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学位論文（要約）

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（内側前頭前野の神経回路に関する解剖学的研究

および光学的研究のための新規デバイス開発）

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Introduction

The neocortex coordinates adaptive behaviors by integrating information from the sensory environment, internal goals, and memory. These high-level cognitive and emotional behaviors critically depend on the prefrontal cortex (PFC). PFC dysfunction, especially the ventromedial PFC, is implicated in cognitive deficits in a spectrum of neuropsychiatric disorders, including anxiety, schizophrenia, and bipolar disorders. This ventromedial PFC function is well conserved among animals, and the rodent medial prefrontal cortex (mPFC) has been widely used as a good research model. The mPFC receives a variety of long-range inputs from other brain regions, such as other cortical areas, thalamus, ventral hippocampus (vHPC), and basolateral amygdala (BLA). Among various interactions, the connection between the mPFC and BLA is particularly important for emotional control. Since how projection neurons in the mPFC respond to BLA inputs is strongly shaped by inhibitory interneurons, it is important to elucidate interneuron population and how they connect with the BLA. Thus, it is necessary to capture the interaction between the BLA and mPFC *in vivo*.

To understand how neuronal ensembles cooperate and integrate multiple inputs, it is necessary to capture neuronal networks in large volume with single neuron resolution. Two-photon laser scanning microscopy (TPLSM) is a powerful tool for visualizing dynamic molecular and cellular phenomena in thick biological samples due to its excellent penetration depth and optical cleavage capability into the brain. In the conventional TPLSM, the *z*-scan method is commonly used to visualize three-dimensional (3D) structures by varying the position of the objective lens, sampling along the optical axis. Then, two-dimensional (2D, in the *xy*-plane) images at each position are reconstructed into a 3D stacked image. In volume imaging methods, the time delay between each scan plane is usually the rate-limiting factor. Thus, the temporal resolution depends primarily on the mechanical speed of *z*-scanning, which typically requires at least a few seconds. However, 3D visualization of fast intracellular signalling requires higher temporal resolution. To meet these requirements, several state-of-the-art microscopic techniques have been proposed to increase the scanning speed for ultrafast volume imaging.

The upcoming popular technique to visualize 3D image is a Bessel beam based TPLSM, which extends the effective depth of field and that allows to convert the traditional 2D frame rate into a 3D volumetric rate. Using the spatial light modulator to generate Bessel foci, a previous study has demonstrated the calcium dynamics in neurons and synapses within a volume over 160 μm-in-depth at up to 30 Hz volume rate. Using a Bessel beam scanning system, a single elongated focus is possible to acquire all the structures along the *z* axis within a certain depth of field. Thus, we can adjust the 3D volume rate by simply enhancing the 2D frame rate. However, it requires complex components to build up a Bessel beam scanning system on the commercially available TPLSM, hindering the widespread use.

In this dissertation, I conducted an anatomical and optical study on the intercommunication between the BLA and the mPFC that underlies cognitive and emotional functions. In Chapter 1, anterograde and retrograde neuronal labeling and immunofluorescence

was employed to characterize this reciprocal connection. Also, I examined whether the conventional TPLSM system can be used to capture how BLA inputs regulate neuronal activity of inhibitory interneurons in the mPFC. In Chapter 2, I provided an easy-to-use Bessel beam generator, which allowed volume imaging in a single scan. I evaluated if the volume imaging system can be used for capturing structural information from paraformaldehyde-fixed brain slices, and calcium responses from *ex vivo* and *in vivo* samples.

Materials and Methods

Anatomical experiments

Adult C57/BL6N mice aged more than 2 months were used. Retrograde and anterograde neuronal labelling was performed to characterize the connections between BLA and mPFC. For tracer injection, mice were anesthetized with 1%–2% isoflurane. For retrograde labelling, FluoroGold was injected into the BLA or the mPFC. For anterograde labelling, either of adeno-associated virus (AAV) vector encoding enhanced green fluorescent protein (EGFP) or GCaMP7b was injected into the BLA. After allowing for expression, mice were transcardially perfused with 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.2) for 10 min. After post-fixation, 50-μm-thick brain sections were prepared. Tracer signals were enhanced by immunofluorescence for FluoroGold or EGFP. Subcellular structures labelled with tracer were characterized with immunofluorescence for presynaptic and postsynaptic molecules. To identify three mutually exclusive subtypes of interneurons, immunofluorescence for somatostatin (SST) or parvalbumin (PV), or fluorescent in situ hybridization for serotonin receptor 3a (5HT3a) mRNA, was performed. Images were taken with an Olympus confocal microscope FV1000.

In vivo calcium imaging

Following mice aged more than two months were used: C57/BL6N mice, Thy1-G-CaMP7-DsRed mice, and SST-Cre mice. Several lines of AAV vectors encoding genetically encoded calcium indicators or Designer Receptors Exclusively Activated by Designer Drug (DREADD) were used as follows: For imaging and stimulating from BLA boutons in the mPFC, AAV1-hSyn-jGCaMP8s or AAV2-hSyn-hM3Dq-mCherry was injected into the BLA. For imaging and manipulating from SST neurons, AAV-hSyn-FLEX-jGCaMP7s, AAV-hSyn-FLEX-YCaMP1s or AAV1-hSyn-DIO-hM3Dq-mCherry was injected into the mPFC. A craniotomy window was implanted above mPFC and all imaging data were obtained from the window using a Nikon A1R-MP+ TPLSM system equipped with a 25x water-immersion objective lens and a Ti:Sapphire laser.

Designing and testing of the light-needle creating device

The light-needle creating device composed of a custom-made concave axicon and a plano convex lens was placed at a filter turret of a TPLSM system. The concave axicon refracted an incident Gaussian beam on its conical surface and converted it into an annular beam at the

focus of the convex lens. The performance of volumetric imaging using the present device was evaluated by using fluorescent beads and paraformaldehyde-fixed brain slices, which were prepared from Thy1-H-YFP mice and treated with optical clearing reagents. Applicability to ex vivo and in vivo calcium measurements was examined using Thy1-G-CaMP7-DsRed and SST-Cre mice.

Results

In Chapter 1, I first showed that the BLA was reciprocally connected to both the prelimbic (PL) and infralimbic (IL) cortices of mPFC in a layer- and region-specific manner. In the PL, BLA axons are densely distributed in layer 2 (L2), while they are broadly distributed in the IL. Projections to the BLA mostly originate from L2 in the PL, and from layer 5 (L5) in the IL. Furthermore, SST neurons were identified as the principal interneurons in the mPFC by immunofluorescence and in situ hybridization analysis. SST neurons predominate in L2/3 and L5/6, and thus are located where they are likely to receive inputs from the BLA. BLA inputs are glutamatergic and form excitatory synapse on SST neurons. Finally, I captured *in vivo* calcium responses from BLA terminals and SST neurons using a conventional TPLSM system. From a series of experiments, I identified targets for improvement in the conventional system.

In Chapter 2, I developed a light-needle creating device composed of a concave axicon lens and a convex lens, aiming to capture calcium responses from SST neurons *in vivo*. This device was installed to a filter turret in an existing TPLSM system, and successfully extended the depth of field beyond 200 μm. This system enabled a single Bessel scan to capture structural information from thick biological specimens around 250-μm-thick, yielding comparable images to those obtained with the Gaussian volume scanning. The zooming effect and peripheral distortion were observed in a distance-dependent manner. However, the zooming factor can be utilized to predict the depth position of the target without referring to the Gaussian z-stack image that is usually necessary for most of other Bessel scanning techniques. Moreover, a combination with major tissue clearing protocols and immunofluorescence allowed 2D Bessel scan to visualize subcellular structures such as dendritic shaft and spines. Although the present system provided high spatial and temporal resolutions for capturing spontaneous and evoked calcium transients from acute brain slices, the spatial resolution dropped considerably *in vivo*.

Discussion

Observations in Chapter 1 substantiate the previous findings on the wiring diagram in the mPFC. The mPFC and the BLA were reciprocally connected and that this connection was biased towards distinct subregions and layers. The present results also support that the composition of inhibitory interneuron in the mPFC was distinct from that in the sensory cortex. There were fewer parvalbumin neurons (PV) and more SST neurons, which received excitatory inputs from BLA neurons. In Chapter 2, I developed a new system, which allowed a conventional TPLSM to perform a volumetric imaging in a single frame scan. When tested on fixed slices and acute slices, it yielded high spatial and temporal resolutions. However, it was

difficult to capture neuronal activity at high resolution *in vivo*, suggesting that it requires further optimization, including shortening the laser beam to reduce out-of-field signals and improve signal-to-background ratio, and reducing light scattering with higher laser power and red-shift fluorescent labelling.

Conclusion

1. The BLA and the PL/IL are reciprocally connected in a layer- and region-specific manner. In the PL, BLA axons are densely distributed in L2, while they are broadly distributed in the IL. Projections to the BLA mostly originate from L2 in the PL, and from L5 in the IL.

2. Among three major types of GABAergic interneurons, SST neurons are the most abundant cell type in the PL and IL. SST neurons predominate in L2/3 and L5/6, and thus are located where they are likely to receive input from the BLA.

3. BLA axons are glutamatergic and form excitatory synapse on SST neurons; however, calcium imaging from SST neurons in vivo requires more robust and physiological stimulation of BLA axons other than DREADD stimulation.

4. The light-needle creating device is composed of a concave axicon lens and a convex lens. This device can be installed to a filter turret in an existing TPLSM system, and successfully extend the DOF beyond 200 μm.

5. This system enables a single Bessel scan to capture structural information from thick biological specimens around 250-μm-thick, yielding images comparable to those obtained from the Gaussian volume scanning. However, zooming effect and peripheral distortion are observed in a distance-dependent manner.

6. Combination with tissue clearing and immunofluorescence allows 2D Bessel scan to visualize subcellular structures, such as dendritic shaft and spines. Major protocols of tissue clearing are compatible with the 2D Bessel scanning.

7. The 2D Bessel volumetric imaging can capture spontaneous and evoked calcium transients from acute brain slices from adult mouse brains at 7.5 Hz temporal resolution. However, application to in vivo imaging requires further optimization.

The present findings confirm the layer- and region-specific organization of BLA–mPFC circuit, and provide anatomical and physiological evidence that helps understand how these connections drive specific circuits in the PFC. Also, the present system would be a promising solution for capturing the neuronal dynamics of this circuit at high spatial and temporal resolution.

In future studies, I have to take into account the heterogeneity of SST neurons, which show diverse molecular, anatomical and electrophysiological properties. Thus, it is important to elucidate how the diversity of SST neuron subtypes relates to specific behaviors. Also, I

must consider the role of other interneurons and their interaction in the mPFC. For example, SST neurons inhibit PV neurons, while VIP neurons suppress SST neurons. Also, SST and PV neurons have been shown to mediate feedforward inhibition and disinhibition, thereby regulating distinct BLA-mPFC connections. Therefore, I have to identify possible cell ensembles that might be recruited together and interact during specific behaviors.

The present light-needle creating device can be easily installed to an existing TPLSM system, enabling Bessel beam scanning based volumetric imaging. To improve the performance of in vivo 3D imaging, the laser beam generated by the optical modeling system must be further shortened to reduce the out-of-field signal. In addition, higher laser power is required for in vivo 3D imaging, along with red-shift fluorescent labeling, to increase SBR and reduce light scattering. With the advantages of the easy-to-use optical needle generator, it can be extended to other microscopes to investigate three-dimensional cellular structures and network dynamics.