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DIRECT PROJECTIONS FROM HYPOTHALAMIC OREXIN NEURONS TO BRAINSTEM CARDIAC VAGAL NEURONS

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Abstract—Orexin neurons are known to augment the sympathetic control of cardiovascular function, however the role of orexin neurons in parasympathetic cardiac regulation remains unclear. To test the hypothesis that orexin neurons contribute to parasympathetic control we selectively expressed channelrhodopsin-2 (ChR2) in orexin neurons in orexin-Cre transgenic rats and examined postsynaptic currents in cardiac vagal neurons (CVNs) in the dorsal motor nucleus of the vagus (DMV). Simultaneous photostimulation and recording in ChR2-expressing orexin neurons in the lateral hypothalamus resulted in reliable action potential firing as well as large whole-cell currents suggesting a strong expression of ChR2 and reliable optogenetic excitation. Photostimulation of ChR2-expressing fibers in the DMV elicited short-latency (ranging from 3.2 ms to 8.5 ms) postsynaptic currents in 16 out of 44 CVNs tested. These responses were heterogeneous and included excitatory glutamatergic (63%) and inhibitory GABAergic (37%) postsynaptic currents. The results from this study suggest different sub-population of orexin neurons may exert diverse influences on brainstem CVNs and therefore may play distinct functional roles in parasympathetic control of the heart. **© 2016 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: optogenetic, neurons, orexin, brainstem, cardiac, parasympathetic.

INTRODUCTION

Preganglionic cardiac vagal neurons (CVNs) in the dorsal motor nucleus of the vagus (DMV) project directly to the cardiac ganglia and play a substantial role in the cardiac regulation (Sullivan and Connors, 1981; Ciriello and Calaresu, 1982; Cheng et al., 1999). Electrical stimulation of the cardiac branches of the vagus nerve antidromically activates neurons in the DMV and electrical stimulation of the DMV reduces heart rate and myocardial contractility (Calaresu and Pearce, 1965; Nosaka et al., 1979; Ciriello and Calaresu, 1982). Increasing the activity CVNs in the DMV protects the heart from ischemia/reperfusion injury independent of changes in heart rate (Mastitskaya et al., 2012). The activity of CVNs in the DMV are strongly influenced by neurotransmission from other neurons in the brainstem, as well as pathways from the locus coeruleus and oxytocin neurons in the hypothalamus (DePuy et al., 2013; Dergacheva et al., 2014; Pinol et al., 2014; Wang et al., 2014). The results from immunohistochemical studies indicate orexin neurons could be another important source of innervation to neurons in the DMV (Peyron et al., 1998; Date et al., 1999). However, the DMV is a heterogeneous nucleus and it is unknown whether there are direct connections between orexin neurons and the selective population of CVNs in the DMV that play a major role in controlling heart rate and cardiac function.

Compelling evidence indicates orexin neurons and receptors play an important role in the regulation of cardiovascular function (Peyron et al., 1998; Ciriello and de Oliveira, 2003; Ciriello et al., 2003; Dergacheva et al., 2005, 2013; Carrive, 2013). Orexin is well known to exert sympathoexcitatory effects such as increases in heart rate, blood pressure and sympathetic nerve activity (Samson et al., 1999; Shirasaka et al., 1999; Chen et al., 2000; Antunes et al., 2001; Matsumura et al., 2001). However, little is known about the role of orexin neurons in parasympathetic control of the heart and the few studies that have examined this issue are conflicting. For example, microinjection of orexin-A into the rostral ventral medulla produces tachycardia mediated in part by inhibition of parasympathetic activity to the heart (Ciriello et al., 2003), whereas microinjection of orexin-A into the nucleus ambiguus elicits bradycardia mediated by excitation of parasympathetic activity to the heart (Ciriello and de Oliveira, 2003).

Thus, this study was undertaken to identify and characterize the synaptic pathway from orexin neurons to CVNs in the DMV. To accomplish this goal, we utilized a transgenic strain of orexin-Cre rats that allows us to photoexcite channelrhodopsin-2 (ChR2) in orexin fibers in the brainstem DMV while recording synaptic

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Abbreviations: aCSF, Artificial cerebrospinal fluid; ChR2, channelrhodopsin-2; CVNs, cardiac vagal neurons; DMV, dorsal motor nucleus of the vagus; EPSCs, excitatory postsynaptic currents; EYFP, enhanced green fluorescent protein; IPSCs, inhibitory postsynaptic currents; NMDG, 110 N-methyl-d-glucamine.

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events in fluorescently identified CVNs in the DMV in an *in vitro* slice preparation.

EXPERIMENTAL PROCEDURES

Animals

Orexin-EGFP-2A-Cre rats (both males and females) were used in this study. The generation of these transgenic animals in which Cre recombinase and green fluorescent protein are exclusively expressed in hypothalamic orexin neurons has been described previously (Dergacheva et al., 2016). Rats were housed in the George Washington University animal care facility. All animal surgeries and experiments were approved by the George Washington University Institutional Animal Care and Use Committee. We made all efforts to minimize number of rats used in this study and reduce animal discomfort.

Cardiac neuron labeling and viral injections into the lateral hypothalamus

Parasympathetic CVNs were labeled as described previously (Dergacheva et al., 2013; Dergacheva, 2015). At postnatal days 4–5 orexin-Cre rats were anesthetized with hypothermia, the heart was exposed and 0.05 ml of 1–5% rhodamine (XRITC; Molecular Probes, Eugene, OR) was injected into the pericardial sac. The previous study showed the specificity of the cardiac vagal labeling (Bouairi et al., 2004).

ChR2 fused to enhanced yellow fluorescent protein (EYFP) was targeted to the plasma membrane of orexin neurons and axons as shown in Fig. 1. Adeno-associated viral vector with "FLEX-switch" ChR2 construct (AAV1-ChR2-EYFP, Penn Vector Core, Philadelphia, PA, catalog number AV-1-20298P) was injected into the lateral hypothalamus of orexin-Cre rats using a stereotactic apparatus with a neonatal adapter (Stoelting, Wood Dale, IL). A pulled calibrated pipette (VWR, Radnor, PA) containing viral vector was positioned at the following coordinates: 1.7–1.9 mm posterior and 0.4 mm lateral relative to the bregma. The viral vector (60 nL) was slowly injected 5.2 mm lower the dorsal surface of the brain. The pipette was left in the lateral hypothalamus for 5 min after injection, then slowly retracted. To reduce pain and discomfort caused by the surgery, buprenorphine was administered after surgery, and animals were carefully monitored until ambulatory.

Slice preparation and electrophysiology

Animals (21–24 days old) were anesthetized with isoflurane and sacrifice by transcardial perfusion of glycerol-based artificial cerebrospinal fluid (aCSF, 4 °C) that contained (in mM): 252 glycerol, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 MgCl, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 11 glucose. Coronal slices of the brainstem (300-μm) were obtained with a vibratome. In another set of experiments 300-μm-thick coronal slices of the hypothalamus that included orexin neurons were made using a vibratome. The slices were then allowed to recover for 15 min in a solution containing (in mM): 110 N-methyl-d-glucamine (NMDG), 2.5 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 110 HCl, 0.5 $CaCl_2$, and 10 mM $MgSO_4$ equilibrated with 95% O_2-5% CO_2 (pH 7.4, at 34 °C). For performing electrophysiological experiments, the slices were transferred to a recording chamber containing (in mM) 125 NaCl, 3 KCl, 2 $CaCl_2$, 26 $NaHCO_3$, 5 glucose, 5 HEPES and equilibrated with 95% O_2-5% CO_2 (pH 7.4, at 25 °C).

CVNs in the DMV and orexin neurons in the lateral hypothalamus were identified by the presence of the retrograde tracer and EYFP-expression, respectfully. Infrared-sensitive video detection cameras and differential interference contrast optics were then used to image these identified CVNs and orexin cells. We patched CVNs with patch pipettes (2.5–3.5 MΩ) filled with a solution consisting of (in mM) 150 KCl, 2 $MgCl_2$, 2 EGTA, 10 HEPES, and 2 Mg-ATP, pH 7.3. In experiments that examined activity in orexin cells, the patch pipettes were filled with a solution consisting of 135 mM K-gluconic acid, 10 mM HEPES, 10 mM EGTA, 1 mM CaCl2, and 1 mM MgCl2, pH 7.3. We performed voltage clamp whole-cell recordings at a holding potential of −80 mV. Firing activity of orexin neurons

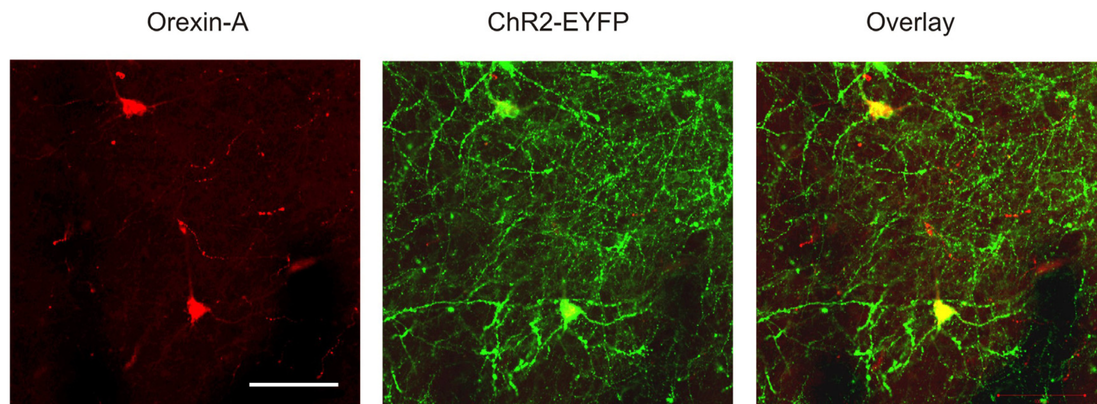

Fig. 1. Localization of orexin-A immunoreactivity (red, left panel) with ChR2-EYFP (green, middle panel) driven by orexin-Cre selective expression in the lateral hypothalamus. Co-localization is shown in the right panel. Representative example of n = 4 animals. Scale bar, 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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