Whole-brain mapping of efferent projections of the anterior cingulate cortex in adult male mice

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Abstract
The anterior cingulate cortex (ACC) is a key cortical region that plays an important role in pain perception and emotional functions. Previous studies of the ACC projections have been collected primarily from monkeys, rabbits and rats. Due to technological advances, such as gene manipulation, recent progress has been made in our understanding of the molecular and cellular mechanisms of the ACC-related chronic pain and emotion is mainly obtained from adult mice. Few anatomic studies have examined the whole-brain projections of the ACC in adult mice. In the present study, we examined the continuous axonal outputs of the ACC in the whole brain of adult male mice. We used the virus anterograde tracing technique and an ultrahigh-speed imaging method of Volumetric Imaging with Synchronized on-the-fly-scan and Readout (VISoR). We created a three-dimensional (3D) reconstruction of mouse brains. We found that the ACC projected ipsilaterally primarily to the caudate putamen (CPu), ventral thalamic nucleus, zona incerta (ZI), periaqueductal gray (PAG), superior colliculus (SC), interpolar spinal trigeminal nucleus (Sp5I), and dorsal medullary reticular nucleus (MdD). The ACC also projected to contralateral brain regions, including the ACC, reuniens thalamic nucleus (Re), PAG, Sp5I, and MdD. Our results provide a whole-brain mapping of efferent projections from the ACC in adult male mice, and these findings are critical for future studies of the molecular and synaptic mechanisms of the ACC and its related network in mouse models of brain diseases.

Keywords
Anterior cingulate cortex, Efferent projections, Whole-brain mapping, VISoR, 3D reconstruction, Mice

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Introduction

The anterior cingulate cortex (ACC) has been shown to play a critical role in pain perception and emotional responses.1–4 The ACC exerts different functions through its projection to different cortical and subcortical areas.5,6 Anatomical studies of the ACC projections have predominantly been collected from monkeys,7,8 rabbits,9–11 cats,12 and rats.13,14 For example, previous studies in monkeys have found that the ACC projected to the temporal cortex, striatum, thalamus, and basolateral amygdala (BLA).7,8 Recently, studies of the ACC molecular and synaptic mechanisms, such as long-term potentiation (LTP) and long-term depression (LTD), have been performed in adult mice.1–3,6 Chen et al. reported that stimulation of the ACC directly enhanced spinal excitatory synaptic transmission through the direct ACC-spinal projection pathway.15,16 Less is known about the ACC efferent in adult mice.

In the past, fluorescence imaging of serial thin brain slices was used to study brain connections.17 This method requires proportional sampling due to the thin and large number of sections, which results in a lack of continuity and resolution of axonal tracing. Recently, Bi group developed an efficient whole-brain imaging method of Volumetric Imaging with Synchronization on-the-fly-scan and Readout (VISoR) for brain mapping. By semi-automated three-dimensional (3D) reconstruction, versatile high-throughput cell-type-specific 3D whole-brain mapping can be achieved quickly. This method has been used to study thalamocortical projection mapping of monkey brains and whole-brain imaging of neuronal activation in mice under the forced swimming conditions.18,19 The VISoR technique overcame several key technical challenges to enable high resolution, high throughput and scalability whole-brain mapping, and provided a better view of long-range projections.

Recently, we used rabies virus-based retrograde virus and combined the VISoR method to map thalamic-ACC monosynaptic inputs in adult mice.20 In the present study, we mapped the whole-brain outputs of the ACC in adult male mice by applying the anterograde virus into the unilateral ACC and following by VISoR imaging technique and 3D reconstruction. By tracing the ACC efferents, we found that the ACC mainly projected to ipsilateral nuclei, while a small number of fibers projected to some contralateral nuclei in adult male mice.

Materials and Methods

Animals

Adult (8–12 weeks old) male C57BL/6 mice were purchased from the Experimental Animal Center of Xi’an Jiaotong University. All animals were randomly housed under an artificial 12 h light/dark cycle (9 a.m.–9 p.m. light) with food and water provided ad libitum. All experimental protocols were approved by the Ethics Committee of Xi’an Jiaotong University.

Virus anterograde tracing

To analyze the efferent projections from the ACC in mice, we micro-injected 200 nL of the rAAV-hSyn-EGFP-2a-WPREs-pA (AAV2/9, 2.0×10^{12} genomes copies per mL; Brainvitaa company, Wuhan, China) into the unilateral ACC. Briefly, four mice were anesthetized with 2% isoflurane and placed in a stereotaxic device. The virus was injected by a glass pipette and micro-syringe pump (23 nL/min, once every 10 s; Nanoject II #3-000-205/206, DRUMMOND) into the right ACC according to the 4th edition of the mouse brain atlas (0.9 mm anterior to bregma, 0.3 mm lateral to the midline, 1.4 mm deep from the cerebral surface). After injection, the surgical wound was carefully sutured and disinfected. The animals were monitored until waking up before returning to their home cages. Mice were allowed to survive for approximately 3–4 weeks for virus expression.16,21

Perfusion and slice preparation

Four weeks after viral expression, mice were deeply anesthetized with 2% isoflurane and perfused intracardially with 0.01 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% w/v paraformaldehyde (PFA, pH 7.4). The brain was removed and incubated in 4% hydrogel monomer solution (HMS, 4% w/v acrylamide, 0.05% w/v bisacrylamide, 1× PBS, 4% w/v PFA, 0.25% VA-044 thermal initiator, and distilled water) at 4°C for 2 days, to allow penetration of fixation solution.

The sample was embedded with an equal volume mixture of 4% HMS and 20% bovine serum albumin (BSA) at 37°C for 4 h, and washed three times with PBS. The embedded block was trimmed to ensure that the sample was vertically glued to the base of the vibroslicer (Compressstome VF-300, Precisionary Instruments). Then, the brain was cut into 300-μm-thick coronal slices.

Sample clearing

Brain slices were then treated following the two-step PuClear clearing method to obtain uniform optical transparency through the 300-μm thickness.18 Firstly, brain slices were placed in 5% PBS-Triton at 37°C and gently shaken for 24 h to increase membrane permeability. Next, these brain slices were placed on quartz slides and incubated in PuClear solution (50% iohexol, 23% urea, 11% 2,2’,2”-nitrolotriethanol, and 16% distilled water) with a refractive index of 1.52 for 4 h.

Fluorescence imaging and whole-brain image reconstruction

Cleared brain slices were imaged at 1×1×2.5 μm^3 voxel resolution using the VISoR2 technique as described previously.18 Synchronized beam-scan illumination and camera-frame readout generated a stack of image frames of the 45° oblique optical sections of the sample with the sample stage moving linearly in the X-direction.

Image volumes of each mouse brain slice were stitched with the custom software to automatically reconstruct the
whole brain as described previously. The image volumes of the whole brain were then converted to the Imaris file format (IMS) for visualization and video rendering in Imaris software (v9.5, Oxford Instruments).

Results
To examine the whole-brain efferents of the ACC, we injected the anterograde virus into the right ACC of four adult male mice. This procedure is illustrated in Figure 1(a). The virus was expressed in the rostral to caudal ACC neurons (Figure 1(b)). These results suggested that the following brain regions with projection fibers were the targets of the ACC outputs. Following the areal nomenclature for cingulate regions was from the fourth edition of the mouse brain atlas. The general correspondence of nomenclature with the brain areas was as follows: area 25 corresponds to the infralimbic cortex (IL), area 32 to the prelimbic cortex (PrL), and area

Figure 1. Expression of virus at the injection site and distribution of anterograde labeled fibers from the ACC in the CPu. (a) The procedure for anterograde tracing projections from the ACC. The rAAV-hSyn-EGFP-2a-WPREs-pA was injected unilaterally into the ACC of the mice. Three weeks after the injection of the virus, the whole mouse brain was sampled and sectioned for VIsoR and 3D reconstruction. (b) Fluorescence images of the brain slices containing ACC after virus-injection into the ACC. (c) Virus-infected fibers were distributed in the fmi of the slice including the rostral ACC. The white box was augmented in 1. (d) Virus-infected fibers were distributed in the bilateral cc, gcc, and ipsilateral CPu. White boxes were augmented in 2 and 3. (e) Virus-infected fibers were distributed in the cc and CPu of the slice containing the caudal ACC. White boxes were augmented in 4 and 5. ACC, anterior cingulate cortex; fmi, forceps minor of the corpus callosum; ipsi, ipsilateral; cont, contralateral; gcc, genu of the corpus callosum; cg, cingulum; CPu, caudate putamen (striatum); cc, corpus callosum. Scale bars: b, top of c, left of d and e, 500 μm; 1, 80 μm; 2, 150 μm; 3–5, 100 μm.
24a/24b to the ACC. The retrosplenial cortex (RSC) includes the retrosplenial agranular (RSA) and the retrosplenial granular cortex (RSG), corresponding to areas 30 and 29, respectively.

**Distribution of projection fibers in the caudate putamen (CPu) and contralateral ACC from the ACC**

The CPu is a key brain region that receives projections from the cortex. The ACC to CPu inputs mediate histaminergic itch sensation. Here, we observed the direct projection from the ACC to the CPu by whole-brain mapping of the ACC efferents in the adult mice. In the brain slice containing the rostral ACC, we found mildly labeled ACC projection fibers at the forceps minor of the corpus callosum (fmi) and its junction with the ACC (Figure 1(c)). In the middle ACC slice, the ipsilateral cingulum (cg), genu of the corpus callosum (gcc) and CPu displayed moderate labeling after the injection of anterograde virus into the ACC. In addition, mild labeling was observed in the contralateral cg and gcc, and very sparse labeling was also shown in the contralateral ACC (Figure 1(d)), indicating that the ACC sent direct projections to the contralateral ACC. In the slice containing the caudal ACC, the neurons of superficial and deep layers in the ACC were obviously infected by the anterograde virus. We also observed strong labeling in the ipsilateral corpus callosum (cc)
Compared with the middle ACC slice, the labeled fibers of the CPu were denser and more ventral in the caudal ACC slice (Figures 1(d) and (e)). These results indicate that the ACC projects to the ipsilateral cc and CPu, with a small number of projections to the contralateral ACC and cc.

**Distribution of projection fibers in the thalamus and superior colliculus (SC) from the ACC**

The thalamus and SC are two main targets of the ACC outputs. In the brain slice containing the rostral thalamus, light labeling was found in the bilateral cc. The internal capsule (ic) received dense inputs from the ACC. The ACC projection fibers were lightly labeled in the reticular thalamic nucleus (Rt) (Figure 2(a)). In the brain slice containing the ventral thalamic nucleus, the projection fibers from the ACC divided into two major branches at the ic: the ventral branch ran along and across the ic, and the dorsal bundle of fibers distributed in the ventral thalamic nuclei, including the ventral posterolateral (VPL), ventrolateral (VL), and ventromedial (VM) thalamic nuclei (Figure 2(b)). In the more caudal brain slice, moderately labeled projection fibers were found in the VL, mediodorsal part of the lateral posterior (LPMR), paracentral (PC), lateral part of mediodorsal (MDL), and centrolateral (CL) thalamic nucleus from the dorsal branch of the ic (Figure 2(c)). In the brain slice containing the caudal thalamus, in addition to light-labeled projection fibers were distributed in the cp, STh and ZI. The white box was augmented in 1. (b) Virus-infected fibers were distributed in the ZI, pretectal area, and cp. White boxes were augmented in 2 and 3. (c) Virus-infected fibers were distributed in the ZI, thalamus, pretectal area and cp. White boxes were augmented in 4 and 5. (d) Virus-infected fibers were distributed in the pretectal area, SC, PAG and cp. White boxes were augmented in 6 and 7. cp, cerebral peduncle; STh, subthalamic nucleus; ZI, zona incerta; ZIV, zona incerta, ventral part; ZID, zona incerta, dorsal part; APTD, anterior pretectal nucleus, dorsal part; MPT, medial pretectal area; Eth, ethmoid thalamic nucleus; APTV, anterior pretectal nucleus, ventral part; REth, retroethmoid nucleus; ml, medial lemniscus; SNR, substantia nigra, reticular part; APT, anterior pretectal nucleus; PAG, periaqueductal gray; SC, superior colliculus; InG, intermediate gray layer of the superior colliculus; InWh, intermediate white layer of the superior colliculus. Scale bars: left of a-d, 500 μm; 1, 3, 5, 7, 100 μm; 2, 4, 6, 150 μm.
fibers in the LPMR and CL of the thalamus, moderate labeling was also observed in the dorsal part of the anterior pretectal nuclei (APTD) (Figure 2(d)). Finally, the dorsal branch terminals were found in the SC, including the intermediate gray layer (InG), intermediate white layer (InWh), deep gray layer (DpG), and deep white layer (DpWh) of SC. The periaqueductal gray (PAG) also received mild ACC projections (Figures 2(e) and 2(f)). Furthermore, the ACC projection fibers were also found in the ipsilateral anterior and posterior thalamic nuclear groups. We also found a small number of the ACC efferent fibers in the contralateral reuniens thalamic nucleus (Re) and PAG. Taken together, these

Figure 4. Distribution of anterograde labeled fibers from the ACC in the brainstem. (a) Virus-infected fibers were distributed in the cp, Pn, ml and PnO. The white box was augmented in 1. (b) Virus-infected fibers were bilaterally distributed in the PnC and RtTg. The white box was augmented in 2. (c) Virus-infected fibers were distributed in the ipsilateral py, SP5I, Mx, MVeMC, and contralateral SP5I, Mx, and SpVe. The white boxes were augmented in 3-7. (d) Virus-infected fibers were distributed in the pyx and MdD of contralateral. The white box was augmented in 8. cp, cerebral peduncle; Pn, pontine nuclei; ml, medial lemniscus; PnO, oral part of the pontine reticular nucleus; PnC, caudal part of the pontine reticular nucleus; RtTg, reticulotegmental nucleus of the pons; ipsi, ipsilateral; cont, contralateral; py, pyramidal tract; Sp5I, interpolar part of the spinal trigeminal nucleus; Mx, matrix region of the medulla; MVeMC, magnocellular of the medial vestibular nucleus; SpVe, spinal vestibular nucleus; pyx, pyramidal decussation; MdD, dorsal part of the medullary reticular nucleus; MdV, ventral part of the medullary reticular nucleus; IRt, intermediate reticular nucleus. Scale bars: left of a-d, 500 μm; 1, 150 μm; 2, 3, 100 μm; 4–7, 50 μm; 8, 80 μm.
results suggest that the ACC projection fibers produce two major branches at ic, the dorsal branch passing through the ventral, medial, and central thalamus, and terminating to SC and PAG.

**Distribution of projection fibers in the zona incerta (ZI) and cerebral peduncle (cp) from the ACC**

ZI connects with many brain areas, which involves the modulation of behaviors and physiological states. The cp consists of descending fibers from the cerebral cortex to the cerebellum, medulla oblongata, and spinal cord. In the brain slice containing the caudal thalamus, we found that the ZI and cp received dense labeled fibers from the ACC, belonging to the ventral branch of the ic. The subthalamic nucleus (STh) between the ZI and cp was also labeled with dense fibers (Figure 3(a)). In the more caudal slice compared with Figure 3(a), the labeled ACC projection fibers were divided into two branches and projected to ZI and cp, respectively. In the ZI branch, the ventral (ZIV) and dorsal parts (ZID) of the ZI displayed strongly labeled fibers. In addition, APTD and medial pretectal nuclei (MPT) also received moderate inputs. The other branch had dense labeling at the cp (Figure 3(b)). In the more caudal slice compared with Figure 3(b), light-labeled fibers were found in the ZI, APTD, and ventral part of anterior pretectal nuclei (APTV). The medial lemniscus (ml), retrolenticular nucleus (REth), and ethmoid thalamic nucleus (Eth) showed dense labeling (Figure 3(c)). In the more caudal slice, InG and InWh of SC received moderate inputs, and the anterior pretectal nuclei (APT) and PAG received light inputs from the ACC (Figure 3(d)). In the cp branch of Figures 3(c) and (d), the cp and substantia nigra (SNR) displayed dense labeled fibers (Figures 3(c) and (d)). Furthermore, in the ZI branch, the ACC projection fibers were also found in the parafascicular thalamic nucleus (PaF) and posterior thalamic nuclear group. These results show that the ventral branch projection fibers at ic project to ZI and cp, respectively. In the ZI branch, ACC efferent fibers project to the posterior thalamus and APTD, and terminate in the SC and PAG. In the cp branch, ACC fibers project to the cp and SNR, and continue into the brainstem.

**Distribution of projection fibers in the brainstem from the ACC**

According to previous studies, the ACC had outputs to the midbrain, pons and medulla oblongata of the brainstem. In Figure 3, the cp received strong ACC projections and continued to transmit the ACC outputs to other areas of the brainstem. In Figure 4(a), the cp, pontine nuclei (Pn), ml, and oral part of the pontine reticular nucleus (PnO) showed moderately labeled fibers from the ACC (Figure 4(a)). The brain slice containing the brainstem showed that the bilateral caudal part of the pontine reticular nucleus (PnC) and reticulotegmental nucleus of the pons (RtTg) received sporadic inputs from the ACC (Figure 4(b)). In the more caudal slice compared with Figure 4(b), the ipsilateral pyramidal tract (py) displayed moderately labeled projection fibers from the ACC. Sporadic ACC projection fibers were transmitted to the bilateral interparietal spinal trigeminal nucleus (Sp5I), matrix region of the medulla (Mx), and spinal vestibular nucleus (SpVe) (Figure 4(c)). In the caudal brainstem, a dense bundle of the ACC fibers was observed in the contralateral pyramidal decussation (pyx), bilateral dorsal (MdD) and ventral (MdV) parts of the medullary reticular nucleus (Figure 4(d)). Furthermore, the ACC projection fibers were also found in the ventral pontine reticular nucleus (PnV), raphe magnus nucleus (RMg), gigantocellular reticular nucleus (Gi), paramedian reticular nucleus (PMn), and tegmental areas of midbrain and pons, and all of these projections were bilateral. The results suggest that the ACC projects to the brainstem through the cp and py, and the main targets are bilateral pons and reticular structures. ACC projection fibers are observed in the contralateral pyx, and it is likely that the ACC projects to the contralateral spinal cord through the pyx.

**Distribution of projection fibers in the other cortical areas from the ACC**

The ACC has direct connections with many other cerebral cortices, such as the motor cortex and visual cortex. In the present study, many cortical areas had labeled efferent fibers from the ACC. The orbitofrontal cortex (OFC) is divided into three parts, including the medial (MO), ventral (VO), and lateral (LO) orbital cortices. Sparse labeling was observed in the OFC after injecting the anterograde virus into the ACC. The frontal association cortex (FrA) and PrL also displayed sparsely labeled projection fibers (Figure 5(a)). In the coronal slice shown in Figure 2(b), the RSA, RSG, primary somatosensory cortex (S1), primary (M1) and secondary (M2) motor cortex received light inputs from the ACC (Figures 5(b) and (c)). In addition, light labeling was found in the primary (V1) and secondary (V2) visual cortices (Figures 2(d) and 5(d)). These results show that the ACC projection fibers are found in many cortical areas, including orbitofrontal, prefrontal, retrosplenial, motor, somatosensory, and visual cortices, but the projection fibers are sparse and ipsilateral.

**Discussion**

The results of our present study show the whole-brain efferent projections of the ACC in the adult male mice by applying anterograde virus into the unilateral ACC. Through fluorescence imaging and 3D reconstruction, we found that a bundle of dense ACC projecting axons produces three major branches through the CPu and ic. The two branches project to the SC and PAG through the ventral thalamus and posterior
thalamus, respectively. The other branch through the cp and py targets to the bilateral pons and medulla oblongata. In addition to subcortical projections, the ACC slightly projects to the cortical areas, such as the ipsilateral orbitofrontal, prefrontal (PFC), motor, somatosensory, visual cortices, and the contralateral ACC (Figure 6).

Ipsilateral and contralateral projection from ACC

In our present study, most of the ACC fibers projected to the ipsilateral brain regions of the virus injection site, and a small amount to the contralateral ACC, Re, PAG, SpS1, and MdD. Major targets of the ACC ipsilateral projection in mice included the CPu, ventral thalamic nuclei, ZI, PAG, SC, SpS1, and MdD. Among them, the ACC projection to bilateral MdD is consistent with a previous study of rats. In addition to MdD, ACC projections to other ipsilateral or contralateral brain regions have been reported in previous studies in mice (Table 1). These anatomical connections may provide new perspectives for further study of the functions and mechanisms of ACC efferent pathways. For example, our results show that the ACC projects to the contralateral ACC. Hu et al. revealed a circuit mechanism in which the cross-callosal projection of ACC-contralateral ACC contributes to mirror-image pain. However, other functions and molecular mechanisms of the ACC-contralateral ACC pathway are not clear, so it is worth more research in the future.

ACC projection to the BLA and hippocampus

The BLA is an important brain region for emotional fear and anxiety. The ACC projection to the BLA has been found in monkeys, rabbits, and rats. Previous studies have reported that the ACC inputs to the BLA control the fear response in mice. Tang et al. also demonstrated that direct stimulation of the ACC in mice produces fear behavior, and induces long-term auditory fear memory, which requires the activation of N-methyl-D-aspartate (NMDA) receptors in the BLA. The hippocampus is an important brain region associated with memory. A direct projection from the ACC to the hippocampus has also been found in rats and mice. The ACC projection to the ventral hippocampus mediates contextual fear generalization. Optogenetic manipulation of the ACC to hippocampus elicits contextual memory retrieval. However, we did not observe any ACC projection to the BLA or hippocampus in the present study. We adjusted the fluorescent brightness and contrast, and found that the BLA and hippocampus still had no signal. Upon analysis, these inconsistent results may be
Figure 6. The 3D map and summary of anterograde labeled fibers from the ACC in the whole brain. (a, b, and d) The 3D (a), vertical (b), lateral (d) views of whole-brain distributions from ACC axons projected by VISoR and 3D reconstruction. (c and e) Shown are major brain areas and pathways in the ACC project network. Ipsi, ipsilateral; cont, contralateral. d, dorsal; a, anterior; r, right; ACC, anterior cingulate cortex; OFC, orbitofrontal cortex; M1, primary motor cortex; M2, secondary motor cortex; RSC, retrosplenial cortex; S1, primary somatosensory cortex; V1, primary visual cortex; V2, secondary visual cortex; CPu, caudate putamen (striatum); Re, reuniens thalamic nucleus; PAG, periaqueductal gray; SC, superior colliculus; Sp5I, interpolar part of the spinal trigeminal nucleus; MdD, dorsal part of the medullary reticular nucleus; py, pyramidal tract; pyx, pyramidal decussation; ic, internal capsule; cp, cerebral peduncle; ZI, zona incerta.

| Brain regions | Monkeys$^{7,8}$ | Rabbits$^{9-11}$ | Rats$^{13,14,28}$ | Mice$^{17,21,22}$ (previous studies) | Mice (present study) |
|---------------|-----------------|-----------------|-----------------|-------------------------------|-------------------|
|               | Ipsi | Cont | Ipsi | Cont | Ipsi | Cont | Ipsi | Cont | Ipsi | Cont |
| ACC           | +    |    - | +    |    - | +    |    - | +    |    - | +    |    - |
| M1/M2/CPu     | +    |    - | +    |    - | +    |    - | +    |    - | +    |    - |
| BLA           | +    |    - |     |    - | +    |    - | +    |    - | +    |    - |
| LS/MS/VDB     |     |    - |     |    - |     |    - | +    |    - | +    |    - |
| CI            |     |    - |     |    - | +    |    - | +    |    - | +    |    - |
| Re/AM/IAD     | +    |    - | +    |    - | +    |    - | +    |    - | +    |    - |
| PAG/VTA/MnR   | +    |    - | +    |    - | +    |    - | +    |    - | +    |    - |
| LC/Pn/LDTg/CG | +    |    - | +    |    - | +    |    - | +    |    - | +    |    - |
| MdD/MdV       |     |    - |     |    - | +    |    - | +    |    - | +    |    - |

+: ACC efferent projections have been reported; --: ACC efferent projections have not been reported; Ipsi: ipsilateral; Cont: contralateral; ACC, anterior cingulate cortex; M1, primary motor cortex; M2, secondary motor cortex; CPu, caudate putamen (striatum); BLA, basolateral amygdaloid nucleus; LS, lateral septal nucleus; MS, medial septal nucleus; VDB, nucleus of the vertical limb of the diagonal band; CI, claustrum; Re, reuniens thalamic nucleus; AM, anteromedial thalamic nucleus; IAD, interanterodorsal thalamic nucleus; PAG, periaqueductal gray; VTA, ventral tegmental area; MnR, median raphe nucleus; LC, locus coeruleus; Pn, pontine nuclei; LDTg, laterodorsal tegmental nucleus; CG, central gray; MdD, dorsal part of the medullary reticular nucleus; MdV, ventral part of the medullary reticular nucleus.
due to the insufficient infectiousness and expression time of the anterograde virus. Differences in the injection site and volume of the virus or tracer may also contribute to the inconsistent results. The specific reason is still unclear and requires more experiments to be explored and confirmed in the future.

**ACC projection to the brainstem and spinal cord**

The brainstem is one of the main target areas of the mouse ACC projections. In the brainstem, we found labeled fibers from the ACC in the SC, PAG, ventral tegmental area (VTA), and rostral ventromedial medulla (RVM). These results are consistent with many previous studies in rabbits, rats and mice. The pathway of ACC to SC participates in avoidance behaviors and visual performance. The PAG and RVM play important roles in the descending modulation of chronic pain. The RVM is thought to be one of the key relays for descending modulation of spinal sensory transmission. Descending facilitation from the ACC, caused by electrical stimulation or chemical activation, apparently relayed at the RVM. The projection from the ACC to the VTA contributes to neuropathic pain-evoked aversion, which has also been described in rats. We show that the ACC neurons also form connections with the ventrolateral (Pr5VL) and dorsomedial parts (Pr5DM) of the principal sensory trigeminal nucleus, interpolar (Sp5I) and oral parts of the spinal trigeminal nucleus (Sp5O). It has been reported that the spinal trigeminal nucleus receives afferents from the orofacial nociceptor, which is important to the modulation of chronic neuropathic orofacial pain. Our results showed that labeled fibers from the ACC in the MdD and MdV act as supraspinial pain regulatory areas to regulate the pain sensation process in the spinal cord. Furthermore, a direct projection from the ACC to the spinal cord has been reported in rats and mice. ACC output mapping was not performed in the spinal cord in our study. However, we found that the ACC projects to the contralateral pyx. It is likely that ACC projects to the contralateral spinal cord through pyx.

**ACC projection to the other cortical areas**

We observed that the ACC sends projections to many cortical areas, including ipsilateral orbitofrontal, prelimbic, retrosplenial, motor, somatosensory, and visual cortices (Figure 5). A previous study in monkeys showed that the pathway of the ACC to OFC affects perception and emotions. The ACC projection to the PFC may influence sleep and cognition. The ACC-M1 pathway regulates the motor activity of the orofacial and forelimb in rats. A projection from the ACC to the visual cortex facilitates visual processing and improves visual discrimination. In summary, the ACC connects with many other cortical areas, and these pathways regulate a variety of different functions.

**Technical comparison**

In the present study, we mapped the efferent projections from the ACC in mice using the VISoR system and 3D reconstruction. Previous reports have used 3D reconstructions to study brain structure and projection. For example, Zhang et al. performed a 3D reconstruction of the brain region or pathway by continuously playing two-dimensional (2D) brain slices. However, this method does not produce a complete 3D image, which means it is impossible to observe the activity trace of the projection fibers. In addition, a previous study digitized projection fibers and then performed 3D reconstruction. It is important to note that this method focuses on the anatomical distribution of the projection fibers, and thus, the intensity of the signal and details of the synaptic organization are not represented. We optimized tissue slicing and clearing, ultrahigh-speed imaging techniques, and efficient analysis tools. The resulting 3D image clearly showed the details and pathway direction of axonal fibers. In summary, our results provide a more thorough understanding of the ACC efferent network in the mouse brain. This study creates an anatomical foundation for future studies on the pathways and functions of the ACC.

**Authors’ contributions**

W.S., X.H.L., G.Q.B., J.S.L. and M.Z. designed the experiments. W.S., M.X., F.W. and K.F. preformed experiments and analyzed data. W.S., X.M., Q.Y.C., F.X., X.H.L., J.S.L. and M.Z. drafted the manuscript and finished the final version of the manuscript. All authors read and approved the final manuscript.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Code availability**

Codes for VISoR2 image reconstruction are openly accessible at https://github.com/SMART-pipeline/Volume-reconstruction.

**Data-availability statement**

Original data is available from the corresponding authors upon reasonable request.

**ORCID iDs**

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