



# Role of nitric oxide and WNK-SPAK/OSR1-KCC2 signaling in daily changes in GABAergic inhibition in the rat dorsal raphe neurons

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## ABSTRACT

Serotonergic neurons in the dorsal raphe nucleus (DRN) act as wake-inducing neurons in the sleep-wake cycle and are controlled by gamma-aminobutyric acid (GABA) synaptic inputs. We investigated daily changes in GABAergic inhibition of the rat DRN neurons and the role of nitric oxide (NO) and cation-chloride co-transporters in the GABAergic action. Neuronal NO synthase (nNOS) was co-expressed in 74% of serotonergic DRN neurons and nNOS expression was higher during daytime (the sleep cycle) than that during nighttime (the wake cycle). GABAergic hyperpolarization of DRN neurons produced by GABA<sub>A</sub> receptor agonist muscimol was greater and the equilibrium potential of muscimol showed a hyperpolarizing shift during daytime compared to that during nighttime. Expression levels of potassium-chloride co-transporter 2 (KCC2) were higher during daytime than that during nighttime, whereas there were no changes in sodium-potassium-chloride co-transporter 1 (NKCC1) expression. With-no-lysine kinase (WNK) isoform 1 was more highly expressed during daytime than that during nighttime. Total Ste20-related proline alanine rich kinase (SPAK) and oxidative stress response kinase 1 (OSR1) were also higher during daytime than during nighttime, while there were no changes in phosphorylated SPAK and OSR1. Consistent with the findings during the sleep-wake cycle, *ex vivo* treatment of DRN slices with a NO donor sodium nitroprusside (SNP) increased the expression of KCC2, WNK1, WNK2, WNK3, SPAK, and OSR1, whilst decreasing phosphorylated SPAK. These results suggest that GABAergic synaptic inhibition of DRN serotonergic neurons shows daily changes during the sleep-wake cycle, which might be regulated by daily changes in nNOS-derived NO and WNK-SPAK/OSR1-KCC2 signaling.

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## 1. Introduction

Normal regulation of the sleep-wake cycle is critical for the maintenance of physical, mental, and emotional health (Foster et al., 2013; Goldstein and Walker, 2014; Xie et al., 2013). The sleep-wake cycle is regulated by two main processes: the circadian and homeostatic processes (Borbely, 1982; Brown et al., 2012). The circadian process of sleep-wake cycle regulation is controlled by the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore,

2007). In contrast, homeostatic processes are regulated by the balance of neural circuits promoting sleep and wakefulness (Fuller et al., 2006). Sleep-inducing neurons are primarily located in the ventrolateral preoptic nucleus and inhibit wake-inducing neurons via the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Schwartz and Roth, 2008). Wake-inducing neurons including serotonergic neurons of the dorsal raphe nucleus (DRN) suppress sleep-inducing neurons and excite the cerebral cortex during the wake cycle (Schwartz and Roth, 2008).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated as byproducts of cellular metabolism during the wake state and recover to lower levels during the sleep state (Gyongyosi and Kaldi, 2014; Vyazovskiy and Harris, 2013). RNS have been posited as important signaling molecules in various

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cellular functions (Calabrese et al., 2007; Droge, 2002). Nitric oxide (NO) has been reported to act as a sleep-inducing factor in the homeostatic regulation of the sleep-wake cycle (Cespuglio et al., 2012; Gautier-Sauvigne et al., 2005); yet, the exact mechanism of this effect is unclear.

The DRN is a main site of serotonin release that includes serotonergic, GABAergic, and nitric oxide synthase (NOS)-expressing neurons (Monti, 2010; Vasudeva and Waterhouse, 2014), suggesting a role for NO in serotonergic function. The activities of serotonergic neurons in the DRN are controlled by GABAergic synaptic inputs from the lateral preoptic area and ventral periaqueductal gray (Gervasoni et al., 2000). GABAergic synaptic activity is influenced by the concentration of intracellular  $\text{Cl}^-$ , which is regulated by cation-chloride cotransporters (CCC) such as the  $\text{K}^+\text{-Cl}^-$  cotransporter (KCC) and  $\text{Na}^+\text{-K}^+\text{-Cl}^-$  co-transporter (NKCC) (Kaila, 1994; Kaila et al., 2014; Payne et al., 2003). Among the four KCC and two NKCC isoforms, the major isoforms expressed in brain neurons are KCC2 and NKCC1 (Blaesse et al., 2009; Russell, 2000). Under certain conditions of decreased KCC2 function and/or increased NKCC1 function, increased intracellular  $\text{Cl}^-$  concentrations shift the function of GABA from inhibitory to excitatory (Kim et al., 2011; Lee et al., 2015; Rivera et al., 1999). In SCN neurons,  $\text{Cl}^-$  transporters and intracellular  $\text{Cl}^-$  concentrations vary throughout the day so that GABA input is excitatory (depolarizing) during daytime and inhibitory (hyperpolarizing) during nighttime (Wagner et al., 1997, 2001). Given that serotonergic neurons in the DRN receive GABAergic input, we hypothesized that daily changes in the expression of KCC2 and NKCC1 during the sleep-wake cycle alter GABAergic inhibition of the DRN serotonergic neurons as a regulatory mechanism.

With-no-lysine kinase (WNK) is a serine-threonine kinase that has four known subtypes (WNK1, 2, 3, and 4) expressed in the brain (Kahle et al., 2006; Wilson et al., 2001). WNKs phosphorylate KCC to inhibit its activity as well as NKCC to enhance its activity (Kahle et al., 2006, 2010). The regulatory effect of WNK on KCC and NKCC is usually through the phosphorylation of Ste20-related proline alanine rich kinase (SPAK)/oxidative stress response kinase 1 (OSR1) (Alessi et al., 2014; Richardson and Alessi, 2008). Therefore, we hypothesized that WNK-SPAK/OSR1 signaling plays a role in the daily changes of KCC2 and NKCC1 in DRN neurons during the sleep-wake cycle.

In the present study, we investigated the daily change in GABAergic inhibition of the DRN serotonergic neurons during the sleep-wake cycle and the role of NO and WNK-SPAK/OSR1-CCC signaling as regulatory mechanisms of the GABAergic action. Here, we found that both nNOS expression in DRN neurons and GABAergic inhibition of DRN neurons were increased during daytime (the sleep cycle) than during nighttime (the wake cycle). Moreover, increased concentration of NO inhibited SPAK/OSR1 phosphorylation and increased the expression of KCC2 in the DRN. These findings suggest that daily changes in GABAergic inhibition of DRN neurons might be regulated by daily changes in nNOS-derived NO and WNK-SPAK/OSR1-KCC2 signaling.

## 2. Materials and methods

### 2.1. Animals

Sprague Dawley rats (either sex, 3 weeks old, Orient Bio, Seongnam, South Korea) were used in this study. Rats were maintained under a 12-h light/dark cycle (lights on at 08:00) for at least one week before experiments. All experimental procedures were approved by the Kyung-Hee University Animal Research Policies Committee and conformed to the guidelines of the Council of the Korean Physiological Society and the National Institutes of Health

*Guide for the Care and Use of Laboratory Animals*. All possible efforts were made to minimize the number of animals as well as any pain or suffering.

### 2.2. Preparation of brain slices

Brain slices including the DRN were prepared as described previously (Kim et al., 2011). Briefly, animals were anesthetized with urethane (1.2 g/kg, i.p., Sigma-Aldrich, St. Louis, MO, USA) (Ruusuvirta et al., 2015) and decapitated. The brains were rapidly removed from the skull and immersed in an ice-cold artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 1.3 mM  $\text{MgSO}_4$ , 3 mM KCl, 1.25 mM  $\text{NaH}_2\text{PO}_4$ , 26 mM  $\text{NaHCO}_3$ , 2.4 mM  $\text{CaCl}_2$ , and 10 mM glucose (bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ ). Coronal midbrain slices (thickness, 400  $\mu\text{m}$ ) containing the DRN were cut using a Vibratome (VT1200, Leica Microsystems, Nussloch GmbH, Nussloch, Germany) in ice-cold aCSF. For Western blotting, DRN-containing tissues were collected from the slices.

For western blotting analysis to examine the changes in protein expression during the sleep-wake cycle, DRN slices were prepared at 4-h intervals over a 24-h period. For daytime data, slices were prepared at 12:00 (first daytime time point), 16:00 (second daytime time point), and 20:00 (third daytime time point). For nighttime data, slices were prepared at 24:00 (first nighttime time point), 04:00 (second nighttime time point), and 08:00 (third nighttime time point).

For western blotting analysis to examine the effects of sodium nitroprusside (SNP) as a NO donor, DRN slices were prepared at 12:00 and incubated in aCSF solution (bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ ) containing 1  $\mu\text{M}$  SNP for 4-h at room temperature. After the incubation period, DRN tissues were collected for Western blotting.

For slice patch-clamp recordings, DRN slices were prepared as follows: coronal midbrain slices (thickness, 300  $\mu\text{m}$ ) containing the DRN were cut using a Vibratome (VT1200, Leica Microsystems, Nussloch GmbH, Nussloch, Germany) in a solution with the following composition: 248 mM sucrose, 1.3 mM  $\text{MgSO}_4$ , 3 mM KCl, 1.25 mM  $\text{NaH}_2\text{PO}_4$ , 26 mM  $\text{NaHCO}_3$ , 2.4 mM  $\text{CaCl}_2$ , and 10 mM glucose. Slices were maintained at 37 °C in a submerged chamber containing aCSF (sucrose was replaced with 124 mM NaCl for maintenance) equilibrated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Individual slices were then transferred to a recording chamber and superfused continuously (2 mL/min) with aCSF during electrophysiological recording.

### 2.3. Western blotting

According to the rat brain atlas (Paxinos and Watson, 2007), the DRN region was identified under a binocular microscope (SZ-ST, Olympus, Tokyo, Japan) and was punched out from brain slices (Fig. 1). Because the DRN area is relatively small, DRN tissues obtained from brain slices of four rats were pooled for each Western blotting sample of 6 time points (12:00, 16:00, 20:00, 24:00, 04:00, and 08:00). Western blotting data of “Whole-Day” indicate all data obtained from the DRN slices at 12:00, 16:00, and 20:00 time points. “Whole-Night” data indicate all data obtained from the DRN slices at 24:00, 04:00, and 08:00 time points; “Early-Day” data indicate all data obtained from the DRN slices at 12:00 and 16:00 time points; “Early-Night” data indicate all data obtained from the DRN slices at 24:00 and 04:00 time points; “Late-Day” data indicate all data obtained from the DRN slices at 16:00 and 20:00 time points; and “Late-Night” data indicate all data obtained from the DRN slices at 04:00 and 08:00 time points.

Samples containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA) were extracted with the PRO-PREP protein extraction kit (iNtRON Biotechnology,

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