主論文の要旨

Functional Interaction Between GABAergic Neurons in the Ventral Tegmental Area and Serotonergic Neurons in the Dorsal Raphe Nucleus

（腹側被蓋野のGABA作動性神経と縫線核のセロトニン神経間の機能連関について）

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【Introduction】

Ventral Tegmental Area (VTA) is a well-characterized midbrain structure containing dopamine (DA), GABAergic and glutamatergic neurons. γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in the adult mammalian brain, is synthesized from glutamate by a catalytic enzyme, glutamic acid decarboxylase (GAD). Two isoforms of GAD, GAD67 and GAD65, are found in mammals. Recently, our research group found that VTA Gad67+ (VTA_{Gad67+}) neurons are critically involved in sleep/wakefulness regulation. VTA_{Gad67+} neurons have distal projections to different areas of the brain, including to brainstem dorsal raphe nucleus (DRN). DRN also comprises multiple cell type including serotonergic (5-HT), glutamatergic, GABAergic and DA neurons. Among them, 5-HT neurons make up around half of the total neuronal population in the DRN. GABAergic neurons are another cell type in the DRN (DRN_{Gad67+}), and they regulate 5-HT neuron activity through a feedforward inhibition. Thus, to better understand the cellular underpinnings of behavior and physiology, it is critical to clarify the neural circuitry that regulates these 5-HT and GABAergic neurons in the DRN. Here, I searched for evidence of a functional innervation from VTA_{Gad67+} neurons to the CeA, DRN and LC. Among the neurons in these regions, only those in the DRN were found to be functionally connected to the VTA. Combining optogenetics with electrophysiological recordings, I found that both the $DRN_{5\text{-}HT}$ and DRN_{Gad67+} neurons were directly innervated and inhibited by the VTA_{Gad67+} neurons. I also discovered that the $DRN_{5\text{-}HT}$ neurons were inhibited by local GABAergic neurons. Furthermore, I explored how VTA_{Gad67+} neurons are modulated by the neurotransmitters serotonin, noradrenaline, dopamine, histamine, carbachol and orexin A, which are all involved in sleep/wakefulness regulation.

【Methods】

To assess the activity of VTA_{Gad67+} neurons upon application of various substances, I performed slice patch clamp recording in loose cell-attached mode where I used a six-channel perfusion valve controller for substances application. To understand the functional connections between VTA_{Gad67+} and $DRN_{5\text{-}HT}$ neurons, I performed in-vitro whole cell current clamp and voltage clamp recording in combination with optogenetics after the confirmation of expression by doing immunohistochemical studies.

【Results】

To visualize VTA_{Gad67+} neurons, Gad67-Cre mice were bilaterally injected with a Cre-dependent AAV expressing humanized renilla GFP (hrGFP) into the VTA. Immunostaining results confirmed that most of the hrGFP-expressing neurons in the VTA colocalized with Gad67, but not with TH (tyrosine hydroxylase, marker of DA neurons). Coronal brain sections of hrGFP-expressing VTA_{Gad67+} neurons from Gad67-Cre mice were prepared and substances were sequentially and locally applied for 4 s under a loose cell-attached mode slice patch clamp

recording. At first, artificial cerebrospinal fluid (aCSF) was applied as a control and then glutamate (Glu, 100 μM) and GABA (100 μM) were applied to confirm responsiveness and positioning of the local application. After that, serotonin (5-HT, 50 μM), noradrenaline (NA, 100 μM), dopamine (DA, 100 μM), histamine (HA, 100 μM), carbachol (CCh, 100 μM) and orexin A (OX-A, 100 nM) were applied in random order. We found that 5-HT, DA and HA application significantly reduced, whereas CCh increased, the firing rate (Figure 1).

To reveal the downstream target neurons of VTA_{Gad67+} neurons, a Cre-dependent AAV carrying the blue light–gated cation channel channelrhodopsin-2 (ChR2) bilaterally injected into the VTA of Gad67-Cre mice. Then I recorded from neurons in the CeA, DRN and LC by stimulating the VTA_{Gad67+} nerve terminals. Current clamp recording from neurons of the DRN showed that a complete cessation of spontaneous firing following a 20-Hz blue light stimulation, whereas activity in CeA or LC neurons was not affected (Figure 2).

As 5-HT neurons are a major population in the DRN, I focused on the DRN 5-HT neurons. I used Gad67-Cre; Tph2-tTA; TetO YC mice where tTA was exclusively expressed in 5-HT neurons under the control of the Tph2 promoter and tTA binds to the TetO sequence for inducing YC expression. A Cre-dependent AAV carrying ChR2 injected into the VTA of trigenic mice. In vitro slice patch clamp recording from ChR2-expressing VTA_{Gad67+} neurons confirmed that these neurons were depolarized by a 10-Hz blue light pulse. Then I recorded from the YC-expressing $DRN_{5\text{-}HT}$ neurons by stimulating VTA_{Gad67+} nerve terminals. In whole-cell current clamp recording, I found that a 5-, 10- and 20-Hz blue light, not yellow light decreased the firing rate (Figure 3).

To further clarify the mechanism of action, I recorded from the 5-HT neurons in whole-cell voltage clamp mode at a −60 mV holding potential. Activation of nerve terminals from VTA_{Gad67+} neurons in the DRN induced post synaptic currents (PSCs) in response to a 5-ms blue light pulse. These light-induced PSCs were blocked by gabazine (10 μM), but not by combined AP5 (50 μM) and CNQX (20 μM). The mean response delay from light onset was 4.0 ± 0.2 ms. To rule out indirect connections, TTX (1 μM) was applied. TTX blocked the light-induced PSCs. The combination of TTX and 4AP (1 mM) rescued the light-induced PSCs, which was again blocked by gabazine, but not by CNQX (Figure 4).

In addition to 5-HT neurons, GABAergic neurons are also distributed throughout the DRN. To elucidate the connection between VTA_{Gad67+} and DRN_{Gad67+} neurons, I used Gad67-Cre mice and injected a Cre-dependent AAV expressing ChR2-eYFP into the VTA and another Cre-dependent AAV expressing tdTomato into the DRN to visualize GABAergic neurons in the DRN. Then I recorded from the tdTomato-expressing DRN_{Gad67+} neurons by stimulating ChR2 positive VTA_{Gad67+} nerve terminals. In whole-cell current clamp recordings from DRN_{Gad67+} neurons, I found that a 20-Hz blue light pulse also silenced them, but not yellow light. Next, to explore the connection patterns, I recorded from DRN_{Gad67+} neurons in whole-cell voltage clamp mode at a −60 mV holding potential. Activation of VTA_{Gad67+} nerve terminals induced

PSCs in response to a 5-ms blue light pulse. These light-induced PSCs were blocked by the combination of TTX (1 μM), 4AP (1 mM) and gabazine (10 μM), but not by TTX and 4AP, confirming a monosynaptic GABAergic connection. Mean response delay was 3.7 ± 0.4 ms (Figure 5).

Next to explore the input mechanism of local GABAergic neurons on $DRN_{5\text{-}HT}$ neurons, I used Gad67-Cre; Tph2-tTA mice and inject a mixture of AAV, Cre-dependent ChR2-eYFP and tTA-dependent TetO-tdTomato. Then I recorded from the tdTomato-expressing $DRN_{5\text{-}HT}$ neurons by stimulating the ChR2 positive local DRN_{Gad67+} nerve terminals. In whole-cell current clamp, 20-Hz blue light stimulation significantly reduced the firing frequency compared with the 20-Hz yellow light stimulation. Next, to explore the connection patterns, I recorded from $DRN_{5\text{-}HT}$ neurons in whole-cell voltage clamp mode at a −60 mV holding potential. Activation of DRN_{Gad67+} nerve terminals in the DRN induced PSCs in response to a 5-ms blue light pulse. These light-induced PSCs were blocked by the combination of TTX, 4AP and gabazine, but not by TTX and 4AP, confirming a monosynaptic GABAergic connection. Mean synaptic delay was 3.7 ± 0.4 ms (Figure 6).

【Conclusion】

In this study, I discovered that the DRN receives dense projections from VTA_{Gad67+} neurons. I found that $DRN_{5\text{-}HT}$ neurons were directly innervated and inhibited by not only VTA_{Gad67+} neurons but also by DRN_{Gad67+} neurons. Further, I showed that DRN_{Gad67+} neurons also receive monosynaptic inhibitory input from VTA_{Gad67+} neurons. In vitro recording from VTA_{Gad67+} neurons revealed that the cholinergic agonist CCh activated them, while DA, HA and 5-HT inhibited them.