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Sleep State Dependence of Optogenetically evoked Responses in Neuronal Nitric Oxide Synthase-positive Cells of the Cerebral Cortex

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Abstract—Slow-wave activity (SWA) in the electroencephalogram during slow-wave sleep (SWS) varies as a function of sleep-wake history. A putative sleep-active population of neuronal nitric oxide synthase (nNOS)-containing interneurons in the cerebral cortex, defined as such by the expression of Fos in animals euthanized after protracted deep sleep, may be a local regulator of SWA. We investigated whether electrophysiological responses to activation of these cells are consistent with their role of a local regulator of SWA. Using a Cre/loxP strategy, we targeted the population of nNOS interneurons to express the light-activated cation channel Channelrhodopsin2 and the histological marker tdTomato in mice. We then performed histochemical and optogenetic studies in these transgenic mice. Our studies provided histochemical evidence of transgene expression and electrophysiological evidence that the cerebral cortex was responsive to optogenetic manipulation of these cells in both anesthetized and behaving mice. Optogenetic stimulation of the cerebral cortex of animals expressing Channelrhodopsin2 in nNOS interneurons triggered an acute positive deflection of the local field potential that was followed by protracted oscillatory events only during quiet wake and slow wave sleep. The response during wake was maximal when the electroencephalogram (EEG) was in a negative polarization state and abolished when the EEG was in a positive polarization state. Since the polarization state of the EEG is a manifestation of slow-wave oscillations in the activity of underlying pyramidal neurons between the depolarized (LFP negative) and hyperpolarized (LFP positive) states, these data indicate that sleep-active cortical neurons expressing nNOS function in sleep slow-wave physiology.

Key words: evoked potential, electroencephalogram, mice, optogenetic, slow-wave activity.

INTRODUCTION

High-amplitude slow-wave activity (SWA; < 4 Hz) in the cerebral cortical electroencephalogram (EEG) is a defining feature of slow-wave sleep (SWS) and is thought to mediate functional consequences of SWS (Tononi and Cirelli, 2006; Puentes-Mestril and Aton, 2017; Siclari and Tononi, 2017; Walsh et al., 2006; Tasali et al., 2008; Aton et al., 2014). We previously demonstrated that type I neuronal nitric oxide synthase (nNOS) cells in the cerebral cortex are activated during episodes of SWS associated with increased SWA (Gerashchenko et al., 2008). Further, we performed injections of neurokinin 1 (NK1) receptor agonists and antagonists into the cerebral cortex and found that SWA was locally enhanced by NK1 receptor agonists and reduced by NK1 receptor antagonists (Zielinski et al., 2015). Because NK1 receptors are expressed exclusively in nNOS neurons in the cerebral cortex (Dittrich et al., 2012), this result suggests that modulation of the SWA production is caused by changes in the activity of nNOS neurons. Collectively, these results suggest the important role of nNOS neurons in the cerebral cortex in the production of SWA and emphasize the importance of studying the mechanisms by which the sleep-active cells affect SWA production. Herein, we used a pharmacologically activated double transgenic mouse model to measure the effects of activating these cells optogenetically on neuronal activity at the EEG level. We report that a Channelrhodopsin2 (ChR2) construct (Feng, 2012) driven by the nNOS promoter (Taniguchi et al., 2011) is expressed

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Abbreviations: ANOVA, analysis of variance; AW, active wake; ChR2, channelrhodopsin2; DAB, 3′-diaminobenzidine; EEG, electroencephalogram; EMG, electromyogram; ER, estrogen receptor; LD, light–dark; LED, light emitting diode; LFP, local field potential; NADPH, nicotinamide adenine dinucleotide phosphate; NK1, neurokinin 1; nNOS, neuronal nitric oxide synthase; PBS, phosphate-buffered saline; QW, quiet wake; Rec, recovery sleep; REMS, REM sleep; RFP, red fluorescent protein; SEM, standard error of the means; SD, sleep deprivation; SWA, slow-wave activity; SWS, slow-wave sleep; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

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in cortical nNOS-positive cells and that the optogenetic activation of this cell population induces EEG potential fluctuations more robustly and reliably during wake than during SWS. The magnitude of evoked EEG responses is polarization-state dependent and is abolished when the cortex is at a positive potential, akin to its state during a physiological slow wave. These observations are compatible with a role for type I nNOS cells in the cortex regulating local SWA homeostatic dynamics.

EXPERIMENTAL PROCEDURES

All *in vivo* experimentation was approved by the institutional animal care and use committee and was performed in accordance with National Institutes of Health guidelines. Immunohistochemical studies were performed at the Harvard Medical School in the West Roxbury VA Medical Center. Optogenetic studies were performed at Elson S. Floyd College of Medicine and Department of Integrative Physiology and Neuroscience in Washington State University. Thirty-five adult male mice were used in these studies.

Transgenic mouse generation

A *loxP*-regulated genetic construct was previously reported as a tool for selectively targeting a neuronal population of interest for optogenetic excitation with the light-sensitive ion channel, ChR2 (Feng, 2012). These mice of the *B6;129-Gt(ROSA)26Sortm1(CAG-COP4*E123T*H134R,-tdTomato)Gfng/J* strain are referred to hereafter as loxP-ChR2. The transgene construct contains a CAG promoter, a *loxP* site-flanked STOP fragment and *pGK-NEO-pA* cassette, 2 copies of ChR2 with the E123T mutation (ChETA variant) and the H134R mutation, a P2A oligopeptide sequence, the tdTomato variant of enhanced red fluorescent protein (RFP), and the WPRE (Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element) sequence at the *Gt(ROSA)26Sor* locus. In the absence of *in vivo* recombination, expression of ChR2 and tdTomato is blocked by the *loxP*-flanked STOP fragment inserted between the *Gt(ROSA)26Sor* promoter and the ChR2/tdTomato sequence. Mice that are homozygous for the targeted mutation are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities (http://jaxmice.jax.org/strain/017455.html).

In order to target ChR2 expression to a population of interest in loxP-ChR2 mice, it is necessary to activate ChR2 expression by Cre recombinase-mediated excision of the *loxP* stop site. Cre recombinase-mediated recombination removes the *loxP*-flanked STOP fragment and results in functional ChR2 and tdTomato expression (separately, not as a fusion protein). A line of mice (Taniguchi et al., 2011) has been made public through JAXMice.org (*B6;129S-Nos1tm1.1(cre/ERT2)Zjh/J*; JAX stock # 14541; genotype referred to hereafter as nNOS-CreER) in which Cre recombinase expression is driven by the nNOS promoter. The transgene construct is integrated at the endogenous nNOS locus as a 'knock-in' and accordingly, homozygous *nNOS-Cre* mice are nNOS-deficient. The animals used in the current study were heterozygous for the transgene. The transgene construct is a Cre recombinase coding sequence fused to a triple mutant human estrogen receptor (ER). In the absence of an activating ligand, the ER-dependent construct is silent. The three mutations collectively render the receptor non-responsive to estradiol at physiological concentrations but inducible by the ER ligand tamoxifen. Tamoxifen was mixed with corn oil (20 mg/mL) and administered by oral gavage at a dose of 200 mg/kg/day for 3 days. Mice undergoing this protocol are referred to hereafter as nNOS-ChR2 mice—this protocol has been used with no side effects (Taniguchi et al., 2011). Control mice (referred to hereafter as non-expressor) received equivalent volumes of corn oil via gavage.

Male B6.Cg-Tg (*Thy1-COP4/eYFP)18Gfng/J* mice (JAX strain #7612) were used only in the anesthetized preparation studies. In these mice, the "*mouse thymus cell antigen 1 (thy1)*" promoter drives expression of ChR2 in cortical pyramidal cells (Arenkiel et al., 2007). We therefore refer to them hereafter as pyramidal-ChR2 mice. Founders were bred with CD1 mice to produce hemizygous transgenic pyramidal-ChR2 males, as previously described (Wisor et al., 2013). Non-expressor and nNOS-ChR2 mice used in the anesthetized preparation studies were also males.

Local field potentials in anesthetized mice

nNOS-ChR2 mice, non-expressor mice and pyramidal-ChR2 mice, were used in the experiments in which local field potentials were measured under isoflurane anesthesia (5% induction, 1.5% maintenance) with perfluoroalkoxy alkane (PFA)-coated tungsten electrodes (A-M Systems, Sequim, WA, catalog No. 797000, 0.008″ bare diameter, AWG 32). A 0.5-mm ball burr bit was used to drill an initial hole through the skull for placement of the electrodes. Holes were placed in left vibrissal motor cortex (0.86 mm anterior/1.5 mm lateral from bregma) and frontal association cortex (2 mm anterior/2 mm lateral from bregma). A 90 degree bend was made in each electrode wire 2.0 mm from the tip. Electrodes were inserted until the bend rested on the skull. Insulation was removed only from the tip of the electrode, thus field potentials were measured at a depth of approximately 1.5 mm into the brain, corresponding to infragranular cortex. A screw placed in the contralateral parietal cortex served as a cable ground. A screw placed in contralateral occipital cortex and connected by wire to the surgical table served as a body ground. Fast curing, 2-part orthodontic acrylic resin was used to secure and insulate all electrodes. The electrophysiological signal was collected at 5000 Hz, amplifier gain 1000X, input voltage range = +/−81 9.2 mV (Multichannel Systems, Reutlingen, Germany; products # MPA81, SC8x8; MC_Rack software version 4.4.8). The signal was processed through a Butterworth 2nd Order filter with a high pass of 0.5 Hz. Data files were converted to raw binary files for data analysis in the MATLAB programing environment (Mathworks, Natick, MA, USA).

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