Cell-type-specific whole-brain direct inputs to the anterior and posterior piriform cortex

Cell-type-specific whole-brain monosynaptic inputs to the anterior and posterior piriform cortex

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ABSTRACT

The piriform cortex (PC) is a key region in the brain that is involved in both processing and coding of olfactory information. It is implicated in various brain disorders, such as epilepsy, Alzheimer's disease and autism. The PC consists of anterior (APC) and posterior (PPC) parts, which are largely different both in their anatomy and functions. However, the monosynaptic input networks to specific neural populations within APC and PPC remain poorly understood. Here, we mapped the whole-brain monosynaptic inputs to the two major neural populations, the excitatory glutamatergic principal neurons and the inhibitory γ-aminobutyric acid (GABA)-ergic interneurons within the APC and PPC using the rabies virus-mediated retrograde trans-synaptic tracing system. We found that for both types of neurons, APC and PPC share some similarities in input networks, with dominant inputs originating from the olfactory areas (OLF), followed by the isocortex, hippocampal formation (HPF), cortical subplate (CTXsp), cerebral nuclei (CNU) and interbrain (IB), whereas the midbrain (MB) and hindbrain (HB) were either blank or sporadically labeled. However, APC and PPC also showed distinct features in their input distribution patterns. For both types of neurons, the APC was innervated more heavily by bilateral OLF and cortical areas compared to the PPC; whereas the input proportions from the HPF to the PPC were higher than to the APC. Overall, our results revealed that

monosynaptic input networks to both excitatory and inhibitory neural populations of different PC subdivisions, may provide the structural architecture for revealing the diverse functions of the PC.

INTRODUCTION

The piriform cortex (PC) is located in the ventrolateral regions of the forebrain and extends broadly along the anterior to posterior (AP) axis in mammals. As one of the primary olfactory cortex, the PC is involved in encoding odor identification (Bekkers and Suzuki, 2013; Courtiol and Wilson, 2017; Gottfried et al., 2006; Howard et al., 2009; Wilson and Sullivan, 2011), odor associated values or contexts (Calu et al., 2007; Gottfried and Dolan, 2003; Roesch et al., 2007), and odor memory (Strauch and Manahan-Vaughan, 2018; Zelano et al., 2011). Besides, the PC is also implicated in various neurological disorders, such as epilepsy (Loscher and Ebert, 1996; Vismer et al., 2015; Young et al., 2019), Alzheimer's disease (Saiz-Sanchez et al., 2015; Samudralwar et al., 1995), autism spectrum disorder (Koehler et al., 2018; Menassa et al., 2017) and Parkinson's disease (Wu et al., 2011).

Previous studies revealed that the PC receives highly converged inputs from distributed glomeruli of the main olfactory bulb (MOB) (Vicente and Mainen, 2011), and further synthesizes these odor features into configural odor objects with the help of abundant association fibers within it (Haberly, 2001; Wilson and Sullivan, 2011). Besides olfactory inputs, the PC also receives extensive inputs from cortical and limbic regions (Haberly and Price, 1978; Illig, 2005; Kowianski et al., 1999; Majak et al., 2004). Through these connections, the PC can integrate multisensory, emotional and memorial information (Courtiol and Wilson, 2017; Wilson and Sullivan, 2011). In addition, the PC neural activities are also regulated by neuromodulatory axons originating from the cholinergic neurons in the horizontal limb of the diagonal band (HDB) (Fletcher and Chen, 2010; Wirth et al., 2000), the noradrenergic neurons in the locus coeruleus (LC) (Bouret and Sara, 2002; Fletcher and Chen, 2010), the serotonergic neurons in the dorsal raphe nucleus (DR) (Fletcher and Chen, 2010; Narla et al., 2015), and the dopaminergic neurons in the ventral tegmental area (VTA) (Loscher and Ebert, 1996; Shipley and Ennis, 1996). Although the anatomical and physiological evidence revealed some basic connectivity features and information processing mechanism of the PC, the comprehensive neural circuit foundation for functional diversities of the PC remain poorly understood.

The PC is a trilaminar paleocortex that is usually divided into anterior (APC) and posterior (PPC) parts along the AP axis. The borderline is defined by the disappearance of the lateral olfactory tract (LOT) and the thickened layer III in the PPC (Loscher and Ebert, 1996). APC and PPC play different roles in olfactory processing including odor response and learning (Calu et al., 2007; Gottfried et al., 2006; Kadohisa and Wilson, 2006; Litaudon et al., 2003). For instance, the APC encodes odor identity and anticipation, and can be activated not only by odor stimuli but also by odor associated values or contextual cues (Gottfried et al., 2006; Kadohisa and Wilson, 2006; Roesch et al., 2007; Zinyuk et al., 2001); whereas the PPC seems to encode more associated information for it to be activated in tasks that require encoding of odor similarity or odor quality (Bao et al., 2016; Calu et al., 2007; Grau-Perales et al., 2019; Howard et al., 2009; Kadohisa and Wilson, 2006; Zelano et al., 2011). In addition, accumulating evidence from research has also revealed distinct susceptibilities of different PC subdivisions to seizure generation (Ekstrand et al., 2001; Loscher and Ebert, 1996; Vismer et al., 2015; Yang et al., 2006). Moreover, the PC comprises glutamatergic principal neurons and γ-aminobutyric acid (GABA)-ergic interneurons. In brief, glutamatergic principal neurons are mainly located in layer II/III in the PC (Suzuki and Bekkers, 2011); GABAergic interneurons, which

serve to provide synaptic inhabitation of principal neurons and shape stimulus receptive fields, scatter more uniformly across all layers (Large et al., 2016; Luna and Schoppa, 2008; Suzuki and Bekkers, 2007, 2012). Since the synaptic inhabitation of principal neurons is distinct between APC and PPC partly because of GABAergic neurons distributed asymmetrically along the AP axis (Loscher et al., 1998; Luna and Pettit, 2010), it reveals the neural connections to specific types of neurons within different PC subdivisions essential to shedding light on the functional diversities and dysfunctions of the PC.

Previous studies using classical tracers have reported many differences in input connectivity between APC and PPC (Haberly and Price, 1978; Kowianski et al., 1999). For instance, the APC receives more inputs from the OB, AON and ORB (Datiche and Cattarelli, 1996; Illig, 2005; Kowianski et al., 1999), whereas the PPC is heavily innervated by the AMY (Johnson et al., 2000; Majak et al., 2004). However, traditional tracers are unable to distinguish synaptic connections from pass-by fibers, let alone to exclusively label direct inputs to specific types of neurons.

In the present study, we mapped the monosynaptic inputs to glutamatergic principal neurons and GABAergic interneurons within APC and PPC using the retrograde trans-synaptic tracing system (Callaway and Luo, 2015; Wall et al., 2010; Wickersham et al., 2007). Our results revealed cell-type-specific input patterns to different subdivisions of the PC in the whole-brain range, and quantitatively compared their input proportions. We found that the APC was related more closely with the olfactory areas (OLF) and isocortex, especially the AON, MOB and ORB; while the PPC was innervated heavily by the emotion and memory coding areas, such as the hippocampal formation (HPF). Our results could provide neural connectivity information for further revealing the functional diversities of the PC and its roles in brain diseases.

MATERIALS AND METHODS

Animals

All surgery and experimental procedures were performed in accordance with the guidelines of the Animal Care and Use Committees at the Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, and all efforts were made to minimize the number and suffering in experimental animals. Both Vglut2-cre and Gad2-cre mice (Jackson # 028863 and Jackson # 028867 respectively, gifts from Prof. Liping Wang) were mated with C57BL/6 mice, which were purchased from Hunan SJA Laboratory Animal Company. All animals were housed under standard conditions of humidity and temperature with a 12/12 h light/dark cycle, and food and water were available ad libitum. Adult transgenic mice (2-4 months) of both sexes were used for the experiments in the present study.

Virus Injections

The virus tools for AAV-Rabies virus based monosynaptic retrograde tracers used in this study were generated by BrainVTA (BrainVTA Co., Ltd., Wuhan, China), and were stored at -80℃ until use. The Cre-dependent AAV helper viruses were composed of AAV- EF1a-Dio-GFP-TVA and AAV-EF1a-Dio-RVG, and packaged into 2/9 serotypes with final titers at about 1.25×10^{12} genomic copies per milliliter. The RV- EnvA-ΔG- dsRed was tittered at 3.00×10^{8} infecting units per milliliter.

The procedure for virus injection was similar to the one used before in biosafety level 2 animal facilities (Zhang et al., 2017). Briefly, the Vglut2-cre or Gad2-cre mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and mounted to a stereotaxic holder (Item: 68030, RWD, Shenzhen, China) for stereotaxic injection of 80 nl AAV-helper viruses into the APC (coordinates:1.50 mm from bregma, 2.60 mm lateral from the midline, -4.75 mm from the bregma surface) or the PPC (coordinates: -1.00 mm from bregma, 3.60 mm lateral from the midline, -5.25 mm from the bregma surface). After three weeks, 150 nl RV- EnvA-ΔG-dsRed was microinjected into the same site. The mice were kept for 6 days, and then perfused for brain slice collection. Sample size: $APC^{Vglut2+}$, n=6 mice; $PPC^{Vglut2+}$, n=6 mice; APC^{Gad2+}, n=4 mice; PPC^{Gad2+}, n=4 mice.

Slice Preparation and Imaging

The mice were overdosed with sodium pentobarbital (100 mg/kg, i.p.), and perfused transcardially with 0.1 M phosphate buffered saline (PBS, PH 7.4, Sinopharm) followed by PBS containing 4% paraformaldehyde (PFA, Sigma). The brain tissues were carefully extracted from the skull for post-fixation and cryoprotection, and were then cut into 40 um coronal sections using the cryostat microtome (Thermo Fisher Scientific) and stored at -20℃.

For input pattern analysis, every sixth section of the brain slices was selected and stained with DAPI (1:4000, Beyotime), then mounted with 75% glycerol (Sinopharm) in PBS and sealed with nail polish. The brain slices were imaged with the Olympus VS120 virtual microscopy slide scanning system (Olympus).

Cell Counting and Data Analysis

The divisions and abbreviations of brain regions were mainly based on the Allen Brain Atlas. **Supplementary Table 1** shows a detailed list of all related abbreviations.

For cell counting, the starter cells (co-expressing the TVA-GFP and EnvA-dsRed) and RV labeled input neurons (only expressing EnvA-dsRed) in each brain region were quantified respectively by the cell counter plugin in ImageJ. To get rid of the potential leakage of TVA near the injection site, the RV-labeled neurons within the target injection site (ipsilateral APC or PPC) were not counted as input neurons. For quantitative comparison, the input proportions of discrete nuclei or intact brain areas across different tracing groups, the input from each brain region was normalized by dividing the number of labeled neurons in the region by the total number of labeled neurons from whole-brain regions (excluding the target injection site).

For statistical analyses, two-tailed unpaired Student's t-tests and Wilcoxon signed rank-sum tests were performed to determine statistical differences using SPSS (version 13.0), with the significance set at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. All data values were presented as mean ± SEM.

RESULTS

Monosynaptic Inputs to Glutamatergic and GABAergic Neurons in Different PC Subregions

To identify input patterns of glutamatergic and GABAergic neurons in APC and PPC, Vglut2-cre mice and Gad2-cre mice were used to genetically target distinct neuronal populations, and the AAV-rabies virus based retrograde trans-synaptic system was used to map the monosynaptic inputs to each

type of neuron (**Figures 1A**,**B**). The starter cells, identified by the co-expression of TVA-GFP and EnvA-dsRed, were observed near the injection sites of the PC subregions (**Figures 1C**, **D**). For each tracing group, the starter cells were highly restricted to the specific PC subregion ($APC^{Vglut2+}$, 97.79%±0.98%; $PPC^{Vglut2+}$, 99.00%±0.38%; APC^{Gad2+}, 100%±0%; PPC^{Gad2+}, 88.66%±4.86%). To examine the specificity of the tracing study, the same viruses were injected in the APC of the wild-type mice (C57BL/6 mice). Despite a very limited number of EnvA-dsRed positive cells near the injection sites within the APC, no RV retrograde labeled neuron outside the injection sites was detected (**Supplementary Figure 1**). These data suggested a high specificity of cre-dependent trans-synaptic property of our viral tracing approach.

When we quantified the whole-brain connections to the APC and the PPC, the results showed that the excitatory and inhibitory neurons in both PC subregions receive extensive inputs from many brain regions along the AP axis (**Figure 2A**), including the OLF, isocortex, HFP, cortical subplate (CTXsp), cerebral nuclei (CNU), interbrain (IB), midbrain (MB) and hindbrain (HB) (**Figures 2B**,**C**). To analyze the input weights from each brain region, RV labeled neuron numbers in eight major brain regions were quantified and normalized by the total inputs for each brain. For all tracing groups, the majority input sources were observed in the OLF, followed by the isocortex, HPF, CTXsp and CNU (consisted of the striatum (STR) and pallidum (PAL)), thalamus (TH) of IB, whereas the hypothalamus (HY) of IB, MB and HB were either blank or sporadically labeled (**Figures 2B**,**C**). Despite similar input patterns from all four tracing groups (**Figure 2C**; $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, P=0.674; APC^{Gad2+} vs PPC^{Gad2+}, P=0.161; $APC^{Vglut2+}$ vs APC^{Gad2+}, P=0.575; $PPC^{Vglut2+}$ vs PPC^{Gad2+}, P=0.779; Wilcoxon signed rank-sum tests), there were distinct input preferences for some brain areas between the APC and PPC tracing groups. For instance, compared with the PPC, the APC receives a higher proportion of inputs from the OLF (77.44%±0.96% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 68.74%±1.43%, P=0.0005; 82.93%±1.54% for APC^{Gad2+} vs PPC^{Gad2+}, 57.07%±4.17%, P=0.0006; Student's t-tests), but a lower proportion of inputs from the HPF (2.00%±0.43% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 8.37%±1.38%, P=0.0013; 0.89%±0.29% for APC^{Gad2+} vs PPC^{Gad2+}, 7.24%±1.74%, P=0.0018; Student's t-tests) and CNU (4.07%±0.34% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 8.44%±0.50%, P<0.0001; 5.80%±0.85% for APC^{Gad2+} vs PPC^{Gad2+}, 15.81%±1.79%, P=0.0022; Student's t-tests) (**Figure 2B**). To further compare the detailed input features among the four tracing groups, the input proportions from subdivided brain nucleus were quantified and analyzed. We found that, for both two cell types, the MOB, PC, AON and Endopriform nucleus (EP) contributed over 5% input proportions and made up the top four input sources to the APC; while the top four inputs to the PPC came from the MOB, PC, EP and RHP (**Figure 2D**). Thus next, we mainly focused on the detailed subdivision-specific analysis in tracing groups using Vglut2-cre mice.

Bilateral Innervation from the OLF to the PC

The OLF contributed bilateral innervation to both APC and PPC, but the RV labeled neurons distributed more densely in the ipsilateral OLF, including the MOB, AOB, AON, PC, TT, NLOT and COA, et al. (**Figures 3, 4A**). Among these brain areas, the PC, AON, MOB and TT made up the top four input sources to both APC and PPC (**Figures 4A**, **B**). Specially, both AON (**Figures 4A**,**B**; 28.55%±2.25% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 10.12%±1.33%; P<0.0001, Student's t-tests), MOB (**Figures 4A**,**B**; 24.68%±1.37% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 17.20%±1.01%; P=0.0013, Student's t-tests) and TT (**Figures 4A**,**B**; 8.45%±1.62% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 3.10%±0.32%; P=0.0089, Student's t-tests) contributed more abundantly with a higher proportion of inputs to the APC compared to the PPC, as well as the AOB (**Figures 4A**,**B**; 2.41%±0.64% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 0.28%±0.16%; P=0.0094, Student's t-tests). In addition, there was no subdivision preference and the AP axis distribution preference for the AON and TT (**Figures 4C**, **E**). The significant inputs from the

MOB, AOB and AON to the APC might suggest a strong link between the APC and peripheral olfactory inputs. By contrast, the PPC received fewer inputs from most olfactory subareas compared to the APC (**Figure 4A**), except the strong inputs from the ipsilateral APC (**Figure 4B**; $PPC^{Vglut2+}$, 64.97%±1.34%). The APC and PPC connected closely with distinct laminar distribution, that the APC was innervated by the PPC neurons mainly arising from layer II/III (**Figures 4D**; layer II, 62.15%; layer III, 35.44%), while the PPC was innervated by the APC neurons mainly arising from layer II (**Figures 4D**; layer II, 86.26%; layer III, 9.73%). It should be noted that, the NLOT and COA, which belong to the olfactory amygdala, also innervated the PC (**Figures 4A**,**B**), and specially, the COA inputs showed obvious spatial distribution differences between the APC and PPC tracing groups as the posteromedial part of the COA (COApm) contributed higher proportion of inputs to the PPC than to the APC (**Figures 4C**,**E**;, 5.06%±2.39% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 28.63%±7.73%; P=0.0155, Student's t-tests).

Both the APC and PPC received commissural inputs from the contralateral hemisphere, and especially, the majority of commissural inputs to both APC and PPC arose from the contralateral OLF (, 95.04%±1.42% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 81.01%±3.94%), with only sparse input neurons found in the contralateral isocortex and AMY, suggesting a possible role of PC in integrating bilateral olfactory information. In the contralateral OLF, the RV labeled neurons were mainly distributed in several specific olfactory subareas, including the AON, PC and NLOT (**Figures 3**, **5A**). Significantly, compared with the PPC, the APC received much higher commissural inputs from the contralateral hemisphere, especially from the contralateral AON, which contributed dominant commissural inputs to the APC (86.63%±1.66% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 9.09%±3.94%) (**Figures 5A**, **B**). Besides, in the $APC^{Vglut2+}$ tracing group, the input strength as well as the subdivision distribution pattern of RV labeled neurons in the contralateral AON were similar to that in the ipsilateral AON (**Figures 5C**,**D**), suggesting that the APC was heavily innervated by bilateral AONs unbiasedly and might play an important role in binasal odor information integration. Besides, the contralateral PC and NLOT contributed fewer commissural inputs to either the APC or PPC (**Figure 5A**). The RV labeled neurons mainly arose from the layer II of the contralateral PC and NLOT with obvious ipsilateral preference in most cases, except the PPC which seemed to receive a higher proportion of the contralateral NLOT inputs than the ipsilateral NLOT inputs (**Figure 5E**, **C**; contra-/ipsi-inputs radio: 0.21±0.03 for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 2.73±0.76; P=0.0074, Student's t-tests). It should be noted that, although in both APC and PPC tracing groups, the commissural inputs from the contralateral PC showed predominantly rostral distribution along the AP axes (**Figures 5E**,**G**), and there were still some differences in the distribution patterns. In the APC tracing group, the RV labeled commissural inputs from the contralateral PC were observed particularly in the rostral part of the APC (rAPC), and scarcely in the PPC (**Figures 5E**, **G**); whereas in the PPC tracing group, the RV labeled commissural inputs from the contralateral PC were distributed both in the rAPC and caudal part of the APC (cAPC) without any obvious difference, 1but sparsely in the PPC (**Figures 5E**, **G**).

Innervation from the isocortex to the PC

Besides the olfactory inputs, the inputs from the isocortex have been identified. In both APC and PPC tracing groups, the RV labeled neurons were mainly found in the orbitofrontal cortex (ORB), agranular insular area (AI), somatomotor area (MO) and perirhinal area (PERI) (Figure 6A). All these isocortex subareas innervated the APC and PPC with similar AP axis distribution (**Figure 6C**), but distinct input strength and subdivision distribution (**Figures 6A**, **B**). The major distinctions between the APC and PPC tracing groups were that, the APC seemed to connect more closely with the isocortex, since the APC received strong and preferred innervation from the ORB

(37.88%±1.84% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 1.34%±0.85%; P<0.0001, Student's t-tests), as well as the MO (11.82%±2.30% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 1.75%±0.43%; P=0.0016, Student's t-tests) (**Figures 6A**,**B**). We also noted that the AI innervated unbiasedly to the APC and PPC (34.47%±3.02% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 49.19%±4.34%; P=0.0934, Student's t-tests), and the input proportion from the PERI was slightly higher in the PPC tracing group (5.02%±1.45% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 15.58%±3.69%; P=0.0211, Student's t-tests) (**Figure 6B**).

Innervation from the HPF to the PC

For both APC and PPC tracing groups, the RV labeled neurons were found in the HPF, especially in the ventral hippocampus (vHIP) and the lateral part of the entorhinal cortex (LEC) of the RHP (**Figure 7A**). The APC and PPC were innervated by the vHIP as well as the LEC with similar strength, layer and AP axis distribution (**Figures 7A**, **C**). But the inputs from vHIP were clearly skewed toward the PPC (**Figure 7B**; 11.81%±4.86% for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 30.04%±2.03%; P=0.0061, Student's t-tests), suggesting that animals' emotional or memory states might play a more dominant role in the neural activities of the PPC.

Innervation from the cerebral nuclei to the PC

In the CNU, the RV labeled neurons were mainly found in the striatum-like amygdala (sAMY), especially the anterior amygdala area (AAA) and medial amygdala nucleus (MEA); and the ventral / medial parts of the PAL (PALv / PALm), specifically the substantia innominata (SI), magnocellular nucleus (MA) and medial septal complex (MSC) (**Figure 8A**). Both the AAA, MEA, MA, MSC and SI showed unbiased innervation to APC and PPC (**Figures 8A**, **B**), and the distribution pattern were similar along the AP axes between the APC and PPC tracing groups (**Figure 8C**).

DISCUSSION

The study reported here was undertaken in order to determine the whole-brain monosynaptic inputs to two main types of neurons in different PC subdivisions. Our results are consistent with many previous tracing studies using traditional tracers, but we revealed cell-type specific inputs to the APC and PPC, and quantitatively compared the input proportions. Our results show that both types of neurons in the APC and PPC integrate extensive inputs from numerous discrete brain areas across the whole brain. In addition, the input patterns are similar for different PC cell types, but they are diverse for different PC subregions. The most prominent differences between the different PC subregions are that the APC received preferential innervation from the OLF and isocortex, while the PPC received preferential innervation from the HPF.

Cell-type-specific Inputs to the PC

The PC comprises glutamate releasing principal neurons and GABA-releasing interneurons. Previous electrophysiology studies demonstrated that, both principal neurons and interneurons in the PC may show consistent excitatory or inhibitory responses to receptor-specific pharmacologic stimuli or pathway-specific photogenetic stimuli (Luna and Morozov, 2012; Sadrian and Wilson, 2015; Tseng and Haberly, 1989). For instance, activating the PPC projecting BLA neurons can induce excitatory postsynaptic currents (EPSC) on both principal neurons and interneurons in the PC (Luna and Morozov, 2012), suggesting that both principal neurons and interneurons in the PC may receive excitatory inputs from the BLA. In our studies, we found that, in both APC and PPC, the excitatory

Vglut2+ neurons and inhibitory Gad2+ neurons share almost similar input sources, signifying that inputs to the PC may target both excitatory and inhibitory PC neurons. The diversity of cellular targets in the PC may contribute to complex effects on information encoding. For instance, it has been reported that activating the MOB or LOT induces rapid excitation and short time delay feedforward inhibition on the PC principal cells, with the feedforward inhibition shaping the stimulus receptive fields of the PC (Large et al., 2016; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2012). However, there is still no clear consensus on how these two types of neurons in the PC are connected by their concurrent inputs. In addition, we also found that the excitatory Vglut2+ neurons and inhibitory Gad2+ neurons in both the APC and PPC share approximately similar input in proportion from most input sources. This is similar to many tracing results from other brain areas, that showed different types of neurons within the same brain regions and received approximately similar inputs from the whole-brain areas (Ahrlund-Richter et al., 2019; Cai et al., 2019; Zhang et al., 2017). It should be noted that different subtypes of PC neurons may be distinct in their cell morphology, layer distribution, neural circuit and neural response characteristics (Diodato et al., 2016; Large et al., 2016; Suzuki and Bekkers, 2006, 2011). In our studies, we were just concerned with the input connectivity of two types of PC neurons, the excitatory Vglut2+ neurons and inhibitory Gad2+ neurons, however, it still needs to be determined if all types of PC neurons share similar input patterns, although different PC subdivisions show distinct features in input patterns.

Input Patterns to Distinct Subdivisions of the PC

The PC is one key cortical region in the brain responsible for olfactory information processing. Our results revealed that the olfactory system contributes dominant inputs to both the APC and PPC, regardless of excitatory or inhibitory neurons. The MOB and AON are two main olfactory inputs to the PC, and also key nodes in the bottom-up olfactory information transfer process (Shipley and Ennis, 1996). Our results showed that, for both types of neurons, the APC receives stronger innervation from both the MOB and AON compared to the PPC, suggesting that the APC may be innervated more heavily by peripheral olfactory inputs. Our results are consistent with previous tracing studies using traditional tracers, for instance, mitral/tufted cells in the MOB send denser axons to the APC than to the PPC (Igarashi et al., 2012), and the APC was innervated heavily by the AON (Kowianski et al., 1999). Similar conclusions were also drawn in some electrophysiology studies, for instance, it has been established that the neurons in the APC show more robust odor responses and are increased in the phase of respiration than the neurons in the PPC (Litaudon et al., 2003). In addition, we also note that, the APC and PPC are heavily innervated by each other, especially the PPC receives dominant olfactory inputs from the APC and more limbic inputs, implying that the PPC may receive more associational inputs. A previous study demonstrated that by using the GABA(B) receptor agonist to attenuate PC associational inputs, pattern separation of within-category odors is interfered with in the PPC (Bao et al., 2016), meaning that the neural activities in the PC, especially the PPC, may strongly be affected by their associational connections. Our tracing results together with those of previous studies indicate that the APC may be inclined to integrate olfactory gestalts from the AON to generate odor perception and is more sensitive to predator or food-related odors (Morrow et al., 2000).It also receives heavy direct peripheral olfactory inputs from the MOB and AOB; whereas the PPC may be more suitable to encode highly integrated and plastic olfactory information, as it strongly depends on the associational network. Besides, it is noteworthy that, although the PC is traditionally defined as a part of the main olfactory pathway, our results showed that the PC receives a considerable amount of inputs from the AOB and COApm, which are the major parts of the accessory olfactory system. The anatomical connection between the AOB and PC showed that the AOB sends sparse axons to the APC (Gutierrez-Castellanos et al., 2014; Kang et al., 2011), thus the APC could respond to some pheromone odorants (Pfaus et al.,

2009; Schneider et al., 2016). We extend on the findings of previous studies that the APC receives more AOB inputs, while the PPC receives more COApm inputs. Our findings provide an anatomical basis that may help elucidate the different roles of APC and PPC in processing vomeronasal information. The main and accessory olfactory systems are believed to function complementarily when they respond to some chemical stimuli. The convergence of olfactory and vomeronasal information in the PC may therefore, help to compose a complete map of the chemical environment and play an important role in the mating and survival for animals (Martinez-Garcia et al., 2009; Martinez-Ricos et al., 2008; Xu et al., 2005).

The PC is not only an information integrator of peripheral olfactory inputs, but also a central node in a larger cognitive network involving cortical network and limbic connections. Consistent with previous axon tracing studies (Illig, 2005; Majak et al., 2004), our results showed that the cortical and limbic inputs innervate differently on the two PC subdivisions, as some subareas of the isocortex prefer to innervate the APC, whereas the limbic system prefers to innervate the PPC. The cortical inputs to the APC mainly arise from the ORB, a high order associative cortex integrating multimodal sensory information (Gottfried and Dolan, 2003), which is involved in learning and represents information about behavior significance and the associated contextual cue (Bowman et al., 2012; Howard and Gottfried, 2014). The innervation from the ORB to the APC has been reported to play a role in promoting information encoding about odor values or nonolfactory contextual cues in olfactory associated behaviors, and modulating odor response properties of APC neurons (Roesch et al., 2007; Schoenbaum and Eichenbaum, 1995; Strauch and Manahan-Vaughan, 2018; Zinyuk et al., 2001). Besides the direct cortical connections, the PC also connects with cortical areas indirectly through the TH, especially the mediodorsal thalamic nucleus (MTN). Similar to cortical inputs, the MTN, which is believed to modulate and coordinate activities in the primary sensory system and high order cortical areas (Courtiol et al., 2019; Mease et al., 2016), also innervated more heavily to the APC than the PPC. It was speculated that the preferential cortical and thalamocortical innervation to the APC may help in forming and recalling associations between odor stimuli, contextual cues, and behavioral outcomes, and multisensory information converging in the APC may facilitate the preprocessing and generating of expectations of incoming olfactory information. In contrast, the limbic areas, including the LEC, ventral Hip and AMY, innervated more heavily to the PPC than the APC. The limbic system has been implicated in a variety of emotional, cognitive and memory processes. For instance, the LEC is involved in olfactory discrimination learning and olfactory related associative multimodal memory integration (Chapuis et al., 2013); while the AMY is thought to encode innate and learned odor values and odor intensity, especially that associated to fear and anxiety (Anderson et al., 2003; Sadrian and Wilson, 2015). Both the LEC and AMY have been proved to modulate odor coding in the PC (Anderson et al., 2003; Chapuis et al., 2013; Mouly and Di Scala, 2006; Sadrian and Wilson, 2015), especially since the ventral HIP innervated strongly to the AON, and has been found to modulate olfactory sensitivity (Aqrabawi et al., 2016). However, the innervation from the ventral HIP to the PC has rarely been mentioned in previous studies, perhaps this is due to the low infection efficiency of the traditional tracers and the difficulty to distinguish the axon terminal with pass-by fibers in axons tracing studies. It is worth to note that, the LEC, ventral HIP and AMY are all known to be susceptible to seizures (Bui et al., 2018; Mohapel et al., 1996; Vismer et al., 2015), and they all connect closely with the PPC, implying that the PPC may be one of the key nodes for seizure spreading (Vismer et al., 2015). Combining the findings of previous studies and our tracing results, it could be speculated that the preference for limbic areas innervation to the PPC may provide a route by which the animals' emotional states guide the information processing and memory formation in the PPC.

In addition, the PC also receives a variety of neuromodulatory innervation, including cholinergic, noradrenergic, dopaminergic, and serotonergic inputs, etc. Consistent with previous tracing studies using traditional tracers (Haberly and Price, 1978; Kowianski et al., 1999), our studies showed that both the APC and PPC were innervated heavily by the BF, but weakly by the LC, DR and VTA. Together with a previous immunochemistry study which reported that most of the PC-projecting neurons in the BF are choline acetyltransferase positive (Woolf et al., 1984), we concluded that the cholinergic system is the main source of neuromodulatory inputs to both the APC and PPC. It has been revealed that the cholinergic system modulates neural excitability and synaptic plasticity of the PC in a state-dependent manner (Barkai and Hasselmo, 1997; Chapuis and Wilson, 2013), high arousal or attention enhance acetylcholine release (Hasselmo and McGaughy, 2004), while disruption of cholinergic activity in the PC impairs odor discrimination and associative memory (Fletcher and Wilson, 2002; Wirth et al., 2000). Except for the cholinergic inputs, the noradrenergic, dopaminergic and serotonergic systems also play a nonnegligible function in shaping information processing and synaptic plasticity in PC (Bouret and Sara, 2002; Fletcher and Chen, 2010; Narla et al., 2015). For instance, the serotonergic system is implicated in a variety of olfactory functions including olfactory associative conditioning and short-term memory (Fletcher and Chen, 2010). Consistent with previous axon tracing studies using traditional tracers (Datiche et al., 1995; De Olmos and Heimer, 1980), we found that the APC receives obviously more DR inputs compared to the PPC (data not shown). Although the role that the serotonergic system plays in olfactory processing within the PC is not well known, it is possible that the serotonergic neuromodulation may be implicated in enhancing the signal-to-noise ratio of odor inputs in the APC (Fletcher and Chen, 2010), because a previous electrophysiology study reported that activation of DR serotonin neurons may inhibit spontaneous activities in the APC, but not influence the odor induced response (Lottem et al., 2016).

Contralateral Inputs to the PC

The PC is a bilateral structure with a strong reciprocal interconnection via the anterior commissure (Martin-Lopez et al., 2018). Another previous electrophysiology study showed that the APC responds to odors presented to either the ipsilateral or contralateral nostril (Wilson, 1997). In our study, we found that both the APC and PPC receive commissural inputs mainly from the contralateral olfactory areas, implying that the PC may integrate olfactory information from bilateral hemispheres. In accordance with previous axons tracing studies (Haberly and Price, 1978), our results revealed that, compared with the PPC, the APC receives more commissural inputs, especially from the contralateral AON, which is believed to generate olfactory gestalts (Brunjes et al., 2005; Shipley and Ennis, 1996), suggesting a role of the APC in odor identity information integration from bilateral hemispheres. In fact, many previous behavioral studies have showed that olfactory information could be shared between the two hemispheres in some innate odor-driven behaviors such as odor habitation, and simple behavior tasks, such as odor associated preference and coarse odor discrimination task (Kucharski and Hall, 1987, 1988; Mainland et al., 2002; Yan et al., 2008), but not in fine odor discrimination task (Feng and Zhou, 2019). This could be due to the coarse odor discrimination or odor identification relying more on the highly commissural APC network, while the fine odor discrimination may be depending more on the highly associative but less commissural PPC network. Besides the contra-AON inputs, we also noted that both the APC and PPC receive heavy commissural inputs from the contralateral PC, although the distribution patterns were different. Our results indicated that the APC is innervated heavily by the control-APC, especially the rostral part; by contrast, the PPC receives commissural inputs from the whole PC, although the contra-PPC inputs was much weaker than the contra-APC inputs. The APC not only encode odor perception, but also encodes odor associated values or context, for it is heavily innervated by the isocortex. The heavy commissural inputs from the control-APC to ipsi-PC may show that not only the odor identity

information, but also the odor associated value or context information could be exchanged between the bilateral hemispheres. Therefore, we speculated that bilateral olfactory information integration in the PC may be crucial for animals to precisely discriminate or localize the odors (Esquivelzeta Rabell et al., 2017; Kucharski and Hall, 1988; Rajan et al., 2006; Yan et al., 2008). Furthermore, the rostral part of the APC is considered as a seizure susceptible area (Piredda and Gale, 1985), thus, the close connections between the bilateral APCs may play a role in seizure spreading.

In summary, the whole-brain monosynaptic inputs to excitatory and inhibitory neurons in different PC subregions were mapped in this study. Although the input patterns were similar for different cell types, they were diverse for different PC subregions. The findings revealed that the PC integrates extensive inputs from numerous discrete brain areas, and the APC and PPC were innervated differently by the olfactory areas, cortex and limbic areas, which may provide new insights for further study into the diverse functions of the PC.

Figure Legends

Figure1. Experimental procedures for cell-type-specific retrograde monosynaptic tracing of different PC subregions. (A) Recombinant AAV strains and rabies virus. (B) Experimental design. (C, D) Representative images of coronal brain sections containing the injection sites and the magnifications of the starter cells in the Vglut2-cre mice (C, $APC^{Vglut2+}$ tracing group; D, $PPC^{Vglut2+}$ tracing group). The starter cells were indicated by co-expressing the TVA-GFP and EnvA-dsRed. Scale bar: 200 μm.

Figure 2. Input patterns to glutamatergic and GABAergic neurons of different PC subdivisions. (A) Quantified distribution of RV labeled neurons along the AP axes. (B) Quantified distribution of RV labeled neurons in eight major brain divisions. (C) Input proportions of eight major divisions in the four tracing groups were ranked and shown. (D) Quantified distribution of RV labeled neurons in twenty detailed brain subareas. The brain subareas with averaged input proportions greater than 1.0% were selected and illustrated here.

Figure 3. Example images showing inputs from bilateral OLFs to the Vglut2+ neurons of different PC subdivisions. (A, B) RV labeled neurons distributed in the MOB, AON, PC, TT and NLOT of ipsilateral OLF, and AON, PC and NLOT of contralateral OLF (A, $APC^{Vglut2+}$ tracing group; B, $PPC^{Vglut2+}$ tracing group). Scale bar: 500 μm.

Figure 4. Distribution patterns of inputs from ipsilateral OLF. (A) Normalized inputs from different ipsilateral OLF subareas. (B) Quantified distribution of input neurons in different ipsilateral OLF subareas. (C-E) Distribution pattern of input neurons in the ipsilateral AON (C), TT (D) and COA (E). (F) Laminar distribution of RV labeled neurons in ipsilateral TT, PC, NLOT and COA. (G) Quantified distribution of input neurons along the AP axes in the ipsilateral MOB, AON, TT, NLOT and COA.

Figure 5. Distribution patterns of inputs from contralateral OLF. (A) Normalized inputs from different contralateral OLF subareas. (B) Quantified distribution of input neurons in different contralateral olfactory OLF subareas. (C) Contralateral-ipsilateral input ratio of the APC and PPC. (D) Distribution pattern of input neurons from bilateral AONs in the $APC^{Vglut2+}$ tracing group. (E) Distribution pattern of input neurons from the contralateral PC. (F) Laminar distribution of input neurons in the contralateral PC. (G) Distribution pattern of input neurons along the AP axes in the contralateral AON, PC and NLOT.

Figure 6. Distribution patterns of inputs from the isocortex. (A) Normalized inputs of different isocortex subareas. (B) Quantified distribution of input neurons in different isocortex subareas. (C) Distribution pattern of input neurons along the AP axes in different isocortex subareas.

Figure 7. Distribution patterns of inputs from the HPF. (A) Normalized inputs of different HPF subareas. (B) Quantified distribution of input neurons in different HPF subareas. (C) Distribution pattern of input neurons along the AP axes in different HPF subareas.

Figure 8. Distribution patterns of inputs from the CNU. (A) Normalized inputs of different CNU subareas. (B) Quantified distribution of input neurons in different CNU subareas. (C) Distribution pattern of input neurons along the AP axes in different CNU subareas.

AUTHOR CONTRIBUTIONS

LW, ZZ, FX designed the experiments. LW and JC performed experiments, LW analyzed the data. LW, ZZ, QL, FX and AM contributed to manuscript writing. LW generated the figures. All authors declare no competing interests in experimental data.

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