主論文の要旨

Optogenetic activation of serotonergic terminals facilitates GABAergic inhibitory input to orexin/hypocretin neurons

（光遺伝学を用いたセロトニン神経の活性化は
オレキシン神経への抑制性入力を亢進させる）

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Introduction

Orexin (also known as hypocretin)-producing neurons (orexin neurons) are exclusively distributed in the perifornical area and the lateral hypothalamic area (LHA) and play a crucial role in the regulation of sleep/wakefulness, primarily in the maintenance of wakefulness. These neurons innervate wide areas of the brain and receive diverse synaptic inputs including those from serotonergic (5-HT) neurons in the raphe nucleus. Ascending serotonin (5-hydroxytryptamine, 5-HT) neurons are mostly concentrated in two important nuclei, the dorsal raphe (DR) and the median raphe (MnR) nuclei in the mammalian brain. However, the function of 5-HT on the sleep/wakefulness cycle is complex and controversial. Although 5-HT was first hypothesized to induce drowsiness and sleep, it is now well-established to be wake promoting and to inhibit rapid eye movement (REM) sleep. By combining genetic engineering, slice electrophysiology and optogenetics in mice, here, I aimed to reveal the regulatory mechanism of orexin neurons by 5-HT neurons.

Materials and Methods

I generated a novel transgenic mouse to express a photo-inductive cation channel called channelrhodopsin (ChR2) in 5-HT neuron and fluorescent protein in orexin neurons (**Fig 1**). By combining optogenetics and electrophysiological experiment using the acute brain slice prepared from this novel transgenic animal, I revealed the mechanism of orexin neuronal regulation by 5-HT neurons.

Results

To manipulate the nerve terminals of 5-HT neurons using optogenetics and to record the postsynaptic effects from orexin neurons, I generated triple transgenic *orexin-EGFP; Tph2-tTA; TetO ChR2* mice (hereafter called triplegenic mice) (**Figure 1a**). These triplegenic mice expressed EGFP in orexin neurons under control of the human prepro-orexin promoter, and also a tetracycline-controlled transactivator (tTA) exclusively in 5-HT neurons in the raphe nucleus under the control of the Tph2 promoter. tTA binds to the tetracycline operator (TetO) sequence and induces ChR2 expression. ChR2 was expressed as a fusion protein with enhanced yellow fluorescent protein (EYFP) to visualize ChR2-expressing neurons. I first confirmed the expression of ChR2 in 5-HT neurons and the expression of EGFP in orexin neurons via immunohistochemical studies. (**Figure 1b**). In the DR, 60.5 ± 2.4% of the total 2,385 counted cells, and in the MnR, 56.0 ± 3.8% of the total 1,223 counted 5-HT-positive neurons expressed ChR2-EYFP (n = 3). Confocal imaging revealed a dense projection of 5-HT nerve endings in the LHA. Moreover, cell bodies of orexin neurons and 5-HT nerve endings were in close apposition, supporting the hypothesis that 5-HT neurons regulate orexin neurons (**Figure 1c**).

Next, I recorded spontaneous EPSCs and IPSCs from postsynaptic orexin neurons at a

holding potential of -60 mV. EPSCs were recorded in the presence of 400 μM of extracellular picrotoxin (PTX), a GABAA receptor antagonist, and IPSCs were recorded in the presence of extracellular AP-5 (50 μM) and CNQX (20 μM), glutamate receptor antagonists. EPSCs and IPSCs were confirmed by adding AP-5 and CNQX (**Figure 2a**) or picrotoxin (**Figure 3a**) in the perfused extracellular solution, respectively. Pipette solutions contained 1 mM of QX-314 to inhibit voltage gated sodium channels to block action potential generation. Optogenetic activation of 5-HT terminals in brain slices did not affect EPSC input to orexin neurons (**Figure 2a-f**). The average EPSC interval at baseline (*pre*) was 100.9 ± 27 ms, whereas that after turning on blue light (*light*) was 97.9 ± 21.9 ms; $n = 14$, $p = 0.93$. Again, the average *pre* EPSC amplitude was 16.1 ± 1.1 pA whereas that during *light* was 15.4 ± 1.1 pA; $n = 14$, $p = 0.62$ (**Figure 2c-f**). I recorded EPSCs from 21 orexin neurons obtained from 3 different mice and randomly analysed 14 of them. In contrast to excitatory system findings, when I recorded inhibitory GABAergic inputs to orexin neurons and activated 5-HT nerve endings by illuminating blue light, I found a dramatic increase in IPSCs in orexin neurons (**Figure 3b**). The inter-event intervals of IPSC inputs were decreased (*pre* was 1264.4 ± 191.8 ms and *light* was 684.1 ± 93.6 ms; $n = 20$, $p < 0.05$), implying an increase in total inhibitory input (**Figure 3e**), while the amplitudes of IPSC inputs were also increased (*pre* was 39.3 ± 3.8 pA and *light* was 52.1 ± 4.6 pA; $n = 20$, $p < 0.05$) (**Figure 3f**).

I next tested the complete effect of activation of 5-HT nerve endings on the activity of orexin neurons. I recorded postsynaptic currents from orexin neurons in brain slices of triplegenic mice at a holding potential of -60 mV in the presence of extracellular tetrodotoxin (TTX, 1 μM), which blocks voltage-gated sodium channels and the generation of action potentials, thus eliminating spontaneous events generated by neuronal firings. I found a small but robust effect on orexin neurons by 5-HT terminal activation (**Figure 4a and 4c**). Blue light illumination (50% intensity) induced an outward current in orexin neurons (8.7 ± 1.1 pA; $n = 12$, $p < 0.001$ vs *pre*). However, the inhibitory effect was reduced to 1.02 ± 0.4 pA when acute brain slices were treated with the 5-HT1A receptor selective antagonist, WAY100635 ($n = 21$, $p<0.001$ vs vehicle; **Figure 4b-c**). Brain slices were pretreated with 100 nM of WAY100635 for 2 min prior to illumination. I then performed loose cell-attached recordings from orexin neurons to reveal the effects on firing frequency in the absence of TTX. Blue light illumination (50% intensity) significantly decreased the firing frequency compared with before (*pre*) or after (*post*) light illumination (70.1 ± 7.2%; $n = 14$, $p < 0.01$ vs *pre*) (**Figure 4d-e**). Taken together, these findings clearly indicate a direct inhibitory effect of 5-HT neurons on orexin neurons mediated by the 5-HT1A receptor.

Using acute coronal brain slices generated from triplegenic mice, I next recorded EPSCs and IPSCs from orexin neurons at -60 mV_{hold} and locally applied 5-HT (100 μM), dissolved

in the bath solution. I found that 5-HT application significantly increased IPSC frequency and amplitude but did not affect EPSCs in orexin neurons (**Figure 5a-j**). The average EPSC interval at baseline was 104.1 ± 25.6 ms whereas that during local application of 100 μM of 5-HT was 126.0 ± 34.1 ms; $n = 9$, $p > 0.05$ (**Figure 5d**). Again, the average EPSC amplitude at baseline was recorded as 13.4 ± 1.4 pA whereas that observed during 5-HT application was 13.8 ± 1.6 pA; $n = 9$, $p > 0.05$ (**Figure 5e**). In the case of IPSC recordings, the average interval at baseline was 1526.5 ± 276.3 ms whereas that during 5-HT application was 716.9 ± 12.5 ms; $n = 22$, $p < 0.01$ (**Figure 5i**). Similarly, the average IPSC amplitude also increased significantly. At baseline it was 35.4 ± 3.3 pA whereas during 5-HT it was 52.0 ± 5.8 pA; $n = 22$, $p < 0.01$ (**Figure 5j**). Thus, the regulation of orexin neurons by 5-HT showed a strong resemblance to that induced by photoactivation of 5-HT nerve terminals. These data indicate that 5-HT is the major neurotransmitter released by 5-HT neurons to regulate orexin neuron function in the LHA.

Conclusion

Our previous study showed that pharmacologically applied 5-HT directly hyperpolarized orexin neurons via 5-HT1A receptors and GIRK channels. Using optogenetic activation of 5-HT nerve endings in the hypothalamus, here, I revealed that 5-HT neurons directly inhibit orexin neurons and also indirectly inhibit them by increasing functional GABAergic input from interneurons in the LHA.