Hippocampal plasticity after a vagus nerve injury in the rat

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Abstract
Stimulation of the vagus nerve has been previously reported to promote neural plasticity and neurogenesis in the brain. Several studies also revealed plastic changes in the spinal cord after injuries to somatosensory nerves originating from both the brachial and lumbo-sacral plexuses. However, the neurogenic responses of the brain to the injury of the viscerosensory innervation are not as yet well understood. In the present study, we investigated whether cells in the dentate gyrus of the hippocampus respond to a chemical and physical damage to the vagus nerve in the adult rat. Intraperitoneal capsaicin administration was used to damage non-myelinated vagal afferents while subdiaphragmatic vagotomy was used to damage both the myelinated and non-myelinated vagal afferents. The 5-bromo-2-deoxyuridine (BrdU) incorporation together with cell-specific markers was used to study neural proliferation in subgranular zone, granule cell layer, molecular layer and hilus of the dentate gyrus. Microglia activation was determined by quantifying changes in the intensity of fluorescent staining with a primary antibody against ionizing calcium adapter-binding molecule 1. Results revealed that vagotomy decreased BrdU incorporation in the hilus 15 days after injury compared to the capsaicin group. Capsaicin administration decreased BrdU incorporation in the granular cell layer 60 days after the treatment. Capsaicin decreased the number of doublecortin-expressing cells in the dentate gyrus, whereas vagotomy did not alter the expression of doublecortin in the hippocampus. Both the capsaicin- and the vagotomy-induced damage to the vagus nerve decreased microglia activation in the hippocampus at 15 days after the injury. At 30 days post injury, capsaicin-treated and vagotomized rats revealed significantly more activated microglia. Our findings show that damage to the subdiaphragmatic vagus in adult rats is followed by microglia activation and long-lasting changes in the dentate gyrus, leading to alteration of neurogenesis.

Key Words
vagus injury; hippocampus; vagotomy; capsaicin; vagal afferents; microglia; rat

Abbreviations
BrdU, 5-bromo-2-deoxyuridine; CNS, central nervous system; VNS, vagus nerve stimulation; DCX, doublecortin; Iba1, ionizing calcium adapter binding molecule-1

INTRODUCTION
The communication between the brain and the periphery is a two-way road. The hypothesis that visceral afferents exert a significant influence on the central nervous system (CNS) plasticity has been tested by many investigators. Recent studies have already showed that vagus nerve stimulation (VNS) can induce neurogenesis and plasticity in the hippocampus11. In
Following such insults, microglia rapidly proliferate\textsuperscript{[16-17]} and undergo morphological alterations\textsuperscript{[18-19]}, becoming hypertrophic and increasing or expressing de novo a plethora of surface markers\textsuperscript{[20-21]}. In the present study, we investigated whether damage to the subdiaphragmatic vagus induces neural plasticity in the dentate gyrus of the hippocampus. The neurotoxin capsaicin was used to destroy unmyelinated axons of small primary afferent neurons\textsuperscript{[22]}. Capsaicin treatment of adult rats was shown to produce extensive degeneration of vagal afferent axons and terminals\textsuperscript{[23]}. We also performed a subdiaphragmatic vagotomy to destroy myelinated and non-myelinated axons of the vagus nerve. To study cell proliferation and differentiation, we evaluated the BrdU incorporation and DCX-immunoreactivity. Microglia activation was determined by quantifying changes in the ionizing calcium adapter binding molecule-1 (Iba1) immunoreactivity.

**RESULTS**

**Capsaicin treatment and vagotomy verification**

All the vehicle treated rats immediately wiped the eye where drop of 1% ammonium hydroxide was applied. Systemic administration of capsaicin abolished eye wipe reflex in all the i.p. injected rats. All the vagotomized rats met the criteria of complete vagotomy described previously\textsuperscript{[24]}.

**The number of BrdU-labeled cells changes in the hilus and granule cell layer of the dentate gyrus after vagus nerve injury**

To study if an injury applied peripherally to the vagus nerve could cause changes in the number of newly generated cells in the hippocampus, BrdU-labeled cells were quantified at several time points (3, 15, 30 and 60 days) after injury (Figure 1). No significant differences in BrdU immunoreactivity (BrdU-ir) were detected in the subgranular zone (SGZ) and molecular layer (Mol) layers between capsaicin treated, vagotomy and control groups at each considered time point (data not shown). Interestingly, intraperitoneal capsaicin administration significantly decreased BrdU incorporation in the granule cell layer (GCL) of the dentate gyrus after 60 days from treatment ($P < 0.05$, Figure 1D). Moreover, a significant difference ($P < 0.05$) in BrdU-ir between vagotomized animals and capsaicin-treated animals was found in the hilus at 15 days, where the vagotomized group showed significantly fewer BrdU-positive cells compared to the capsaicin-treated group. Capsaicin treatment slightly increased while vagotomy slightly decreased the number of BrdU-labeled cells; however these differences were statistically insignificant (Figure 1F).
To visualize the different layers of the dentate gyrus (DG), including SGZ, GCL, Mol and hilus, hippocampal sections were immunostained for a nuclear marker of mature neurons (NeuN). All hippocampal sections revealed multiple layers of NeuN-immunoreactive nuclei, forming the GCL of the DG. Hippocampal sections collected from vehicle- and capsaicin-treated rats as well as from vagotomized rats at 30 minutes after BrdU injection revealed no BrdU and NeuN double-labeled cells.

Doublecortin expression decreases after neurotoxic damage to the vagus nerve
To address the neuronal development, we used DCX, a microtubule-associated protein expressed by neuronal precursor cells and immature neurons. Neuronal precursor cells begin to express DCX while actively dividing, and their neuronal daughter cells continued to express DCX for 2–3 weeks as the cells matured into neurons[25-27]. The data indicated that capsaicin administration significantly decreased the number of doublecortin-positive cells in the DG after 3, 30 and 60 days from the injury (\(P < 0.05\)). At 15 days, the number of DCX-positive cells was not significantly different from that of control animals. No significant differences in DCX-positive cell numbers were seen between vagotomy group and control group for each considered time point (Figure 2).
Microglia are activated after a vagus nerve injury

To investigate a possible change in glial cell activity, we focused our attention on the microglia since injury is often associated with an increase in microglial activation. We evaluated the relative absorbance of ionized calcium-binding adapter molecule 1 (iba1) immunoreactivity, a specific marker of microglial activation at 3, 15 and 30 days after injury. Sections collected from vehicle treated rats revealed microglia in maintenance state. Their morphology was indicated by cells with small perikarya and radially branching processes. Sections collected 30 days after capsaicin treatment and vagotomy revealed microglia with larger perikarya and fewer and shorter processes, characteristics of activation.

Quantification analysis of iba1 immunoreactivity revealed that the intensity of staining in the DG was significantly decreased ($P < 0.05$) 15 days after injury in both experimental groups. At 30 days, iba1 immunoreactivity was significantly increased ($P < 0.05$) in both capsaicin-treated and vagotomy groups. The increased overall staining seemed to reflect the increased activation of labeled microglia rather than an increased number of labeled cells (Figure 3).

DISCUSSION

Studies using neuroanatomical, biochemical, electrophysiological and behavioural techniques proved that peripheral injuries trigger alterations of several areas of the CNS, including spinal cord, brainstem, thalamus, cortical and subcortical regions$^{[28-29]}$. The peripheral and central nervous systems are functionally integrated regarding the consequences of a nerve injury: a peripheral nerve lesion always results in intense and long-lasting central plasticity and reorganization$^{[28, 30-32]}$. Moreover, recent experiments on VNS, a well-established adjunct to medical therapy for refractory epilepsy$^{[33-34]}$ and depression$^{[35-37]}$, demonstrated that acute VNS induces neuronal plasticity in the rat hippocampus. Mechanisms of the plasticity and reorganization occurring after a peripheral nerve injury are still very complex and have not been completely understood.

Since the VNS is stimulating vagal neurons without a damage, in this study, we tested the neurogenic responses of the hippocampus following the damage to the vagus nerve. In the age of obesity, bariatric surgeries, while damaging the vagus, have been proven to be an effective treatment. Our studies revealing the effect of the vagus injury on hippocampal plasticity shed the light on possible complications related to memory, learning and depression following the bariatric surgeries.

In our study, cell proliferation was revealed by using BrdU administered with a single i.p. injection 30 minutes before animal sacrifice. No BrdU/NeuN dual labeled cells were observed. This fact was rather expected because we sacrificed animals 30 minutes after BrdU injection, and since a new generated cell takes more than 2 weeks to differentiate into a mature cell expressing NeuN marker$^{[39]}$. Moreover, it is highly unlikely that mature neuron will enter the cell cycle and will incorporate BrdU. BrdU incorporates into the nuclear DNA during cell division and labels mitotically active cells; a single injection of BrdU labels all the cells in the S-phase, whereas the proliferating cells in the other phases of the cell cycle remain unlabeled$^{[39]}$. Thus, since the tissue was collected after a short time, mitotic figures can be seen,
and this time point has been proposed as a true measure for the quantification of cells in the S-phase. The results obtained from the distinct examined hippocampus layers showed that 15 days after injury, capsaicin treatment slightly increased while vagotomy slightly decreased the number of BrdU-labeled cells in the hilus. Vagotomized rats showed fewer BrdU-positive cells in the hilus, compared to the capsaicin-treated group. Moreover, intraperitoneal capsaicin administration significantly decreased BrdU incorporation in the GCL of the DG 60 days after treatment. Increased neural proliferation following capsaicin-induced damage to the vagus nerve was previously reported in the nodose ganglion. VNS has been shown to increase the number of BrdU-positive cells in the DG, demonstrating a rapid effect of VNS on rat hippocampal progenitor proliferation. Therefore, we conclude that the specific VNS (via the TRPV1 receptor or electric stimuli) rather than just the nerve damage is required to release the neurogenic potential and induce neural proliferation. As a next step, we performed DCX-immunohistochemistry to follow the development of proliferating cells in the hippocampus. DCX is a 40 kDa microtubule-associated protein that is associated with migration of neuronal precursors of the developing CNS. In the two neurogenic areas of adult mammalian brain (the SGZ in the DG of the hippocampus and the subventricular zone of the lateral ventricles), DCX is normally expressed by developing neurons. During adult neurogenesis, DCX starts to be expressed when neuroblasts are generated, peaking during the second week, and then decreases, leaving the path to other mature neuronal markers, such as NeuN. After acute VNS, an increase of both the total amount of DCX immunoreactivity and the number of DCX-positive neurons in the DG has been shown. In the present study, we found that capsaicin administration significantly decreased the number of DCX-expressing cells in the DG after 3, 30 and 60 days from the injury. No significant differences in DCX-positive cell numbers were seen between the vagotomy group and control group for each considered time point. This fact may suggest that capsaicin-induced damage to vagal afferents may alter the local environment in the hippocampus and accelerate the maturation of newborn neurons, leading to a faster switch from DCX-expressing cells to NeuN-expressing cells. The possible role of capsaicin on neural proliferation has been recently reviewed; however, the molecular mechanisms of its action need to be elucidated to support this hypothesis. Activation of microglia commonly occurs in an early response of the CNS to a wide variety of pathological stimuli, including axotomy, trauma, inflammation, degeneration, and ischemia. They also play a central role in chronic degenerative conditions, such as Alzheimer's disease, Parkinson's diseases, multiple sclerosis, amyotrophic lateral sclerosis, and many other diseases. Since microglial activation usually increases after injury, we evaluated the intensity of iba1, a specific marker of microglia activation. Our results showed that both the capsaicin- and the vagotomy-induced damage to the vagus nerve first decreased microglia activation in the hippocampus 15 days after the injury. At 30 days post injury, capsaicin-treated and vagotomized rats revealed significantly more activated microglia compared to control animals. These findings suggest that damage to the subdiaphragmatic vagus in adult rats activates the microglia after a period of delay, probably due to the fact that in the first range of time after the injury, the microglia may migrate to other regions of the brain. This hypothesis is also supported by our recently published data showing that subdiaphragmatic vagotomy significantly activated microglia in all the vagal nuclei in the hindbrain 2 weeks post-vagotomy. However, the prolonged microglia activation was only observed in the dorsal motor nucleus of the vagus. Results of the present study together with previously published data suggest that damage to the vagus nerve induces microglia activation progressing retrogradely from the periphery to the CNS. This prolonged activation of microglia in the CNS may have a significant effect on vagal structures through cytokine release and alterations of the microenvironment, leading to changes in adult neurogenesis and neural plasticity. The results of our study suggest that peripheral nerve injury induces a cascade of events that alter adult neurogenesis in the hippocampus. Observed changes in cell proliferation, differentiation and microglia activation may have significant functional consequences. Therefore, consequences to memory and special navigation that result from PNS damage should be investigated in the future.

MATERIALS AND METHODS

Materials
All animal procedures were approved by the Washington State University Institutional Animal Care and Use Committee and conformed to National Institutes of Health guidelines for the use of vertebrate animals (publication No. 86-23, revised 1985). All efforts were made to minimize both the number of animals used and animal suffering. Experiments were performed on adult male Sprague-Dawley rats (6 weeks old; Simonsen Laboratories, Gilroy, CA, USA). Animals were housed in individual hanging cages in a temperature-controlled
vivarium with ad libitum access to food (Harlan Teklad F6 Rodent Diet W; Harlan Laboratories, Madison, WI, USA) and water. Rats were maintained under standard laboratory conditions with a 12-hour light/dark cycle. Rats were divided into three groups. The first two groups received intraperitoneal injections of capsaicin or vehicle; the third group underwent subdiaphragmatic vagotomy.

Methods
Capsaicin and vehicle treatment
Forty rats were anesthetized with isoflurane anaesthetic (3% induction 1–2% maintenance). Twenty rats (five rats for each time point: 3, 15, 30, and 60 days) were injected i.p. with capsaicin (catalogue No. M2028; Sigma-Aldrich, St.Louis, MO, USA) while another twenty rats (five rats for each time point: 3, 15, 30, and 60 days) were injected i.p. with vehicles (10% ethanol in 10% Tween-80; catalog No. P1754; Sigma-Aldrich) in 0.9% saline solution. Three different doses of capsaicin were used in this study (25, 50, and 50 mg/kg; total dose 125 mg/kg) at 0, 6, and 24 hours, respectively, at an injection volume of 1 mL/kg. Twenty minutes before the first injection, animals were injected i.p. with atropine sulfate (3 mg). Artificial ventilation was provided, as required, during the 3- to 5-minute period of respiratory arrest that typically occurred after the first capsaicin injection. Vehicle injections followed the same protocol and schedule as capsaicin treatment.

Finally, to assess the effectiveness of the capsaicin treatment, rats were tested for the corneal chemosensory response (eye wipe) to mild corneal irritation, mediated by the capsaicin-sensitive trigeminal innervation of the cornea. A drop of 1% ammonium hydroxide was applied on the corneal surface of one eye, and the number of eye wipes was measured during the 15 seconds immediately after the application. None of the capsaicin-treated rats had any eye-wipe response.

Subdiaphragmatic vagotomy
An additional twenty animals were subjected to a total surgical resection of both the dorsal and ventral subdiaphragmatic vagal trunks in a manner consistent with previously described procedures. Briefly, after the animals were anesthetized with a drug cocktail containing ketamine, acepromazine, and xylazine (0.1 mL/100 g body weight), a ventral midline incision was made and the vagus was exposed by gentle retraction of the stomach and liver. A 5-mm section of each vagus nerve was transected and removed from the dorsal trunk above the celiac and gastric branches and from the ventral trunk above the hepatic and accessory celiac branches, as close to the esophageal hiatus of the diaphragm as possible. Verification of completeness of vagotomy was performed with criteria described previously. Rats were then maintained on the powdered rodent chow for a few days.

BrdU administration
Labelling of proliferating cells was performed by a single i.p. injection of the thymidine analogue BrdU (Sigma, Steinheim, Germany) at 100 mg/kg body weight 30 minutes before sacrifice. This post-BrdU survival time is sufficient to label cells in S-phase but not to allow the labelled cells to divide, thus providing a measure of cell proliferation.

Tissue fixation and sectioning
At the appropriate time points, animals were deeply anesthetized with ketamine/acepromazine/xylazine cocktail and transcardially perfused with 0.1 M phosphate buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde. After the perfusion, brains were immediately removed, post-fixed with 4% paraformaldehyde for 2 hours and immersed overnight in a cryoprotectant solution of 30% sucrose in PBS with 0.05% sodium azide at 4°C. Free-floating coronal sections (30 µm thick) were obtained using a freezing microtome through the entire hippocampus (~1.80 to ~7.64 mm from bregma; Paxinos and Watson, 1997). Sections were stored in cryoprotectant at −20°C until immunohistochemical labelling.

Immunohistochemistry
For BrdU staining, every tenth free-floating section of the hippocampus (13 sections per animal) was used. For Iba1, the marker of activated microglia, five hippocampal sections from each animal were used. The sections were immersed for 15 minutes in a 1% Triton X-100 in Tris buffered saline. Antigen retrieval was performed by incubating for 90 minutes at 65°C in a solution 50% 0.3 M NaCl/30 mM citrate buffer/50% formamide, and then rinse with 0.3 M NaCl/30 mM citrate buffer. The sections were then incubated in 2 N HCl at 37° for 30 minutes and rinsed in 0.1 M borate buffer for 10 minutes. The sections were incubated for 30 minutes in a blocking solution consisting of 3% normal horse serum in 0.1% Triton X-100 solution. The blocking solution was used to wash the tissues, and each section was incubated overnight at room temperature with primary antibody. Subsequently, the sections were incubated for 2 hours at room temperature with appropriate secondary antibodies. Sections were mounted in ProLong (Invitrogen, Carlsbad, CA, USA) to reduce photo bleaching. Sections stained for BrdU were counterstained with NeuN, a neuronal nuclear marker of mature neurons.

For DCX staining, five sections from each rat were used. Blocking of endogenous peroxidase activity was performed with 0.3% H2O2 for 30 minutes. After washing...
with PBS, the slides were incubated in 1.5% horse serum in PBS for 30 minutes. Goat polyclonal antibody against doublecortin (1: 1 000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) in 0.04% Triton-X100 in PBS was applied overnight at room temperature. The slides were subsequently exposed to biotinylated anti-goat IgG and streptavidin peroxidase complex (1:800 dilution). They were then visualized by staining with 3,3-diaminobenzidine (Sigma-Aldrich, Deisenhofen, Germany). The slides were finally mounted with Permount after dehydration.

Quantification analysis

Images of the DG were viewed and captured under ×200 and ×400 magnification with a Nikon 80 i imaging photomicroscope (Nikon, Tokyo, Japan) equipped with a digital camera (Nikon Digital Sight DS-Q1Mc). The number of positive-stained profiles for BrdU was counted with the NIS-Elements AR 3.0 Imaging System (Nikon, Tokyo, Japan).

The number of BrdU-positive cells was determined for each rat by the optical fractionator method. Briefly, the counts were collected from every tenth hippocampal section throughout the anterior–posterior extent of the hippocampus (~1.80 to ~7.64 mm from bregma; Paxinos and Watson, 1997). Resulting cell counts were multiplied by the fraction of the hippocampus examined (e.g., 10)[65-67]. Four discrete regions of the DG were examined [68]: the SGZ, GCL, Mol, and the hilus.

The number of DCX-positive cells was determined on five sections evenly spaced throughout the entire hippocampus. For quantification of Iba1 expression in the DG of each rat, the total fluorescence intensity was measured by the NIS-Elements AR 3.0 Imaging System in five evenly spaced sections throughout the entire hippocampus; the intensity values were then averaged for each group of animals and expressed as the mean densitometric gray scale values.

Statistical analysis

Data were analyzed by one-way analysis of variance and preplanned comparisons with the controls performed by the post hoc Fisher and Bonferroni protected least significant difference test via StatView software (SAS Institute, Cary, NC, USA). Significance was set at P < 0.05. All values are presented as mean ± SEM.

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