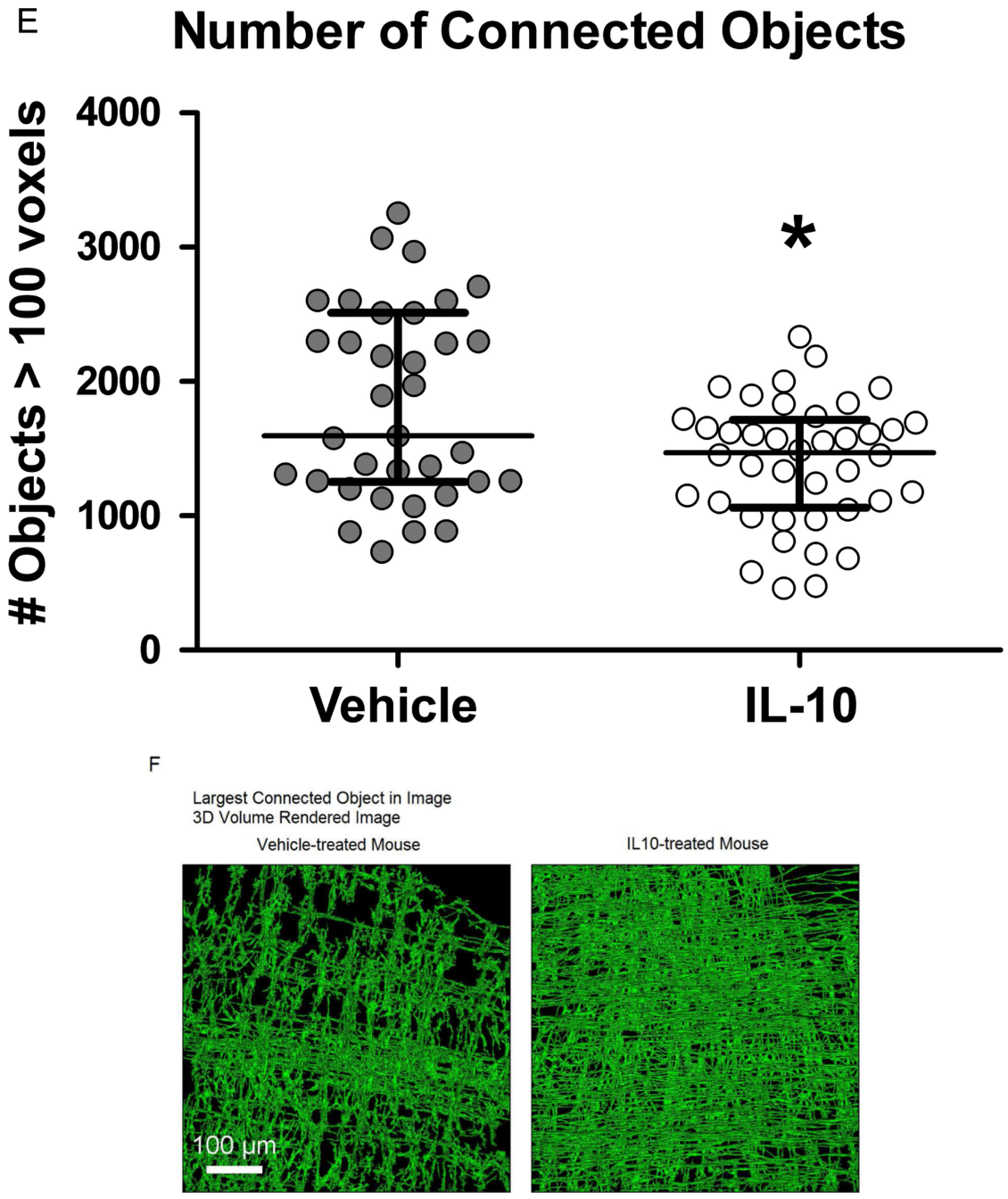


Figure 7.

ICC networks. Image stacks from vehicle (A) and IL10 (B) mice. C) Network density scores. Means±SD of scores for all fields, n=5, *-P<0.05, ttest. D) Network volume from recording sites in antrum. E) Count of independent connected structures after morphological opening in reconstructed images. Data are for 75 fields from 5 mice in each group. (*-P=0.026, ttest) F) 3D volume rendered bitmap of the largest connected Kit-positive ICC networks in two fields with similar total Kit volumes. Note that most of the Kit-positive structures in the field from the IL10-treated mouse are connected into a single object.

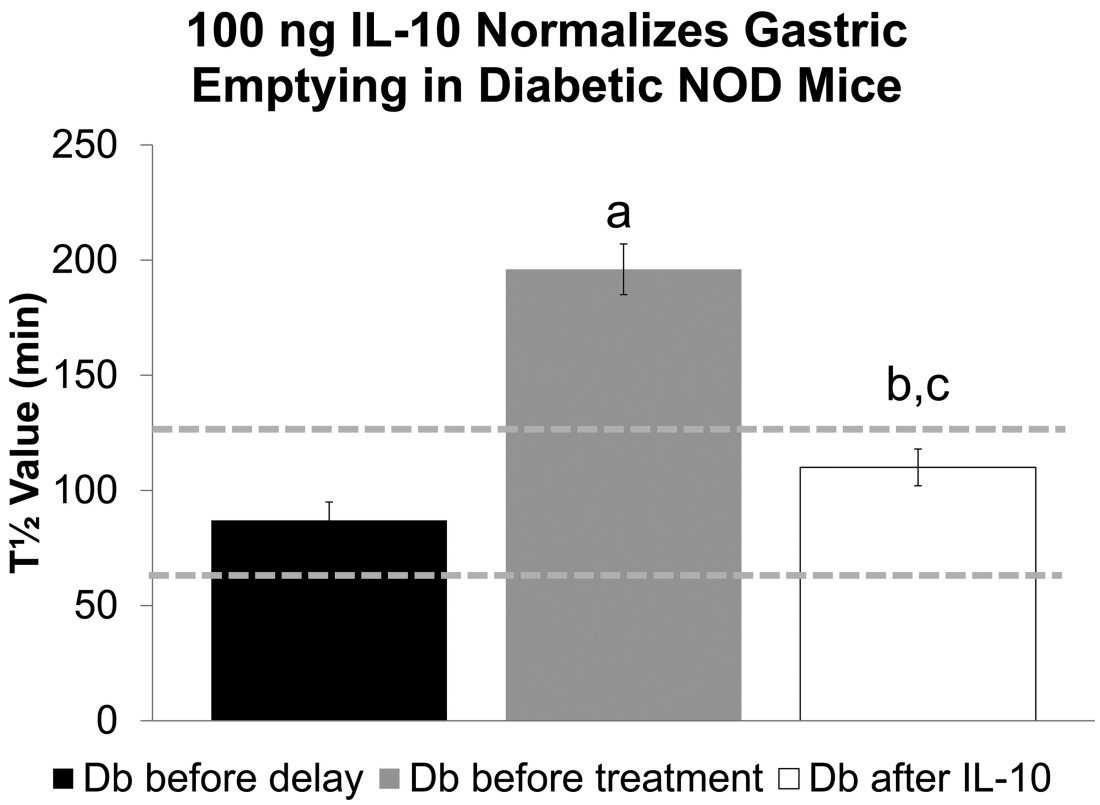


Figure 8.

A low dose of IL10 (100 ng) also reversed delayed gastric emptying in diabetic NOD mice. Data are the mean T½ values for gastric emptying ± SEM. $P < 0.001$ – one way ANOVA, (a) $P<0.05$ vs “db before delay”, (b) $P<0.05$ vs “db before treatment”, (c) not significantly different vs “db before delay”.