Preferential subcortical collateral projections of pedunculopontine nucleus-targeting cortical pyramidal neurons revealed by brain-wide single fiber tracing

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Abstract
The pedunculopontine nucleus (PPN), a heterogeneous midbrain structure involved in various brain functions, such as motor control, learning, reward, and sleep. Previous studies using conventional tracers have shown that the PPN receives extensive afferent inputs from various cortical areas. To examine how these cortical axons make collateral projections to other subcortical areas, we used a dual-viral injection strategy to sparsely label PPN-targeting cortical pyramidal neurons in CaMKIIα-Cre transgenic mice. Using a high-speed volumetric imaging with on-the-fly-scan and Readout (VISoR) technique, we visualized brain-wide axonal projections of individual PPN-targeting neurons from several cortical areas, including the prelimbic region (PL), anterior cingulate area (ACA) and secondary motor cortex (MOs). We found that each PPN-projecting neuron had a unique profile of collateralization, with some subcortical areas being preferential targets. In particular, PPN-projecting neurons from all three traced cortical areas exhibited common preferential collateralization to several nuclei, with most neurons targeting the striatum (STR), lateral hypothalamic area (LHA) and periaqueductal gray (PAG), and a substantial portion of neurons also targeting the zona incerta (ZI), median raphe nucleus (MRN) and substantia nigra pars reticulata (SNr). Meanwhile, very specific collateralization patterns were found for other nuclei, including the intermediate reticular nucleus (IRN), parvicellular reticular nucleus (PARN) and gigantocellular reticular nucleus (GRN), which receive collateral inputs almost exclusively from the MOs. These observations provide potential anatomical mechanisms for cortical neurons to coordinate the PPN with other subcortical areas in performing different physiological functions.

Keywords: Pedunculopontine nucleus, CaMKIIα-positive neuron, Collateral projection, Anterior cingulate area, Prelimbic region, Motor cortex, VISoR whole-brain imaging

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known for their critical roles in reward and motivation [5, 6]. Meanwhile, cortical neurons are known to branch out and project to multiple targets [7]. Thus, it is possible that PPN-targeting axons send collaterals to other subcortical areas, especially those with related functions. In this study, we performed dual-AAV injections to sparsely label PPN-projecting cortical pyramidal neurons in several cortical areas of CaMKIIα-Cre mice. We then used a high-speed volumetric imaging with on-the-fly-scan and Readout (VISoR) technique to obtain the brain-wide 3D morphology of individual neurons targeting the PPN and to characterize their collateral projections.

We first labeled PPN-projecting excitatory neurons in bulk by injecting rAAV2-retro-EF1α-DIO-EGFP into the PPN of CaMKIIα-Cre mice (Additional File 1: Materials and Methods, Fig. S1). Three weeks after injection, the distribution of EGFP fluorescence was examined across the whole brain using our high-throughput VISoR system, with EGFP-positive neurons observed in many cortical and subcortical regions, indicating that the PPN received inputs from these regions (Fig. 1a; Additional file 1: Fig. S2; Additional file 2: Video S1). In addition to the secondary motor cortex (MOS), which is responsible for their roles in various functions, including attention, emotion, reward, and learning (Fig. 1b; Additional file 1: Materials and Methods). Four weeks after injection, bright EYFP fluorescence was observed in a small set of neurons in the corresponding cortical areas with 3D VISoR imaging at 1 μm resolution. The sparseness of the labeling allowed us to trace the full morphology of individual axons, including all branches and terminal arborizations for some of these cortical pyramidal cells, which, in addition to the PPN, also make collateral projections to other subcortical areas, including the periaqueductual gray (PAG), ventral tegmental area (VTA) and striatum (STR) (Fig. 1f–h; Additional file 3: Video S2, Additional file 4: Video S3, Additional file 5: Video S4, Additional file 6: Video S5, Additional file 7: Video S6, Additional file 8: Video S7). In total, 11 PPN-targeting pyramidal neurons from three imaged animals were traced and verified, with 4 neurons from the ACA, 4 from PL and 3 from MOSs (Fig. 1i). Each of these traced neurons formed at least 2 axonal termini in the PPN. For quantification purposes, we defined a subcortical area to be a collateral target of a cortical neuron if two or more axonal termini were formed in this area.

The 11 traced neurons exhibited diverse collateralization profiles, with each individual neuron targeting 6 to 24 subcortical areas (Fig. 1i). Overall, they made collateral projections to nearly 50 subcortical nuclei, some of which emerged as preferential collateralization targets (Fig. 1j). Three subcortical nuclei, including the PAG, STR, and lateral hypothalamic area (LHA), received extensive collateral inputs from a supermajority of PPN-projecting neurons (67% or more, marked “+++” in Fig. 1j) in all three traced cortical areas, with at least two termini formed by each input neuron. Among them, the LHA and PAG both received collateral inputs from all 11 neurons. The STR, LHA, and PAG are known to be important for diverse functions, such as movement...
Fig. 1 (See legend on previous page.)
control, arousal, reward, and learning [8–11], which the PPN also participates in. Some other subcortical regions received extensive collateral inputs from one or two cortical areas, together with collateral inputs from a smaller but still substantial fraction of neurons (34%–66%, marked “++” in Fig. 1j) in the other cortical areas. These include the zona incerta (ZI), which is involved in eating and defensive behavior; the reticular part of substantia nigra (SNr), which is a major output nucleus of the basal ganglia; and the midbrain reticular nucleus (MRN), which is involved in motor control. Several cortical neurons, such as MOs-2 and PL-3, sent collaterals to all 6 aforementioned nuclei (Fig. 1i). The broad collateral inputs into these nuclei from different cortical areas could play a role in coordinating their related functional activity to support complex behavior.

On the other end of the spectrum, some subcortical regions received collateral inputs primarily from one of the three cortical areas. For example, the intermediate reticular nucleus (IRN), parvicular reticular nucleus (PARN), and giantocellular reticular nucleus (GRN) all received extensive collateral inputs from the MOs. However, only a small fraction (no more than 33%, marked “+” in Fig. 1j) or none (marked “−” in Fig. 1j) of the neurons in the ACA and PL made collateral inputs to these three nuclei. The specificity of the collateralization pattern here is in accordance with the rather particular role of these nuclei in motor-related functions [12–14].

Our observations regarding the collateralization patterns were primarily based on a limited number of traced neurons. To further validate these results, we explored the recently published mouse brain projectome database (https://mouse.braindatacenter.cn) [15], from which we searched for PPN-projecting neurons with soma in the three cortical areas above that collateralize to the aforementioned subcortical targets. Even though the projectome data are not strictly pyramidal specific, analysis of the search results largely confirmed the commonality and specificity of collateralization as described above (Additional file 1: Table S1). Through these distinctly preferential collateral projections, different cortical neurons may work synergistically to coordinate the activity of different subcortical neuronal ensembles to control complex cognitive functions and behaviors.

Supplementary Information

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Additional file 1: Materials and Methods, Supplementary figures S1, S2 and Table S1. Fig. S1. Microinjection of AAV2-retro to the PPN of a CaMKIIα-Cre mouse. Fig. S2. Brain-wide distribution of PPN-projecting neurons. Table S1. Verification of preferred subcortical collateralization of PPN-projecting cortical areas based on mouse brain projectome data.

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Author contributions

P.-M.L. and G.-Q.B. supervised the research. Q.-Q.L., P.-M.L. and G.-Q.B. conceived and designed the experiments, Q.-Q.L., Y.-X.C., and Q.J. performed the experiments. Q.-Q.L., Y.-X.C., Q.J., K.-M.Z., L.-F.D., X.-W.F., C.-H.J. and F.X. analysed the data. Q.-Q.L., P.-M.L. and G.-Q.B. interpreted the data and wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

All data presented are available upon reasonable request.

Declarations

Ethics approval and consent to participate

All animal experiments were performed with approval from the Animal Use and Care Committee of the University of Science and Technology of China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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