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Substance P and the neurokinin-1 receptor regulate electroencephalogram non-rapid eye movement sleep slow-wave activity locally

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Abstract

The neuropeptide substance P is an excitatory neurotransmitter produced by various cells including neurons and microglia that is involved in regulating inflammation and cerebral blood flow—functions that affect sleep and slow-wave activity (SWA). Substance P is the major ligand for the neurokinin-1 receptor (NK-1R), which is found throughout the brain including the cortex. The NK-1R is found on sleep-active cortical neurons expressing neuronal nitric oxide synthase whose activity is associated with SWA. We determined the effects of local cortical administration of a NK-1R agonist (substance P-fragment 1, 7) and a NK-1R antagonist (CP96345) on sleep and SWA in mice. The NK-1R agonist significantly enhanced SWA for several hours when applied locally to the cortex of the ipsilateral hemisphere as the electroencephalogram (EEG) electrode but not after application to the contralateral hemisphere when compared to saline vehicle control injections. In addition, a significant compensatory reduction in SWA was found after the NK-1R agonist-induced enhancements in SWA. Conversely, injections of the NK-1R antagonist into the cortex of the ipsilateral hemisphere of the EEG electrode attenuated SWA compared to vehicle injections but this effect was not found after injections of the NK-1R antagonist into contralateral hemisphere as the EEG electrode. Non-rapid eye movement sleep and rapid eye movement sleep duration responses after NK-1R agonist and antagonist injections were not significantly different from the responses to the vehicle. Our findings indicate that the substance P and the NK-1R are involved in regulating SWA locally.

Keywords

neurokinin-1 receptor; substance P; local sleep; tachykinin; slow-wave activity; mice

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1.0 Introduction

Substance P is a neuropeptide derived from the preprotachykinin A gene that is produced by many cell types including neurons and microglia (Marriott, 2004; Munoz and Covenas). Substance P acts primarily through the neurokinin-1 receptor (NK-1R; also known as the tachykinin receptor 1) and is found throughout the body including the central nervous system (CNS), peripheral nervous system, pulmonary tissue, and immune and vascular endothelial cells (Steinhoff et al., 2014). Within the CNS, the NK-1R is distributed throughout the brain including regions highly involved in regulating sleep such as the hypothalamus, brainstem, and cortex (Brown et al., 2012; Dam et al., 1988). The NK-1R couples to G protein coupled receptors including $G_{q/11}$, G_{α_s} , and G_{α_0} proteins to activate phospholipase C beta leading to enhanced cytosolic calcium levels, arachidonic acid utilization, and cyclic adenosine monophosphate production (Harrison and Geppetti, 2001). Substance P and the NK-1R are implicated in many functions including the regulation of pain, anxiety, stress, neurogenesis, vasodilation, and inflammation (Munoz and Covenas, 2014).

Non rapid-eye movement sleep (NREMS) electroencephalogram (EEG) delta power (~ 0.5 -4.0 Hz frequency range) [also known as slow-wave activity (SWA)] is an indicator of sleep intensity (Achermann and Borbély, 2003). SWA is increased after acute enhanced waking activity that occurs during sleep deprivation in many species, including mice, rabbits, rats, and humans (Zielinski and Krueger, 2011). Enhanced brain activity from cognitive tasks also enhances SWA (Mölle et al., 2004). Further, dysregulated SWA is prominent in sleep disorders, including insomnia and sleep apnea and many chronic inflammatory conditions such as type 2 diabetes, Alzheimer's disease, cancer, and cardiovascular disease (Zielinski and Krueger, 2011).

The exact mechanisms that regulate SWA are unknown, although the literature indicates that SWA is generated within cortico-thalamic loops (Steriade, 2006). However, a wide literature indicates that pro-inflammatory molecules, including interleukin-(IL)-1 beta (IL-1 β), IL-6, and tumor necrosis factor-alpha (TNF- α), enhance SWA (Zielinski and Krueger, 2011; Imeri and Opp, 2009). Injections of these substances centrally enhance NREMS and SWA. Enhanced waking activity induces increased expression of pro-inflammatory molecules, such as IL-1 β and TNF- α , in the brain including the cortex (Zielinski and Krueger, 2011). In addition, pathogens and related components, such as the gram-negative bacterial cell wall component lipopolysaccharide (LPS), enhance pro-inflammatory brain molecules and also enhance SWA (Zielinski and Krueger, 2011). Evidence indicates that LPS enhances substance P (Takeda et al., 2011), although the effects of LPS on substance P expression in the brain remains unknown. Inhibiting brain inflammatory molecules, including IL-1 β and TNF- α using pharmacology, knockout mice or siRNA inhibits SWA or homeostatic sleep responses to sleep deprivation or somnogenic stimuli (Zielinski and Krueger, 2011; Imeri and Opp, 2009). Nevertheless, the role of the pro-inflammatory molecule substance P and the NK-1R effect on SWA remains unknown.

The 1,7 fragment of substance P is produced by the enzymatic hydrolyzing of the phenylalanine-phenylalanine bond of substance P in the extracellular space and binds to the NK-1R (Zhou Q et al., 2000). Intracerebroventricular injections of substance P fragment 1,7 enhances the expression of N-methyl-D-aspartate (NMDA) receptors in the brain (Zhou Q et al., 2000)—receptors that are known to regulate sleep and the EEG (Brown et al., 2012). Herein, we examined the effects of local cortical hemispheric injections of the NK-1R agonist substance P fragment 1,7 and the NK-1R antagonist CP96345 on sleep and SWA in mice.

2.0 Methods

2.1 Animals

Two-month-old male C57BL/6J mice (N = 8 per treatment group) were purchased from Jackson Laboratories (Bar Harbor, ME) and used for the present experiments. Mice were kept in individual cages in a sound attenuated room maintained at 22 ± 3 °C. Mice were kept on a 12:12 h light/dark cycle and had *ad libitum* access to food and water at all times. All experimental protocols were approved by Harvard University and Veteran Affairs Boston Healthcare system Animal Care and Use Committee and were in compliance with the National Institutes of Health guidelines.

2.2 Polysomnography Surgery and Recording

Mice were anesthetized with a ketamine and xylazine cocktail (80 and 10 mg/kg, respectively) for surgical procedures. Mice were implanted with EEG electrodes over the left somatosensory cortex (1 mm posterior to bregma and 1 mm lateral to the midline) and a reference electrode over the cerebellum (0.5 mm posterior to lambda placed centrally) (Paxinos and Franklin, 2001). Additionally, a cannula was placed into the ipsilateral hemisphere as the EEG electrode (1.5 mm posterior to bregma and 1 mm lateral to the midline; injection syringe dorsal -0.5 mm) and into the contralateral hemisphere as the EEG electrode (1.5 mm posterior to bregma and 1 mm lateral to the midline) for the administration of pharmacological substances. Two electromyogram (EMG) electrodes were implanted into the nuchal muscles to assess muscle activity. The EEG and EMG electrodes were secured to the skull and a pedestal with dental cement. Mice were tethered to wireless transponders (F20-EET transponders; Data Sciences International, St. Paul, MN) using a system (Neurotargeting Systems, Inc., Chestnut Hill, MA) that allows mice to move freely as previously described (Zielinski et al., 2013a). Mice were placed in standard mouse caging on top of receiver plates (PhysioTel receiver RPC-1; Data Sciences International, St. Paul, MN) that detected the FM signals of the transponders. EEG and EMG signals were amplified and recorded.

2.3 Pharmaceutical Substances and Injections

Mice were allowed at least 10 days to recover from the surgical procedure and were acclimated to the tethered system for two days prior to experimental treatments. Mice were injected with 0.2 µL of 0.9 % NaCl (i.e., saline) into the ipsilateral or contralateral hemisphere as the EEG electrode at light onset [zeitgeber (ZT) 0] 24 h prior to each pharmacological injection. Sleep was recorded for 24 h after the saline injection, which

served as a baseline (experiment 1). Thereafter, 5000 nM, 500 nM, or 50 nM diluted in 0.2 μ L of saline concentrations of the NK-1R agonist substance P fragment 1-7 (Sigma-Aldrich, St. Louis, MO)(experiment 2) or the NK-1R antagonist CP96345 (Sigma-Aldrich, St. Louis, MO)(experiment 3) were injected into the ipsilateral hemisphere as the EEG electrode in counter balance order of dosage concentrations at light onset (ZT 0). Sleep was then recorded for 24 h. In addition, the NK-1R agonist substance P fragment 1,7 (500 nM diluted in 0.2 μ L of saline) and the NK-1R antagonist CP96345 (500 nM diluted in 0.2 μ L of saline) were given in the contralateral hemisphere of the EEG electrode and sleep was then recorded for 24 h. 0.2 μ L of the saline vehicle control was injected prior to each dosage of the pharmaceutical that was applied.

2.4 Polysomnography Analysis

Sleep states [NREMS, rapid-eye movement sleep (REMS), and wake] were determined manually off-line in 10-second epochs as previously described (Zielinski et al., 2013b). Sleep state durations were calculated across 2 h time periods. Sleep state episode durations and episode frequencies were determined in 12 h time periods after injections of the vehicle. EEG signal power spectra were determined after vehicle and pharmaceutical injections. Fast Fourier transformation of the EEG signals (μ V²) within the 0.5-20 Hz frequency range in 0.5 Hz bins were made for each epoch over 24 h periods after vehicle and pharmaceutical injections. Additionally, NREMS EEG SWA (0.5-4 Hz frequency range) was determined in 2 h time bins across 24 h periods after vehicle and pharmaceutical injections. SWA data for each 2 h time bin were normalized to mean vehicle injection data over 24 h periods for each individual mouse as previously described (Zielinski et al., 2013a). After vehicle injections, EEG power spectra (0.5-20 Hz) during the dark period (ZT 12-0) were normalized to EEG power spectra (0.5-20 Hz) during the light period (ZT 0-12) for each individual mouse. EEG power (0.5-20 Hz) data after pharmaceutical injections were normalized to the mean of EEG power spectra (0.5-20 Hz) during the respective time-of-day after the vehicle injections for each individual mouse. EEG power spectra time blocks assessed after pharmaceutical injections were chosen based upon corresponding significant differences observed in SWA [i.e., first 2 h after the 5000 nM dosage of NK-1R agonist (ZT 0-2); first 6 h after the 500 nM dosage of the NK-1R agonist (ZT 0-6); 11-18 h after the 500 nM dosage of the NK-1R agonist (ZT 11-18); First 12 h after the 5000, 500, and 50 nM dosage of the NK-1R antagonist (ZT 0-12)].

2.5 Statistics

Independent and paired *t*-tests and analysis of variance (ANOVA) were performed using IBM SPSS software version 22.0 (IBM Corp., Armonk, NY) to determine significant differences in polysomnography data for the experimental treatments (Table 1). Independent and paired *t*-tests were used for post-hoc comparisons when appropriate. Significance was set at $p < 0.05$.

3.0 Results

3.1 Experiment 1

3.1.1 Sleep State Durations after Local Cortical Injections of Saline—Sleep state responses to local cortical injections of the vehicle into the cortex of the ipsilateral and contralateral hemisphere as the EEG electrode are presented in Fig 1. Diurnal variations in NREMS duration were found after vehicle injections into either the ipsilateral or contralateral hemisphere with greater values found during the light period compared to the dark period [ipsilateral hemisphere: $t(1,47) = 12.881, p < 0.001$; contralateral hemisphere: $t(1,15) = 8.179, p < 0.001$] (Fig 1A). The enhanced NREMS duration during the light period occurred, in part, from an enhanced frequency of NREMS episode bouts occurring during the light period compared to the dark period [ipsilateral hemisphere: $t(1,47) = 11.235, p < 0.001$ (light period: 399.67 ± 17.80 ; dark period: 225.06 ± 18.32); contralateral hemisphere: $t(1,15) = 4.699, p < 0.001$ (light period: 403.50 ± 27.39 ; dark period: 265.44 ± 43.00)]. However, no significant differences in NREMS episode durations were found between light and dark cycles after injections of the vehicle into either the ipsilateral (light period: 1.10 ± 0.12 min; dark period: 1.01 ± 0.09 min) or contralateral hemisphere (light period: 1.03 ± 0.16 min; dark period: 0.90 ± 0.19 min). Further, no significant differences in NREMS episode frequencies or episode durations were found between injections of the saline into the cortex of either hemisphere.

A diurnal variation in REMS duration was found after vehicle injections were applied to either the ipsilateral or contralateral hemispheres [ipsilateral hemisphere: $t(1,47) = 10.045, p < 0.001$; contralateral hemisphere: $t(1,15) = 6.840, p < 0.001$] (Fig 1B). Greater REMS durations occurred during the light period vs. the dark period after injections of the vehicle was applied either hemisphere, which was attributed, in part, to enhanced REMS episode frequencies occurring during the light period vs. dark period [ipsilateral hemisphere: $t(1,47) = 9.709, p < 0.001$ (light period: 207.44 ± 15.91 ; dark period: 95.15 ± 11.70); contralateral hemisphere: $t(1,15) = 4.502, p < 0.001$ (light period: 230.94 ± 28.73 ; dark period: 104.31 ± 24.27)]. No significant differences in REMS episode durations were detected between light and dark cycles after injections of the vehicle into either the ipsilateral (light period: 0.38 ± 0.03 min; dark period: 0.37 ± 0.03 min) or contralateral hemisphere (light period: 0.36 ± 0.04 min; dark period: 0.33 ± 0.04 min). Further, REMS episode frequencies and episode durations were not significantly different between vehicle injections into the cortex of either hemisphere.

3.1.2 SWA and EEG Power after Local Cortical Injections of Saline—SWA and EEG power responses to local cortical injections of the vehicle into the cortex of the ipsilateral and contralateral hemisphere as the EEG electrode are exhibited in Figs 1 and 2. SWA was greater during the dark period compared to the light period after injections of the vehicle into the ipsilateral hemisphere [$t(1,47) = 2.123, p = 0.039$] (Fig 1C). Injections of the vehicle into the contralateral hemisphere did not result in a significant enhancement in SWA during the dark period compared to the light period. No significant main effect differences in SWA during light or dark periods were found between hemispheres after vehicle injections, although mice injected with the vehicle into the ipsilateral hemisphere

had significantly enhanced SWA 13-14 h post-injection compared to mice injected the contralateral hemisphere.

NREMS EEG power spectra (0.5-20 Hz frequency range) was greater during the dark period compared to the light period after injections of the vehicle into either the ipsilateral or contralateral hemisphere [$F(1,1880) = 44.238, p < 0.001$; $F(1,600) = 13.619, p < 0.001$, respectively] (Fig 2A). NREMS EEG power frequencies displayed the typical dispersion amplitude pattern across frequencies (0.5-20 Hz) [ipsilateral hemisphere: $F(1,1880) = 104.570, p < 0.001$; contralateral hemisphere: $F(1,600) = 23.009, p < 0.001$]. Post-hoc analysis determined no significant differences in any individual EEG power frequencies bins (0.5-20 Hz) between light and dark periods after vehicle injections in either hemisphere. No significant differences in NREMS EEG power spectra (0.5-20 Hz frequency range) were found between hemispheres during either light or dark periods after vehicle injections.

REMS EEG power spectra values displayed the typical frequency distribution pattern across the 0.5-20 Hz frequency range [ipsilateral hemisphere: $F(1,1880) = 41.411, p < 0.001$; contralateral hemisphere: $F(1,600) = 11.646, p < 0.001$]. REMS EEG power spectra (0.5-20 Hz frequency range) was also greater during the dark period compared to the light period after vehicle injections into either hemisphere [ipsilateral hemisphere: $F(1,1880) = 72.334, p < 0.001$; contralateral hemisphere: $F(1,600) = 48.737, p < 0.001$] (Fig 2B). Post-hoc analysis determined that this effect occurred, in part, due to significant enhancements in REMS EEG power spectra at the 3.0, 7.5, 10.5-11.0, 12.5, 13.0-13.5, 15.5, 17.0, and 20.0 Hz frequency bin ranges after injections of the vehicle into the ipsilateral hemisphere and the 7.0-7.5 Hz frequency bin ranges after injections of the vehicle into contralateral hemisphere. REMS EEG power spectra (0.5-20 Hz frequency range) were not significantly different between hemispheres during the light or dark periods after vehicle injections.

EEG power spectra (0.5-20 Hz frequency range) during wakefulness was greater during the dark period compared to the light period after vehicle injections into either hemisphere [ipsilateral hemisphere: $F(1,1880) = 5.305, p = 0.021$; contralateral hemisphere: $F(1,600) = 10.068, p = 0.002$] (Fig 2C). Differences in waking EEG power spectra occurred over the 0.5-20 Hz frequency range after injections of the vehicle [ipsilateral hemisphere: $F(1,1880) = 171.674, p < 0.001$; contralateral hemisphere: $F(1,600) = 38.785, p < 0.001$]. Post-hoc analysis determined significant enhancements during the dark period vs. light period within the 3.0, 7.5-10.5, 12.5-20 Hz frequency ranges after injections of the vehicle into the ipsilateral hemisphere. In addition, post-hoc analysis found significant enhancements within the 8.5-10.0 Hz frequency range after injections of the vehicle into the contralateral hemisphere. No significant differences in waking EEG power (0.5-20 Hz frequency range) were found between hemispheres after vehicle injections.

3.2 Experiment 2

3.2.1 Sleep State Durations Responses to the NK-1R Agonist—Sleep state duration responses to NK-1R agonist injections are presented in Figs 3 and 4. No significant differences in NREMS or REMS duration during the 24 h period after injections of the NK-1R agonist into the cortex of the ipsilateral hemisphere as the EEG electrode at dosages of 5000 nM, 500 nM or 50 nM were found compared to vehicle injections (Figs 3A, 3B, 3C,

4A, 4B, 4C) with the exception of a significant reduction in NREMS duration 10-12 h after injection (ZT 10-12) of the 500 nM dosage of the NK-1R agonist into the ipsilateral hemisphere. Additionally, no significant differences in NREMS or REMS duration were found during the 24 h period after the 500 nM dosage of the NK-1R agonist was applied to the cortex of the contralateral hemisphere as the EEG electrode (Figs 3D and 4D).

3.2.2. SWA and EEG Power Responses to the NK-1R Agonist—Figs 5 and 6 exhibit the SWA and EEG power responses to the NK-1R agonist. An interaction (dosage x treatment) was found for the dosage of NK-1R agonist (5000, 500, and 50 nm) injected into the cortex of the ipsilateral hemisphere as the EEG electrode enhancing SWA (0.5-4.0 Hz frequency range) during the first 6 h post-injection compared to vehicle injections [F , (5,84) = 2.977, p = 0.16] (Fig 5A, 5B, 5C).

A main effect was found for injections of the 500 nM dosage of the NK-1R agonist injected into the ipsilateral hemisphere enhancing SWA during the first 6 h post-injection (ZT 0-6) compared to the vehicle injections [F (1,54) = 6.925, p = 0.011; 19.5 ± 6.0 % change vs. vehicle](Fig 5B). EEG power in the 0.5-20 Hz frequency range was enhanced during first 6 h post-injection period after the 500 nM dosage of the NK-1R agonist was injected into the ipsilateral hemisphere compared to the vehicle injections [F (1,280) = 99.352, p < 0.001] (Fig 6B). Post-hoc analysis determined significant enhancement in EEG power spectra within the 2.5-4.5, 7.5-11.0, and 13.0-14.0 Hz frequency bin ranges during the first 6 h post-injection after the 500 nM dosage of the NK-1R agonist injections into the ipsilateral hemisphere compared to the vehicle injections. In addition, a reduction in SWA was found 11-18 h post-injection (ZT 11-18) after the 500 nM dosage of the NK-1R agonist was administered to the ipsilateral hemisphere compared to the vehicle [F (1, 54) = 13.242, p = 0.001] (Fig 5B), although this significant effect was most prominent between 14-16 h post-injection. EEG power spectra (0.5-20 Hz frequency range) was reduced 11-18 h (ZT 11-18) after the administration of the 500 nM dosage of the NK-1R agonist into the ipsilateral hemisphere compared to vehicle [F (1,280) = 88.140, p < 0.001] (Fig 6C). EEG power spectra (0.5-20 Hz frequency range) also exhibited a reduction 11-18 h after the 500 nM dosage of the NK-1R agonist was injected into the ipsilateral hemisphere compared to vehicle injections [frequency x treatment: F (39,280) = 2.309, p < 0.001]. Post-hoc analysis determined significant attenuations in EEG power spectra within the 0.5-2.0, 5.5-6.0, and 7.0 Hz frequency bin ranges after injections of the 500 nM dosage of the NK-1R agonist into the ipsilateral hemisphere vs. vehicle injections. However, no significant differences in SWA were found after the 500 nM dosage of NK-1R agonist was injected into the contralateral hemisphere compared with vehicle injections (Fig 5D).

A main effect was found for the 5000 nM dosage of the NK-1R agonist enhancing SWA during first 2 h (ZT 0-2) after it was injected into the ipsilateral hemisphere compared to vehicle injections [F (1, 54) = 4.955, p = 0.030] (Fig 5A), although this effect was not significant over the first 6 h post-injection time period (3.9 ± 1.5 % change vs. saline). Also, there was no observable reduction in SWA after the enhancement occurring during the first 2 h post-injection. NREMS EEG power spectra (0.5-20.0 Hz frequency range) was enhanced during the first 2 h after the 5000 nM dosage of the NK-1 receptor agonist was injected into the ipsilateral hemisphere compared to the vehicle injections [ipsilateral hemisphere:

$F(1,280) = 28.758, p < 0.001$] (Fig 6A). Post-hoc analysis revealed no significant differences within 0.5 Hz frequency bands (0.5-20 Hz frequencies) after injections of the 5000 nM dosage of the NK-1R agonist into the ipsilateral hemisphere compared to the vehicle injections.

No significant differences in SWA were found after the 50 nM dosage of the NK-1R agonist was injected into the ipsilateral hemisphere when compared to vehicle injections (-2.6 ± 2.6 % change vs. vehicle control)(Fig 5C).

3.3 Experiment 3

3.3.1 Sleep State Durations responses to the NK-1R Antagonist—Sleep state duration responses to the NK-1R antagonist are shown in Figs 3 and 4. NREMS and REMS duration responses to the NK-1R antagonist injections into the cortex of the ipsilateral hemisphere as the EEG electrode at dosages of 5000 nM, 500 nM or 50 nM were similar to those found after vehicle injections (Figs 3A, 3B, 3C, 4A, 4B, 4C) with the exception of injections of the 5000 nM dosage injected into the ipsilateral hemisphere significantly enhancing REMS duration during the first 2 h post-injection (ZT0-2). Both NREMS and REMS duration responses after injections of the NK-1R antagonist at the 500 nM dosage into the cortex of the contralateral side of the EEG electrode were also similar to that found after vehicle injections (Figs 3D and 4D).

3.3.2 SWA and EEG Power Responses to the NK-1R Antagonist—Figs 5 and 7 exhibit the SWA and EEG power spectra responses to the NK-1R antagonist. A main effect was found for the NK-1R antagonist at dosages of 5000 nM, 500 nM, and 50 nM injected into the cortex of the ipsilateral hemisphere as the EEG electrode reducing SWA during the first 12 h post-injection compared to the that after the vehicle [$F(1,84) = 4.367, p = 0.04$] (Fig 5A, 5B, 5C). The greatest reduction effect of the NK-1R antagonist injections into the ipsilateral hemisphere occurred with the 500 nM dosage (5000 nM: -15.3 ± 4.4 % change; 500 nM: -22.0 ± 2.6 % change; 50 nM: -11.6 ± 2.5 % change).

The 500 nM dosage of the NK-1R antagonist injected into the cortex of the ipsilateral hemisphere reduced SWA during the first 12 h post-injection compared to the vehicle [$F(1,54) = 31.310, p < 0.001$] (Fig 5B). NREMS EEG power spectra (0.5-20.0 Hz frequency range) was attenuated during the first 12 h after the 500 nM dosage of the NK-1R antagonist was injected into the ipsilateral hemisphere as the EEG electrode [$F(1,280) = 74.500, p < 0.001$] (Fig 7B). Post-hoc analysis determined significant reductions within the 3.0-3.5, 7.5-8.0, 12.5-14.5, and 15.5 Hz frequency range bins during the first 12 h after the 500 nM dosage of the NK-1R antagonist was given into the cortex of the ipsilateral hemisphere as the EEG electrode compared to the vehicle. However, similar SWA values were observed between injections of the 500 nM dosage of the NK-1R antagonist into the contralateral hemisphere and vehicle injections (Fig 5D).

A main effect was found for the 5000 nM dosage of the NK-1R antagonist injected into the ipsilateral hemisphere attenuating SWA during the first 12 h post-injection (ZT 0-12) compared to the vehicle injections [$F(1,54) = 8.015, p = 0.007$](Fig 5A). However, NREMS EEG power spectra (0.5-20.0 Hz frequency range) after the 5000 nM dosage of the NK-1

antagonist was administered to the ipsilateral hemisphere was not statistically different from that after vehicle injections [$F(1,280) = 2.760, p = 0.098$](Fig 7A).

A main effect was found for attenuated SWA occurring during first 12 h after the 50 nM dosage of the NK-1R antagonist was injected into the ipsilateral hemisphere when compared to the vehicle [$F(1,54) = 9.109, p = 0.004$](Fig 5C). EEG power in the 0.5-20 Hz frequency range was attenuated during the first 12 h post-injection period after the 50 nM dosage of the NK-1R antagonist was injected into the ipsilateral hemisphere compared to the saline injections [$F(1,280) = 40.067, p < 0.001$] (Fig 7C). Post-hoc analysis revealed significant reductions within the 5.5-10.5, 12.5-13.0, 17.5, and 19.0 Hz frequency bin ranges during the first 12 h after injections of the 50 nM dosage of the NK-1R antagonist into the ipsilateral hemisphere vs. injections of the vehicle.

4.0 Discussion

Herein, our findings are the first to demonstrate that the pro-inflammatory molecule substance P regulates SWA. We demonstrate that an NK-1R agonist enhanced SWA, whereas an NK-1R antagonist had the opposite effect. We also report that these changes in SWA do not occur when the NK-1R agonist or the NK-1R antagonist are injected into the cortex of the contralateral hemisphere as the EEG electrode indicating local effects of substance P and the NK-1R on SWA regulation and support the role of substance P in the local regulation of sleep. This result is consistent with the literature that suggests that sleep and SWA are regulated not only globally but also locally (Krueger et al., 2013). Overall, the present findings are the first to demonstrate that substance P regulates SWA locally in the cerebral cortex and suggest that cortical cells containing NK-1Rs are, in part, responsible for modulating SWA.

Cortical gamma-aminobutyric acid-ergic neurons expressing neuronal nitric oxide synthase (nNOS) [an enzyme that catalyzes the production of nitric oxide (NO) from L-arginine] are activated during sleep occurring after enhanced waking activity and these cells also express the NK-1R (Gerashchenko et al., 2008; Wisor et al., 2011; Kilduff et al., 2011; Morairty et al., 2013; Dittrich et al., 2012). The extent of activation of these nNOS neurons is proportional to changes in SWA found during NREMS (Morairty et al., 2013; Gerashchenko et al., 2008). nNOS expressing cells are located throughout the cortex, including the somatosensory cortex where the injections of the NK-1R agonist and antagonist were applied in the present study (Gerashchenko et al., 2008; Morairty et al., 2013; Dittrich et al., 2012; Zielinski et al., 2013b). Low-frequency rhythms occurring during SWA are generated by a synchronous firing of neurons in the cortex during the depolarization phase (Steriade, 2004). Further, recent evidence indicates that the NK-1R is depolarized in neurons expressing nNOS within mouse and rat cortical slices after the application of substance P (Dittrich et al., 2012). Consequently, these findings suggest that sleep-active cortical cells co-expressing nNOS and the NK-1R are involved in regulating SWA.

Substance P is known to modulate inflammatory and endothelial cells (Ziche et al., 1990; Ziche et al., 1991; Nakagawa et al., 1995), which occurs, in part, by its induction of NO production (Furchgott et al., 1984). Evidence indicates that nNOS regulates sleep and SWA

(Morairty et al., 2013). nNOS dysregulation is also associated with conditions that affect sleep and SWA including schizophrenia (Salerno et al., 2002), restless leg syndrome (Winkelmann et al., 2008), and sleep apnea (Yuksel et al., 2014). NOS and NO are highly conserved across species as simple as bacteria (Andreakis et al., 2011). NO is a neurotransmitter with multiple functions including long term potentiation, neuroprotection, and the regulation of cerebral blood flow (Hardingham et al., 2013; Bor-Seng-Shu et al., 2012), which is associated with changes in SWA (Gerashchenko and Matsumura, 1996). TNF- α , which enhances nNOS production in the brain after injections and is enhanced with increased waking activity, also increases SWA (Zielinski and Krueger, 2011; Chen et al., 2004). Conversely, mice lacking nNOS have attenuated SWA responses to sleep deprivation compared to wild-type control mice (Morairty et al., 2013), and rats receiving central injections of the nNOS inhibitor 3-bromo-7-nitroindazole exhibit attenuated slow-wave sleep (i.e., slow-wave sleep is comprised of greater SWA vs. light sleep) compared to control injections further supporting the role of nNOS in sleep regulation (Cavas and Navarro, 2006). Our findings that SWA was enhanced and suppressed after injections of the NK-1R agonist and antagonist, respectively, suggests that cortical sleep-active neurons co-expressing nNOS and the NK-1R might regulate SWA. Nevertheless, *in vitro* and *in vivo* studies indicate that the NK-1R is also present on microglia, including within the cortex (Chauhan et al., 2008). Therefore, it is possible that substance P acting through glia expressing the NK-1R within the cortex could also modulate SWA.

Evidence in cell culture indicates that extracellular adenosine tri-phosphate (ATP) acting through the purine type 2 X7 receptor (P2X7R) enhances the release of substance P and the somnogenic cytokine interleukin-1 β (IL-1 β) (Raffaghello et al., 2006). Evidence also indicates that IL-1 β enhances the release of substance P (Grider, 2003). Pharmaceuticals activating and inhibiting the P2X7R and transgenic mice lacking the P2X7R indicate that extracellular ATP induced by enhanced waking activity functions, in part, through the P2X7R to enhance the activation of IL-1 β thus inducing sleep and SWA (Krueger et al., 2010). Additionally, there are circadian fluctuations in substance P within the brain and nociceptive behavioral circadian rhythms that are abolished by NK-1R antagonism (Zhang et al., 2012). Substance P also induces inflammation and other molecules that are well-established to enhance sleep duration and SWA including TNF- α and IL-6 (Koon and Pothoulakis, 2006; Zielinski and Krueger, 2011). Consequently, we hypothesize that waking activity induces substance P to activate sleep-active cortical cells expressing nNOS to regulate SWA. Nevertheless, the injection response dynamics including a reduced SWA response effect with higher concentrations of the NK-1R agonist and antagonist could occur, in part, from the activation of inflammatory molecules downstream that affect SWA or other non-specific mechanisms affected by the pharmaceuticals used such as calcium binding (Guard et al., 1993).

Typically, enhancements in SWA occur within the first few hours after acute sleep deprivation and are followed by attenuations in SWA, which is often referred to as a compensatory negative rebound (Zielinski et al., 2012; Zielinski et al., 2013b; Krueger et al., 2010). The mechanisms that are responsible for this negative rebound are unknown. Regardless, our current findings indicate a reduction in SWA after an enhancement in SWA

induced by the local injections of the NK-1R agonist (Fig 5), which is consistent with SWA responses found after acute sleep deprivation (Zielinski et al., 2012; Zielinski et al., 2013b; Krueger et al., 2010).

Accumulating evidence indicates that sleep occurs locally within brain regions, cortical columns, or cellular groupings, and this occurs, in part, due to molecular substances that regulate sleep including pro-inflammatory molecules (Krueger et al., 2013). The supporting evidence includes birds and marine mammals that have uni-hemispheric sleep (Siegel, 2008), humans that undergo parasomnias such as sleep walking that retain movement functions (Harris and Grunstein, 2009), functional magnetic resonance imaging studies indicating differences in brain region activity (Deco et al., 2013), and rats that have enhanced SWA and enhanced inflammatory sleep regulatory cytokine immuno-positive cell labeling for TNF- α in corresponding cortical columns following stimulation of their whiskers (Rector et al., 2005; Churchill et al., 2008). Further, injections of the somnogenic substances TNF- α or IL-1 β onto ipsilateral side of the somatosensory cortex enhances SWA compared to the injection of saline on the contralateral side (Krueger et al., 2013). Our current findings of enhanced SWA after the NK-1R agonist (500 nM dosage) was injected into the somatosensory cortex on the ipsilateral side of the EEG electrode but not the contralateral side of the EEG electrode is consistent with the rat studies using local injections of TNF- α and IL-1 β enhancing SWA on the ipsilateral hemisphere. The local effects we found on a NK-1R agonist and antagonist altering SWA but not sleep duration likely occurred due to the small volume we applied into the cortex (0.2 μ L). Notwithstanding, individuals with insomnia who received the NK-1R antagonist vestipitant exhibited attenuations in SWA dominant slow-wave sleep (Ratti et al., 2013), which is consistent with the attenuation in SWA we found after local injections of the NK-1R antagonist CP96345. Nevertheless, it remains unknown how substance P and cells possessing the NK-1R in brain areas other than the cortex affect sleep or SWA.

In conclusion, the substance P fragment 1,7 injected into to cortex enhanced SWA locally, while the NK-1 receptor antagonist CP96345 attenuated SWA locally indicating that substance P and the NK-1R regulate SWA. These results provide evidence linking enhanced waking activity and the pro-inflammatory molecule substance P to changes in SWA associated with sleep-active cortical cells that co-express nNOS and the NK-1R and suggest that substance P and the NK-1R might be a potential target for sleep-related disorders.

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Highlights

- Substance P, a neurokinin-1 receptor agonist, enhances EEG slow-wave activity locally
- A neurokinin-1 receptor antagonist inhibits EEG slow-wave activity locally
- A neurokinin-1 receptor agonist enhances EEG power spectra
- A neurokinin-1 receptor antagonist attenuates EEG power spectra

Fig 1.

NREMS and REMS duration and SWA responses to ipsilateral and contralateral hemisphere injections of the vehicle. Mice injected with the vehicle into either the cortex of the ipsilateral hemisphere (○) or the contralateral hemisphere (●) as the EEG electrode exhibited diurnal rhythms of NREMS (A) and REMS (B) duration with significant enhancements during the light period vs. dark period. Significant enhancements in SWA (C) occurred during the dark period vs. light period after injections of the vehicle into the ipsilateral hemisphere. No significant differences in NREMS or REMS duration or SWA responses to saline were observed between hemispheres after injection of the vehicle with the exception of a significantly greater amount of SWA occurring at ZT 14-16 for the contralateral hemisphere treatment vs. the ipsilateral treatment. (#) = significant difference between

hemispheres; (*) = significant difference between light and dark periods; shaded area = dark period. Significance was set at $p < 0.05$.

Fig 2. NREMS, REMS, and waking EEG power spectra within the 0.5-20 Hz frequency range after injections of the vehicle into the cortex of the ipsilateral and contralateral hemisphere as the EEG electrode during the light and dark periods. NREMS (A), REMS (B), and waking (C) EEG power spectra exhibited enhanced values in the 0.5-20 Hz frequency range during the dark period (•) compared to the light period (○) after injections into either the ipsilateral or contralateral hemisphere. No significant differences were observed between hemispheres after vehicle injections. (*) = significant difference between light and dark periods. Significance was set at $p < 0.05$.

Fig 3.

NREMS duration responses after injections of the NK-1R agonist and antagonist and vehicle into the cortex of the ipsilateral and contralateral hemisphere as the EEG electrode. The NK-1R agonist and antagonist injected into the ipsilateral hemisphere at 5000 (A), 500 (B), and 50 (C) nM dosages (•) did not significantly alter NREMS duration compared to vehicle injections (o) with the exception of a significant reduction in NREMS duration 10-12 h after injection (ZT 10-12) of the 500 nM dosage of the NK-1R agonist into the ipsilateral hemisphere. Injections of the 500 nM dosage of the NK-1R agonist and antagonist into the

contralateral hemisphere (D) also did not alter NREMS duration compared with injections of the vehicle. (*) = significant difference between NK-1R agonist and vehicle injections; shaded area = dark period. Significance was set at $p < 0.05$.

Fig 4.

REMS duration responses after injections of the NK-1R agonist and antagonist and vehicle into the cortex of the ipsilateral and contralateral hemisphere as the EEG electrode. Injections of the NK-1R agonist and antagonist into the ipsilateral hemisphere at 5000 (A), 500 (B), and 50 (C) nM dosages (•) did not significantly alter REMS duration vs that after vehicle injections (○), although injections of the NK-1R antagonist into the ipsilateral hemisphere significantly enhanced REMS duration during the first 2 h post-injection (ZT0-2) when compared with the vehicle injections. Compared to the vehicle, the 500 nM

dosage of the NK-1R agonist and antagonist injected into the contralateral hemisphere (D) did not significantly alter REMS. (*) = significant differences between NK-1R agonist or antagonist and vehicle injections; shaded area = dark period. Significance was set at $p < 0.05$.

Fig 5.

SWA responses after injections of the NK-1R agonist and antagonist and vehicle into the cortex of the ipsilateral and contralateral hemisphere as the EEG electrode. Injections of the NK-1R agonist (●) enhanced SWA and the NK-1R antagonist (●) attenuated SWA compared to the vehicle (○). Significant enhancements in SWA occurred during the first 2 h after injection of the 5000 nM dosage (A) of the NK-1R agonist into the ipsilateral hemisphere. During the first 6 h after injection of the 500 nM dosage (B) of the NK-1R agonist into the ipsilateral hemisphere there was a significant enhancement in SWA and attenuation in SWA

11-18 h post-injection (ZT 11-18) compared to vehicle injections. Injections of the 5000 (A), 500 (B), and 50 (C) nM dosages of the NK-1R antagonist significantly attenuated SWA during the first 12 h post-injection (ZT 0-12) compared to vehicle injections. No significant differences in SWA were observed after the 500 nM dosage of either the NK-1R agonist or antagonist were injected into the contralateral hemisphere (D) compared to vehicle injections. (*) = significant difference between NK-1R agonist and antagonist and vehicle injections; shaded area = dark period. Significance was set at $p < 0.05$.

Fig 6.

NREMS EEG power spectra (0.5-20 Hz frequency range) responses after injections of the NK-1R agonist and vehicle into the cortex of the ipsilateral hemisphere as the EEG electrode. The 5000 nM dosage (A) of the NK-1R agonist (●) applied into the ipsilateral hemisphere significantly enhanced NREMS EEG power spectra during the first 2 h post-injection (ZT 0-2) compared to vehicle injections (○). The 500 nM dosage (B and C) of the NK-1R agonist (●) injected into the ipsilateral hemisphere significantly enhanced NREMS EEG power spectra during the first 6 h post-injection (ZT 0-6) followed by an attenuation in NREMS EEG power spectra 11-18 h post-injection (ZT 11-18) compared to vehicle

injections (○). (*) = significant difference between NK-1R agonist and the vehicle. Significance was set at $p < 0.05$.

Fig 7. NREMS EEG power spectra (0.5-20 Hz frequency range) responses after injections of the NK-1R antagonist and vehicle into the cortex of the ipsilateral hemisphere as the EEG electrode. The 5000 (A), 500 (B), and 50 (C) nM dosages of the NK-1R antagonist (●) significantly attenuated NREMS EEG power spectra during the first 12 h post-injection (ZT 0-12) compared to vehicle injections (○). (*) = significant difference between NK-1R antagonist and the vehicle. Significance was set at $p < 0.05$.

Table 1
Types of statistical analysis used for compared measures

Compared Measure	Type of Analysis	Independent Variable(s)	Repeated Variable
NREMS, REMS, SWA (saline)	Paired t-test	Light period vs. dark period	Not applicable
NREMS, REMS, SWA during light and dark periods (saline)	Independent t-test	Hemisphere	Not applicable
NREMS, REMS, waking EEG power spectra (saline)	Two-way ANOVA	Frequency	Light period vs. dark period
NREMS, REMS, waking EEG power spectra (saline)	Two-way ANOVA	Frequency, hemisphere	Not applicable
NREMS, REMS, SWA (treatment)	Three-way ANOVA	Dosage	Time, treatment
NREMS EEG power spectra (treatment)	Two-way ANOVA	Frequency	Treatment