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Vagal cardiac efferent innervation in F344 rats: Effects of chronic intermittent hypoxia

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ABSTRACT

Chronic intermittent hypoxia (CIH), which is a physiological consequence of obstructive sleep apnea, reduces baroreflex control of heart rate (HR). Previously, we showed that the heart rate (HR) response to electrical stimulation of the vagal efferent nerve was significantly increased following CIH in F344 rats. Since vagal cardiac efferent from the nucleus ambiguus (NA) project to cardiac ganglia and regulate HR, we hypothesized that vagal cardiac efferent innervation of cardiac ganglia is reorganized. Young adult F344 rats were exposed either to room air (RA) or to intermittent hypoxia for 35–50 days. Fluorescent tracer DiI was injected into the NA to label vagal efferent innervation of cardiac ganglia which had been counterstained by Fluoro-Gold (FG) injections (i.p). Confocal microscopy was used to examine vagal cardiac efferent axons and terminals in cardiac ganglia. NA axons entered cardiac ganglia and innervated principal neurons (PNs) with robust basket endings in both RA control and CIH animals. In addition, the percentage of PNs which were innervated by DiI-labeled fibers in ganglia was similar. In CIH rats, abnormally large swollen cardiac axon segments and disorganized terminals as well as leaky endings were observed. In general, vagal efferent terminal varicosities around PNs appeared larger and the number of varicosities was significantly increased. Interestingly, some cardiac axons had sprouting-like terminal structures in the cardiac ganglia as well as in cardiac muscle, which had not been found in RA control. Finally, CIH increased the size of PNs and reduced the ratio of nucleus to PN somata. Thus, CIH significantly remodeled the structure of vagal cardiac axons and terminals in cardiac ganglia as well as cardiac PNs.

1. Introduction

Chronic intermittent hypoxia (CIH), a model for obstructive sleep apnea, reduces baroreflex control of heart rate (Gu et al. 2007). The baroreflex arc includes baroreceptor afferent, central, and efferent components as well as cardiac ganglia in the heart. The cardiac ganglia act as the local integration center to coordinate central and local inputs and regulate cardiac function (Armour 2004, 2008). Using CIH F344 rats as a model, we have previously demonstrated that aortic baroreceptor afferent innervation of the aortic arch is increased following CIH (Ai et al. 2009). The increased baroreceptor afferent innervation in the aortic arch is associated with the enhancement of aortic baroreceptor depressor nerve function as measured by whole nerve recordings (Gu et al. 2007). In addition, we found that HR responses to electrical stimulation of the vagal efferent nerve were significantly increased (Gu et al. 2007). Thus, we reasoned that vagal cardiac efferent projections to cardiac ganglia may undergo remodeling in F344 rats. Previously, we have also shown that the nucleus ambiguus (NA) projects to cardiac ganglia very extensively and innervates cardiac principal neurons (PNs) with robust basket-like endings around PNs on the dorsal surface of the left and right atria in both rats and mice (Cheng and Powley 2000, Cheng et al. 2004a, Ai et al. 2007a,b, Li et al. 2010). We also confirmed that the NA plays an important role in baroreflex control of the heart rate (Cheng et al. 2004b). In this study, we injected the fluorescent tracer DiI into the left NA to label vagal cardiac efferent axons and terminals in cardiac ganglia and Fluoro-Gold injection (i.p.) to label cardiac ganglia. We then used confocal microscopy and Neurolucida Tracing and Digitization System to examine the structural changes of vagal cardiac axons and terminals in cardiac ganglia as well as cardiac PNs in the cardiac ganglia following CIH. Our findings indicated that CIH significantly remodeled the structures of vagal cardiac axons and terminals in cardiac ganglia and cardiac PNs.

2. Materials and methods

2.1. Animals

Fischer 344 rats (4–5 months, $n = 30$) were used. Procedures were approved by the University of Central Florida Animal Care and Use Committees and followed the NIH guidelines.

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2.2. Chronic intermittent hypoxia

Animals were housed in hypoxia chambers. The protocol for chronic intermittent hypoxia (CIH) was identical to our previously reported rat models of sleep apnea (Gu et al. 2007, Ai et al. 2009, Yan et al. 2009).

2.3. DiI microinjection into the NA in the brainstem

2.3.1. DiI and FG injections

At 15–25 days of either RA ($n = 15$) or IH ($n = 15$) exposure, rats were taken out from the chambers for injections of DiI (1,1′-dioctadecyl-3,3,3′,3′ tetramethylindocarbocyanine methanesulfonate, Molecular Probes). Animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and placed in a stereotaxic instrument. Depth of anesthesia was regularly checked by pinching the tail. Supplemental dose of 1/5 of the initial dose was given to maintain the anesthesia as needed. DiI injection into left nucleus ambiguus (NA) at 9 different sites (−1600 to +1600 μm relative to the obex; total volume 22.5–112.5 nl) has been established and described by the previous studies (Cheng and Powley 2000, Ai et al. 2007a,b). After completion of all injections, the surgical wound was closed with sutures and the animal was returned to its cage for continuation of RA or intermittent hypoxia exposure (Ai et al. 2009). To prevent infection, a single dose of penicillin G (50,000 U, s.c.) was injected at the end of surgery. To reduce pain, Buprenorphine (0.05 mg/kg) was given (s.c) daily for 2 days after surgery. Only animals that had well placed injections of DiI into the NA were used for analysis (Cheng and Powley 2000, Ai et al. 2007a,b). All animals were also injected with Fluoro-Gold (FG: i.p., 2 ml of 2 mg/ml) to label cardiac ganglia and vagal cardiac motor neurons in NA 3–5 days before they were perfused (Cheng and Powley 2000, Ai et al. 2007a).

2.3.2. Verification of DiI injection sites

Brainstem slices were examined using an epifluorescence microscope. The registrations of DiI injection drops with FG-labeled vagal motor neurons in NA were carefully assessed in the brain slices from −2000 to +2000 μm relative to obex which included the entire rostral and caudal NA in rats (Cheng and Powley 2000, Ai et al. 2007a, b). DiI injection sites along rostral and caudal NA were checked. In addition, the dorsal motor neurons of vagus (DmnX), the nucleus of the solitary tract (NTS), and the left nodose ganglia (ipsilateral site for DiI injection) were also examined to check the potential leakage of the tracer which may cause secondary labeling of vagal efferent axons from NTS or vagal efferent nerve from the nodose ganglia. We used 6 animals/each group which had excellent DiI-labeled vagal efferent axons and terminals in the atria for the final analysis, but without significant labeling in DmnX, NTS and nodose ganglia. Any animals which had poor DiI-labeled vagal efferent axons and terminals due to a mis-targeted DiI injection intended for the NA, or DiI injections that were too small to effectively cover the NA, or provide significant secondary labeling were removed from the final data analysis.

2.4. Tissue preparation

21–25 days were given for the DiI injection to allow tracer DiI to clearly label vagal efferent axons and terminals in the heart (Cheng and Powley 2000). Additional survival time would not have changed quality and the pattern of labeling (previous unpublished observation). Rats were deeply anesthetized with an overdose of pentobarbital sodium (100 mg/kg). When animals were did not respond to the tail-pinch assessment of depth of anesthesia, they were perfused with 0.9% saline (500 ml) and 10% phosphate-buffered (pH = 7.4) formalin (800 ml). Brainstems and the left nodose ganglion were removed, stored in 10% sucrose formalin overnight. The brainstems were sectioned transversely at 100 μm from −2000 to +2000 μm relative the obex on the second day. The atria were then separated and cut open as previously described in rats (see Cheng et al., 1997, 1999, Cheng and Powley 2000). All heart, brain slice, and nodose ganglion tissues were then dehydrated through a graded series (70%, 90%, and 2 × 100%) of glycerin. Finally, the tissues were mounted and coverslipped in 100% glycerin and n-propyl gallate (5%) to prevent fading.

2.5. Vagal efferent axons and terminals in cardiac ganglia: quantification

DiI labeled axons and cardiac ganglia were screened with an epifluorescence microscope equipped with filter cubes appropriate for DiI (rhodamine) and FG (UV). When DiI axons and/or cardiac ganglia were found, their locations were recorded for subsequent detailed confocal microscopic analysis at high magnification (Leica TCS SP5). The excitation wavelength for FG was 405 nm and emission wavelength of FG ranged from 360 to 600 nm. Three major ganglionated plexuses labeled by FG were identified in the epicardium of the atria as previously shown (Cheng et al. 1999 and Ai et al. 2007b). Within each plexus, multiple FG-labeled ganglia were found. In some confocal images (Fig. 6A–C), we showed FG-labeled PNs in gold-yellow colour. Because the background fluorescence of the cardiac PNs could also be visible in the single-channel confocal images scanned using 563 nm laser (excitation) for scanning DiI signals, we used the background fluorescence for somata of cardiac ganglionic PNs to show PNs in the majority of confocal images (e.g., Fig. 2A1′).

After we identified DiI-labeled bundles and fascicles, we could trace them to their targets: the principal neurons (PNs) in cardiac ganglia. DiI-labeled axons formed basket endings around PNs. To quantify the amount of PNs innervated by DiI-labeled axons, 30 frames (5 frames/each animal) of confocal stacks of images with densely DiI-labeled axons and terminals were selected from the ganglia in each group ($n = 6$). The density of PNs being innervated by DiI labeled fibers with basket endings, i.e., the percentage of PNs innerved by DiI-labeled axons vs the total PNs in each frame, was calculated and averaged for each animal, and then averaged within the group.

Vagal cardiac fibers in rats typically made dense ending varicosities (2–5 μm in diameter) around PNs. Previously, we used the varicosity as the candidate for a synapse (Soukhova-O'Hare et al. 2006, Ai et al. 2007a,b). To quantify the amount of DiI-labeled presumed synaptic contacts around each PN, the stack of optical sections which contained the whole vagal terminal varicosities around that PN was then analyzed using the confocal module software available in the Neurolucida 3-D Tracing and Digitization System. The detailed method and the illustrative figure for digitization have been described in detail (Soukhova-O'Hare et al. 2006, Ai et al. 2007a,b). Briefly, each terminal varicosity around a PN soma was marked using a solid circle and the number of varicosities around the PN soma was then obtained for each optical sectioned image (Z-step 1 μm). Care was taken not to count the same varicosity twice which appeared in the adjacent optical sections. The total number of varicosities around this PN was the summation of the total close appositions around that PN from all optical sections. We sampled 100 innervated PNs from 30 frames in 6 animals (5 frames/each animal) of confocal stacks where vagal terminals were most densely labeled by DiI. The number of varicosities around each PN were counted and averaged for each animal, and then averaged within a group and compared between groups.

2.6. Data analysis and expression

Numbers of PNs innervated by tracer DiI and terminal varicosities were calculated for RA and CIH groups. The data were expressed as means ± SEM. To compare the number of PNs innervated and terminal varicosities around PNs across groups, Student's t-tests were used. $P < 0.05$ was considered as achieving statistical significance. All the images scanned by the confocal microscope were first saved as Tiff files and then were processed in Adobe Photoshop 8.0. We used the Photoshop 8.0 to assemble montages to show the DiI-labeled axons and terminals as needed.