Journal of Neuroscience Methods 210 (2012) 238–246

Contents lists available at SciVerse ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Optogenetic approaches to characterize the long-range synaptic pathways from the hypothalamus to brain stem autonomic nuclei

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HIGHLIGHTS

- Lentiviral vectors were injected into PVN to express ChR2-EYFP under oxytocin, vasopressin or synapsin promoter.
- Only synapsin promoter was strong enough to optogenetically stimulate long-range axon endings in brainstem autonomic nuclei.
- All three promoters could generate action potential firing at PVN cell bodies.
- Crossbreeding floxed ChR2 with Sim1-Cre mice allowed optogenetic PVN neuron stimulation, but not at brain stem distal axons.
- Useful approach to study important hypothalamus-brainstem connections; easily modifiable for other long-range projections.

ARTICLE INFO

Article history:
Received 15 December 2011
Received in revised form 12 July 2012
Accepted 19 July 2012

Keywords:
Optogenetics
Channelrhodopsin-2
Cardiac vagal neuron
Paraventricular nucleus of the hypothalamus
Cardiovascular
Electrophysiology

ABSTRACT

Recent advances in optogenetic methods demonstrate the feasibility of selective photoactivation at the soma of neurons that express channelrhodopsin-2 (ChR2), but a comprehensive evaluation of different methods to selectively evoke transmitter release from distant synapses using optogenetic approaches is needed. Here we compared different lentiviral vectors, with sub-population-specific and strong promoters, and transgenic methods to express and photostimulate ChR2 in the long-range projections of paraventricular nucleus of the hypothalamus (PVN) neurons to brain stem cardiac vagal neurons (CVNs). Using PVN subpopulation-specific promoters for vasopressin and oxytocin, we were able to depolarize the soma of these neurons upon photostimulation, but these promoters were not strong enough to drive sufficient expression for optogenetic stimulation and synaptic release from the distal axons. However, utilizing the synapsin promoter photostimulation of distal PVN axons successfully evoked glutamatergic excitatory post-synaptic currents in CVNs. Employing the Cre/loxP system, using the Sim-1 Cre-driver mouse line, we found that the Rosa-CAG-LSL-ChR2-EYFP Cre-responder mice expressed higher levels of ChR2 than the Rosa-CAG-LSL-ChR2-tdTomato line in the PVN, judged by photo-evoked currents at the soma. However, neither was able to drive sufficient expression to observe and photostimulate the long-range projections to brainstem autonomic regions. We conclude that a viral vector approach with a strong promoter is required for successful optogenetic stimulation of distal axons to evoke transmitter release in pre-autonomic PVN neurons. This approach can be very useful to study important hypothalamus-brainstem connections, and can be easily modified to selectively activate other long-range projections within the brain.

1. Introduction

Electrical stimulation of neurons and fibers in-vivo within the central nervous system (CNS) has been used for identifying and assessing the targets and responses of long-range synaptic pathways for many years. Although much seminal work has been done with this approach, it possesses several insurmountable inherent obstacles, including limited control over the spread and spatial resolution of stimulation, confounding stimulation of a heterogeneous population of neurons or axons within the stimulated region, unpredictable effectiveness of stimulation that depends on

Abbreviations: 12, Hypoglossal Nucleus; 3V, Third Ventricle; Amb, Nucleus Ambiguus; AP-5, D-2-amino-5-phosphonovalerate; AVP, Arginine vasopressin; cc, Central Canal; ChR-2, Channelrhodpsin-2; CNS, Central Nervous systems; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CVN, Cardiac vagal Neuron; DMV, Dorsal Motor Nucleus of the Vagus; GABA, Gamma-Aminobutyric Acid; NTS, Nucleus of the Solitary Tract; OXY, Oxytocin; PPR, Paired-pulse response; PVN, Paraventricular Nucleus of the Hypothalamus; Sim1, Single-minded homolog 1; SON, Supraoptic Nucleus; TTX, Tetrodotoxin; XRITC, X-rhodamine-5-(and 6)-isothiocyanate.

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http://dx.doi.org/10.1016/j.jneumeth.2012.07.022

different thresholds for fibers and different neurons within the region, and also the potential for tissue damage. In-vivo studies are also hindered by the inability to apply pharmacological agents in a highly localized area surrounding the target neuron that receives these long-range projections.

In-vitro electrophysiological approaches developed over the last 20 years overcome some of these limitations, such as permitting tight control over the external and internal milieu surrounding cells and allowing more precise stimulation and electrophysiological recordings from neurons. However studies of pathways within in-vitro preparations are limited by the viable depth of the tissue, typically 400–800 μm, and therefore many of the important long-range projections to neurons cannot be studied in-vitro.

A new approach, using optogenetic techniques, overcomes many of these obstacles and potentially enables highly selective stimulation of homogenous neuronal populations, high temporal precision, and activation of synapses originating from neurons distant from the neuron of interest while retaining the high degree of control and precision afforded in-vitro (Fenno et al., 2011). This approach utilizes heterologous expression of the microbial opsin channelrhodopsin-2 (ChR2), a light-activated cation channel that is ubiquitously inserted into the plasma membrane of cells. Brief (millisecond scale) light pulses can depolarize the membrane of cell bodies, dendrites or axons of neurons that express ChR2, allowing for excitation of specific compartments of the plasma membrane, including presynaptic terminals distant from the soma (Petreanu et al., 2007).

Several recent reports have shown the feasibility of this optogenetic method to study the neurophysiology of distant synapses by driving expression of ChR2 using different viral vectors (Cruikshank et al., 2010; Varga et al., 2009) or transgenic mice (Ren et al., 2011). However, a comprehensive comparative assessment of the different available methods has not been done. For instance, it is not readily clear which of the different methods could best drive expression of ChR2 into the plasma membrane of distal synaptic terminals. In order to extend the current knowledge of how to successfully and efficiently employ optogenetic stimulation of distant axons and identified synaptic endings, we tested and compared the usefulness of lentivirus, with neuron- and population-specific promoters, as well as two ChR2 Cre responder transgenic mouse lines to express ChR2 and selectively stimulate the important neurotransmission from paraventricular hypothalamus nucleus (PVN) to brainstem cardiac vagal neurons (CVNs) that control heart rate in the brainstem.

More specifically we tested lentiviral vectors expressing ChR2 under two PVN promoters (minimal promoter regions upstream of the origin of transcription) for vasopressin and oxytocin neurons, and evaluated their ability to drive ChR2 expression in distal axons in autonomic brain stem sites after microinjection in the PVN. We also used a lentiviral vector with a strong neuron-specific promoter (human synapsin I fragment) (Kugler et al., 2003). In addition, we assessed the expression of ChR2 in paraventriculo-autonomic projections using crossbred transgenic mice that express ChR2 in the PVN. To this purpose, we employed the Cre/LoxP system and crossbred two different ChR2 Cre responder lines with a Sim1-Cre driver transgenic mouse. *Sim1* is a gene strongly expressed during embryonic development in PVN neurons, including in vasopressin and oxytocin parvocellular neurons, that project to the nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMV) (Balthasar et al., 2005; Duplan et al., 2009; Fan et al., 1996). In vitro electrophysiology coupled with laser evoked photoactivation of ChR2 was used to assess the functional connectivity of distal autonomic-related PVN projections and to test the effectiveness of each of these approaches in activation of synaptic terminals and transmitter release from PVN neurons onto CVNs.

2. Materials and methods

2.1. Lentiviral vector plasmids and promoter constructs

Lentiviral plasmids pLenti-Syn-hChR2(H134R)-EYFP-WPRE, packaging plasmid pCMV-ΔR8.74 and envelope plasmid pMD2.G were all kindly provided by K. Deisseroth (Stanford University, Stanford, CA). We chose to use the H134R mutant for the current studies for its enhanced photocurrents (Nagel et al., 2005) and higher release probability (Schoenenberger et al., 2011), since our objective was to probe long-range projections and maximize the likelihood of release of neurotransmitter from presynaptic endings. A rat minimal vasopressin promoter fragment containing 527 bp upstream and 30 bp downstream of the start of transcription of the vasopressin gene (UCSC genome browser on rat November 2004 assembly; chr3:118,206,966 to 118,207,522) was de novo synthesized and flanked by multiple cloning sites (Genscript, Piscataway, NJ) (Iwasaki et al., 1997; Kim et al., 2001). Similarly, we synthesized (Genscript, Piscataway, NJ) a rat minimal oxytocin promoter element from −530 bp to +33 relative to the origin of transcription of the oxytocin gene (UCSC genome browser on rat November 2004 assembly; chr3:118,193,690 to 118,194,252) (Chu and Zingg, 1999; Richard and Zingg, 1991). Vasopressin and oxytocin promoter fragments were subcloned into pLenti-Syn-hChR2(H134R)-EYFP-WPRE using the *Xba*I and *Age*I cloning sites to replace the Synapsin promoter, creating the new plasmids pLenti-AVP-hChR2(H134R)-EYFP-WPRE and pLenti-OXY-hChR2(H134R)-EYFP-WPRE.

2.2. Viral vectors

Plasmids pLenti-Syn-hChR2(H134R)-EYFP-WPRE, pLenti-AVP-hChR2(H134R)-EYFP-WPRE and pLenti-OXY-hChR2(H134R)-EYFP-WPRE were transformed into MAX Efficiency Stbl3 Competent Cells for amplification. Plasmids pMD2.G and pCMV-ΔR8.74 were transformed into DH5α cells for amplification and all plasmids were purified using a Maxiprep kit (Qiagen, Valencia, CA). VSVg pseudotyped lentivirus particles were produced according to standard protocols (Szulc et al., 2006). Briefly, we transfected 8–24 150 mm plates of 60–80% confluent HEK 293T cells with 37.5 μg pLenti, 33 μg pCMV-ΔR8.74 and 18 μg pMD2.G using 2.5 M $CaCl_2$ and 2× HBSS for transfection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% FBS (Gibco, Invitrogen), 1% L-glu (Invitrogen) and 1% Pen/Strep (Invitrogen). Eight hours after the transfection, plates were washed with incubation medium and replaced with 13 ml medium. Twenty-four hours after transfection sodium butyrate was added to the plates in a final concentration of 5 mM. The virus was harvested 48 h after the transfection and the supernatant was spun for 15 min at 1500 × *g* and filtered through a 0.22 μm PES bottle top filter. The supernatant was concentrated by ultracentrifuging for 2 h at 20,000 rpm using 38.5 ml thinwall tubes and a Beckmann SW-28 rotor. Viral particles were resuspended in 1–2 ml PBS and pooled for a second 2 h, 20,000 rpm ultracentrifuge spin into one 10 ml thickwall tube. The final viral vector pellet was resuspended in 35–100 μl of PBS, which was stored at −80 °C in 4 μl per aliquot. The viral titer was determined by counting infected HEK 293T cells using FACS (Ramezani and Hawley, 2002). All batches of virus had a titer between 2×10^8 and 1×10^9 transducing units (TU)/ml (see Table 1).

2.3. Stereotactic injections, transgenic mice and cardiac labeling

Neonatal Sprague Dawley rats (postnatal day 4 or 5) were anesthetized by hypothermia and mounted in a stereotactic apparatus with a neonatal adapter (Stoelting, Wood Dale, IL). A midline incision exposed the skull and a small burr hole was made to