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In vivo two-photon imaging of striatal neuronal circuits in mice

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ABSTRACT

Imaging studies of the subcortical striatum *in vivo* have been technically challenging despite its functional importance in movement control and procedural learning. Here, we report a method for imaging striatal neuronal circuits in mice *in vivo* using two-photon microscopy. Cell bodies and intermingled dendrites of GABAergic neurons labeled with fluorescent proteins were imaged in the dorsal striatum through an imaging window implanted in the overlying cortex. This technique could be highly useful for studying the structure and function of striatal networks at cellular and subcellular resolutions in normal mice, as well as in mouse models of neurological disorders.

1. Introduction

The striatum is a part of the subcortical basal ganglia that plays a pivotal role in motor control, motivation and procedural learning (Graybiel, 2005; Nelson & Kreitzer, 2014). Its dysfunction is known to cause neurological disorders, such as Huntington's disease and Parkinson's disease, which are characterized by difficulty in initiating and controlling movement. Research has revealed that the striatal circuits consist of a large number of anatomically intermingled, GABAergic spiny projection neurons and a smaller number of interneurons, such as large cholinergic interneurons, arranged between them (Kawaguchi, 1997). Moreover, the striatum is known to form two distinct parallel pathways: the dopamine D1 receptor-expressing direct pathway and the D2 receptor-expressing indirect pathway. The anatomical organization of the striatum is thus somewhat different from that of the cerebral cortex and hippocampus, where the basic feedforward circuits are constructed by glutamatergic excitatory neurons arranged into layers.

These striking features of striatal circuits have raised the possibility that a principle of neural circuit operation that is different from those of excitatory circuits may be visualized if we could study the structure and function of striatal neuronal circuits using *in vivo* imaging techniques. Despite the technical difficulty in gaining optical access to subcortical striatal neurons, a few studies so far have succeeded in optically recording striatal neural circuit activity by using microprobes or optical fibers (Bocarsly et al., 2015; Cui et al., 2013; Goto et al., 2015; Luo, Volkow, Heintz, Pan, & Du, 2011). To image the neuronal circuits under the neocortex at cellular and subcellular resolutions, two-photon imaging through an implanted window has been performed successfully for the hippocampus (Dombeck, Harvey, Tian, Looger, & Tank, 2010; Mizrahi, Crowley, Shtoyerman, & Katz, 2004; Sato et al., 2015). However, the applicability of this method for striatal imaging has not yet been explored. In this study, we established a procedure for high-resolution two-photon imaging of striatal neurons *in vivo* through an implanted imaging window.

Abbreviation: DTI, direct two-photon imaging through an implanted window.

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2. Materials and methods

2.1. Fluorescent labeling of striatal and cortical neurons

This study was performed in accordance with the institutional guidelines and protocols approved by the RIKEN Animal Experiments Committee.

An adeno-associated virus serotype 9 vector harboring a green fluorescent protein (GFP) gene downstream of the chicken beta-actin (CBA) promoter (AAV9-CBA-GFP, titer 1.08×10^{13} vg/ml, Virovek, Hayward, CA) was diluted 1:50–100 with phosphate-buffered saline (PBS) and was infused into the left dorsal striatum of C57BL/6J mice at 1–2 months of age using a glass pipette connected to a 10 μl syringe via polyethylene tubing at a rate of 0.3 μl/min (1.5–3 μl of total infusion volume). The coordinates were 0.4 mm posterior and 1.8 mm lateral to the bregma, where the primary somatosensory cortex overlies the dorsal striatum (Franklin & Paxinos, 2007), and 2.2 mm deep from the skull surface. After termination of infusion, the pipette was left undisturbed for an additional 2 min to avoid backflow. The glass pipette was then withdrawn from the brain, and the incised scalp was closed with surgical clips. After surgery, the mice were returned to their home cages and allowed to recover until imaging experiments or perfusion. Strong GFP expression in the dorsal striatum was typically observed from approximately 2 weeks after infusion.

Transfection of cortical neurons with plasmids expressing GFP-fused actin (GFP-actin) downstream of the CAG promoter by *in utero* electroporation was performed essentially as described (Tabata & Nakajima, 2001). Briefly, 1 μl of the plasmid DNA solution (dissolved at 2 μg/μl in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA containing 0.05% Fast Green) was pressure injected into lateral ventricles of C57BL/6J embryos on embryonic day 13.5. Four 33-V electrical pulses of 50 ms duration at 950 ms intervals were then delivered to the embryo heads using tweezer-type electrodes (CUY650P3, Nepa Gene Co. Ltd., Chiba, Japan) connected to a pulse generator (CUY21EDIT, BEX Co. Ltd., Tokyo, Japan).

2.2. Immunofluorescence microscopy

The mice were deeply anesthetized with Avertin and were perfused transcardially with PBS, followed by 4% paraformaldehyde (PFA) in PBS. Their brains were removed and further fixed in 4% PFA at 4 °C overnight. Coronal sections were cut using a microslicer to a thickness of 50 μm. The sections were incubated at 4 °C overnight with rabbit anti-Iba1 antibody (1:1000, 019-19741, Wako Pure Chemical Industries, Ltd., Osaka, Japan), rabbit anti-glial fibrillary acidic protein (GFAP) antibody (1:1000, N1506, Dako, Glostrup, Denmark), mouse anti-NeuN antibody (1:500–1:1000, MAB377, Millipore, Billerica, MA), rabbit anti-GABA antibody (1:1000, A2052, Sigma-Aldrich, St. Louis, MO) or goat anti-choline acetyltransferase (ChAT) antibody (1:5000, AB144P, Millipore) diluted in PBS containing 2% normal goat serum, 1% BSA and 0.1% Triton X-100, followed by Alexa Fluor 594-labeled goat anti-mouse, anti-rabbit IgG antibody (1:1000, A-11032 or A-11037, Thermo Fisher Scientific, Waltham, MA) or Cy3-labeled donkey anti-goat IgG antibody (1:1000, AP180C, Millipore) diluted in the same buffer at room temperature for 1 h. The GFP and Venus signals shown in all the images represent their native fluorescence. Fluorescence images were acquired using a Keyence BZ-9000 epi-fluorescence microscope equipped with a 4× objective (Keyence, Osaka, Japan) or an Olympus FV1000 laser-scanning confocal microscope equipped with a 60× water immersion objective (Olympus, Tokyo, Japan).

2.3. Direct two-photon imaging through an implanted window (DTI) for striatal neuronal circuits in vivo

The mice that received viral vector- or *in utero* electroporation-mediated gene transfer or transgenic mice expressing the yellow fluorescent protein Venus under the vesicular GABA transporter promoter (VGAT-Venus mice, Wang et al., 2009) were anesthetized with isoflurane (3% induction, 1.5% maintenance) supplemented with chlorprothixene (1 mg/kg, i.p.) and placed in a stereotaxic frame. Atropine (0.3 mg/kg, s.c.) and dexamethasone (2 mg/kg, s.c.) were administered prior to anesthesia to reduce respiratory secretions and brain edema, respectively. Surgery for implantation of an imaging window was performed as follows (Fig. S1). A circular piece of scalp was removed, and the underlying bone was cleaned and dried (Fig. S1A). A stainless steel head plate with a circular opening (7 mm diameter) was placed over the left parietal bone and attached to the skull with dental acrylic (Fig. S1B). A circular craniotomy was then made on the skull overlying the dorsal striatum. The dura was removed, and the overlying cortex was aspirated in small amounts using a blunted 25-gauge needle connected to a vacuum pump until the surface of the dorsal striatum was exposed (Fig. S1D). To reduce bleeding, aspiration started from a cortical area devoid of large vessels, and bleeding was treated promptly with a small piece of gelatin sponge (Spongel, Astellas Pharma, Tokyo, Japan) wetted with cortex buffer (123 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4). *In vivo* labeling of astrocytes using the red fluorescent dye Sulforhodamine 101 (SR101) was performed by applying the dye solution (dissolved in cortex buffer at 100 μM) to the striatal surface for 5 min, followed by rinsing several times with cortex buffer. An imaging window, which consisted of a stainless steel ring (2.5 mm outer diameter, 2.2 mm inner diameter and 1.0 mm height) with a round coverslip (2.5 mm diameter, 0.17 mm thickness, Matsunami Glass Ind., Osaka, Japan) adhered to the bottom using UV-curable adhesive (NOA81, Norland Products, Cranbury, NJ), was then inserted to the cranial hole to support its surrounding tissue and the striatal surface mechanically. Once positioned, we ensured that the striatal surface was clearly observable through the bottom coverslip, devoid of any trace of bleeding (Fig. S1C and E). The upper rim was then affixed to the skull with dental acrylic.

The AAV infusion targeted to the dorsal striatal surface could inevitably result in GFP labeling of cells in the contiguous deep cortical layers in addition to the striatum (Fig. 1B). To avoid imaging residual cortical tissues, it is important to remove the cortex sufficiently until the underlying white matter is clearly exposed. The exposed white matter should then be gently peeled aside so that the underlying pinkish gray surface of the dorsal striatum is kept intact and visible (Fig. S1D).

After surgery, each mouse was placed under the microscope objective via the head plate. Body temperature was maintained at 37 °C with a heating pad throughout the imaging sessions. Images were acquired using a custom-built microscope equipped with a 25× NA1.05 objective (Olympus) or an Olympus FV1000MPE microscope equipped with a 40× NA0.8 objective (Olympus) in 512 × 512 or 1024 × 1024 pixels. Because the cortex overlying the dorsal striatum is more anterior and thicker than that overlying the dorsal hippocampus, the 40× objective with a longer working distance (3.3 mm) allowed more working space and imaging depth than the 25× objective that we previously used for hippocampal imaging (working distance 2.0 mm; Sato et al., 2015). GFP and Venus were excited using a Ti:sapphire laser (Tsunami or MaiTai DeepSee eHP, Spectra-Physics, Santa Clara, CA) at 910 nm, and the fluorescence was imaged using a 495–540-nm bandpass filter and a GaAsP photomultiplier tube. For dual color imaging, SR101 was simultaneously excited at 910 nm, and the sig-