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

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17 **Keywords: anterior piriform cortex, posterior piriform cortex, monosynaptic inputs,**
18 **glutamatergic neurons, GABAergic neurons.**

19 **ABSTRACT**

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

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

37

38 INTRODUCTION

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

49 Previous studies revealed that the PC receives highly converged inputs from distributed glomeruli
50 of the main olfactory bulb (MOB) (Vicente and Mainen, 2011), and further synthesizes these odor
51 features into configural odor objects with the help of abundant association fibers within it (Haberly,
52 2001; Wilson and Sullivan, 2011). Besides olfactory inputs, the PC also receives extensive inputs
53 from cortical and limbic regions (Haberly and Price, 1978; Illig, 2005; Kowianski et al., 1999; Majak
54 et al., 2004). Through these connections, the PC can integrate multisensory, emotional and memorial
55 information (Courtiol and Wilson, 2017; Wilson and Sullivan, 2011). In addition, the PC neural
56 activities are also regulated by neuromodulatory axons originating from the cholinergic neurons in
57 the horizontal limb of the diagonal band (HDB) (Fletcher and Chen, 2010; Wirth et al., 2000), the
58 noradrenergic neurons in the locus coeruleus (LC) (Bouret and Sara, 2002; Fletcher and Chen, 2010),
59 the serotonergic neurons in the dorsal raphe nucleus (DR) (Fletcher and Chen, 2010; Narla et al.,
60 2015), and the dopaminergic neurons in the ventral tegmental area (VTA) (Loscher and Ebert, 1996;
61 Shipley and Ennis, 1996). Although the anatomical and physiological evidence revealed some basic
62 connectivity features and information processing mechanism of the PC, the comprehensive neural
63 circuit foundation for functional diversities of the PC remain poorly understood.

64 The PC is a trilaminar paleocortex that is usually divided into anterior (APC) and posterior (PPC)
65 parts along the AP axis. The borderline is defined by the disappearance of the lateral olfactory tract
66 (LOT) and the thickened layer III in the PPC (Loscher and Ebert, 1996). APC and PPC play different
67 roles in olfactory processing including odor response and learning (Calu et al., 2007; Gottfried et al.,
68 2006; Kadohisa and Wilson, 2006; Litaudon et al., 2003). For instance, the APC encodes odor
69 identity and anticipation, and can be activated not only by odor stimuli but also by odor associated
70 values or contextual cues (Gottfried et al., 2006; Kadohisa and Wilson, 2006; Roesch et al., 2007;
71 Zinyuk et al., 2001); whereas the PPC seems to encode more associated information for it to be
72 activated in tasks that require encoding of odor similarity or odor quality (Bao et al., 2016; Calu et
73 al., 2007; Grau-Perales et al., 2019; Howard et al., 2009; Kadohisa and Wilson, 2006; Zelano et al.,
74 2011). In addition, accumulating evidence from research has also revealed distinct susceptibilities of
75 different PC subdivisions to seizure generation (Ekstrand et al., 2001; Loscher and Ebert, 1996;
76 Vismar et al., 2015; Yang et al., 2006). Moreover, the PC comprises glutamatergic principal neurons
77 and γ -aminobutyric acid (GABA)-ergic interneurons. In brief, glutamatergic principal neurons are
78 mainly located in layer II/III in the PC (Suzuki and Bekkers, 2011); GABAergic interneurons, which

79 serve to provide synaptic inhibition of principal neurons and shape stimulus receptive fields, scatter
80 more uniformly across all layers (Large et al., 2016; Luna and Schoppa, 2008; Suzuki and Bekkers,
81 2007, 2012). Since the synaptic inhibition of principal neurons is distinct between APC and PPC
82 partly because of GABAergic neurons distributed asymmetrically along the AP axis (Loscher et al.,
83 1998; Luna and Pettit, 2010), it reveals the neural connections to specific types of neurons within
84 different PC subdivisions essential to shedding light on the functional diversities and dysfunctions of
85 the PC.

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

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

101

102 **MATERIALS AND METHODS**

103 **Animals**

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

113 **Virus Injections**

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

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

128 **Slice Preparation and Imaging**

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

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

138 **Cell Counting and Data Analysis**

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

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

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

152

153 **RESULTS**

154 **Monosynaptic Inputs to Glutamatergic and GABAergic Neurons in Different PC Subregions**

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

158 type of neuron (**Figures 1A,B**). The starter cells, identified by the co-expression of TVA-GFP and
 159 EnvA-dsRed, were observed near the injection sites of the PC subregions (**Figures 1C, D**). For each
 160 tracing group, the starter cells were highly restricted to the specific PC subregion (APC^{Vglut2+},
 161 97.79%±0.98%; PPC^{Vglut2+}, 99.00%±0.38%; APC^{Gad2+}, 100%±0%; PPC^{Gad2+}, 88.66%±4.86%). To
 162 examine the specificity of the tracing study, the same viruses were injected in the APC of the wild-
 163 type mice (C57BL/6 mice). Despite a very limited number of EnvA-dsRed positive cells near the
 164 injection sites within the APC, no RV retrograde labeled neuron outside the injection sites was
 165 detected (**Supplementary Figure 1**). These data suggested a high specificity of cre-dependent trans-
 166 synaptic property of our viral tracing approach.

167 When we quantified the whole-brain connections to the APC and the PPC, the results showed that
 168 the excitatory and inhibitory neurons in both PC subregions receive extensive inputs from many brain
 169 regions along the AP axis (**Figure 2A**), including the OLF, isocortex, HFP, cortical subplate
 170 (CTXsp), cerebral nuclei (CNU), interbrain (IB), midbrain (MB) and hindbrain (HB) (**Figures**
 171 **2B,C**). To analyze the input weights from each brain region, RV labeled neuron numbers in eight
 172 major brain regions were quantified and normalized by the total inputs for each brain. For all tracing
 173 groups, the majority input sources were observed in the OLF, followed by the isocortex, HPF,
 174 CTXsp and CNU (consisted of the striatum (STR) and pallidum (PAL)), thalamus (TH) of IB,
 175 whereas the hypothalamus (HY) of IB, MB and HB were either blank or sporadically labeled
 176 (**Figures 2B,C**). Despite similar input patterns from all four tracing groups (**Figure 2C**; APC^{Vglut2+} vs
 177 PPC^{Vglut2+}, P=0.674; APC^{Gad2+} vs PPC^{Gad2+}, P=0.161; APC^{Vglut2+} vs APC^{Gad2+}, P=0.575; PPC^{Vglut2+} vs
 178 PPC^{Gad2+}, P=0.779; Wilcoxon signed rank-sum tests), there were distinct input preferences for some
 179 brain areas between the APC and PPC tracing groups. For instance, compared with the PPC, the APC
 180 receives a higher proportion of inputs from the OLF (77.44%±0.96% for APC^{Vglut2+} vs PPC^{Vglut2+},
 181 68.74%±1.43%, P=0.0005; 82.93%±1.54% for APC^{Gad2+} vs PPC^{Gad2+}, 57.07%±4.17%, P=0.0006;
 182 Student's t-tests), but a lower proportion of inputs from the HPF (2.00%±0.43% for APC^{Vglut2+} vs
 183 PPC^{Vglut2+}, 8.37%±1.38%, P=0.0013; 0.89%±0.29% for APC^{Gad2+} vs PPC^{Gad2+}, 7.24%±1.74%,
 184 P=0.0018; Student's t-tests) and CNU (4.07%±0.34% for APC^{Vglut2+} vs PPC^{Vglut2+}, 8.44%±0.50%,
 185 P<0.0001; 5.80%±0.85% for APC^{Gad2+} vs PPC^{Gad2+}, 15.81%±1.79%, P=0.0022; Student's t-tests)
 186 (**Figure 2B**). To further compare the detailed input features among the four tracing groups, the input
 187 proportions from subdivided brain nucleus were quantified and analyzed. We found that, for both two
 188 cell types, the MOB, PC, AON and Endopriform nucleus (EP) contributed over 5% input proportions
 189 and made up the top four input sources to the APC; while the top four inputs to the PPC came from
 190 the MOB, PC, EP and RHP (**Figure 2D**). Thus next, we mainly focused on the detailed subdivision-
 191 specific analysis in tracing groups using Vglut2-cre mice.

192 **Bilateral Innervation from the OLF to the PC**

193 The OLF contributed bilateral innervation to both APC and PPC, but the RV labeled neurons
 194 distributed more densely in the ipsilateral OLF, including the MOB, AOB, AON, PC, TT, NLOT and
 195 COA, et al. (**Figures 3, 4A**). Among these brain areas, the PC, AON, MOB and TT made up the top
 196 four input sources to both APC and PPC (**Figures 4A, B**). Specially, both AON (**Figures 4A,B**;
 197 28.55%±2.25% for APC^{Vglut2+} vs PPC^{Vglut2+}, 10.12%±1.33%; P<0.0001, Student's t-tests), MOB
 198 (**Figures 4A,B**; 24.68%±1.37% for APC^{Vglut2+} vs PPC^{Vglut2+}, 17.20%±1.01%; P=0.0013, Student's t-
 199 tests) and TT (**Figures 4A,B**; 8.45%±1.62% for APC^{Vglut2+} vs PPC^{Vglut2+}, 3.10%±0.32%; P=0.0089,
 200 Student's t-tests) contributed more abundantly with a higher proportion of inputs to the APC
 201 compared to the PPC, as well as the AOB (**Figures 4A,B**; 2.41%±0.64% for APC^{Vglut2+} vs PPC^{Vglut2+},
 202 0.28%±0.16%; P=0.0094, Student's t-tests). In addition, there was no subdivision preference and the
 203 AP axis distribution preference for the AON and TT (**Figures 4C, E**). The significant inputs from the

204 MOB, AOB and AON to the APC might suggest a strong link between the APC and peripheral
 205 olfactory inputs. By contrast, the PPC received fewer inputs from most olfactory subareas compared
 206 to the APC (**Figure 4A**), except the strong inputs from the ipsilateral APC (**Figure 4B**; $PPC^{Vglut2+}$,
 207 $64.97\% \pm 1.34\%$). The APC and PPC connected closely with distinct laminar distribution, that the
 208 APC was innervated by the PPC neurons mainly arising from layer II/III (**Figures 4D**; layer II,
 209 62.15% ; layer III, 35.44%), while the PPC was innervated by the APC neurons mainly arising from
 210 layer II (**Figures 4D**; layer II, 86.26% ; layer III, 9.73%). It should be noted that, the NLOT and
 211 COA, which belong to the olfactory amygdala, also innervated the PC (**Figures 4A,B**), and specially,
 212 the COA inputs showed obvious spatial distribution differences between the APC and PPC tracing
 213 groups as the posteromedial part of the COA (COApm) contributed higher proportion of inputs to the
 214 PPC than to the APC (**Figures 4C,E**), $5.06\% \pm 2.39\%$ for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, $28.63\% \pm 7.73\%$;
 215 $P=0.0155$, Student's t-tests).

216 Both the APC and PPC received commissural inputs from the contralateral hemisphere, and
 217 especially, the majority of commissural inputs to both APC and PPC arose from the contralateral
 218 OLF ($95.04\% \pm 1.42\%$ for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, $81.01\% \pm 3.94\%$), with only sparse input neurons
 219 found in the contralateral isocortex and AMY, suggesting a possible role of PC in integrating
 220 bilateral olfactory information. In the contralateral OLF, the RV labeled neurons were mainly
 221 distributed in several specific olfactory subareas, including the AON, PC and NLOT (**Figures 3, 5A**).
 222 Significantly, compared with the PPC, the APC received much higher commissural inputs from the
 223 contralateral hemisphere, especially from the contralateral AON, which contributed dominant
 224 commissural inputs to the APC ($86.63\% \pm 1.66\%$ for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, $9.09\% \pm 3.94\%$)
 225 (**Figures 5A, B**). Besides, in the $APC^{Vglut2+}$ tracing group, the input strength as well as the
 226 subdivision distribution pattern of RV labeled neurons in the contralateral AON were similar to that
 227 in the ipsilateral AON (**Figures 5C,D**), suggesting that the APC was heavily innervated by bilateral
 228 AONs unbiasedly and might play an important role in binasal odor information integration. Besides,
 229 the contralateral PC and NLOT contributed fewer commissural inputs to either the APC or PPC
 230 (**Figure 5A**). The RV labeled neurons mainly arose from the layer II of the contralateral PC and
 231 NLOT with obvious ipsilateral preference in most cases, except the PPC which seemed to receive a
 232 higher proportion of the contralateral NLOT inputs than the ipsilateral NLOT inputs (**Figure 5E, C**;
 233 contra-/ipsi-inputs ratio: 0.21 ± 0.03 for $APC^{Vglut2+}$ vs $PPC^{Vglut2+}$, 2.73 ± 0.76 ; $P=0.0074$, Student's t-
 234 tests). It should be noted that, although in both APC and PPC tracing groups, the commissural inputs
 235 from the contralateral PC showed predominantly rostral distribution along the AP axes (**Figures**
 236 **5E,G**), and there were still some differences in the distribution patterns. In the APC tracing group,
 237 the RV labeled commissural inputs from the contralateral PC were observed particularly in the rostral
 238 part of the APC (rAPC), and scarcely in the PPC (**Figures 5E, G**); whereas in the PPC tracing group,
 239 the RV labeled commissural inputs from the contralateral PC were distributed both in the rAPC and
 240 caudal part of the APC (cAPC) without any obvious difference, but sparsely in the PPC (**Figures**
 241 **5E, G**).

242 **Innervation from the isocortex to the PC**

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

250 (37.88%±1.84% for APC^{Vglut2+} vs PPC^{Vglut2+}, 1.34%±0.85%; P<0.0001, Student's t-tests), as well as
 251 the MO (11.82%±2.30% for APC^{Vglut2+} vs PPC^{Vglut2+}, 1.75%±0.43%; P=0.0016, Student's t-tests)
 252 (**Figures 6A,B**). We also noted that the AI innervated unbiasedly to the APC and PPC
 253 (34.47%±3.02% for APC^{Vglut2+} vs PPC^{Vglut2+}, 49.19%±4.34%; P=0.0934, Student's t-tests), and the
 254 input proportion from the PERI was slightly higher in the PPC tracing group (5.02%±1.45% for
 255 APC^{Vglut2+} vs PPC^{Vglut2+}, 15.58%±3.69%; P=0.0211, Student's t-tests) (**Figure 6B**).

256 **Innervation from the HPF to the PC**

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

264 **Innervation from the cerebral nuclei to the PC**

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

271

272 **DISCUSSION**

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

282 **Cell-type-specific Inputs to the PC**

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

291 Vglut2+ neurons and inhibitory Gad2+ neurons share almost similar input sources, signifying that
 292 inputs to the PC may target both excitatory and inhibitory PC neurons. The diversity of cellular
 293 targets in the PC may contribute to complex effects on information encoding. For instance, it has
 294 been reported that activating the MOB or LOT induces rapid excitation and short time delay
 295 feedforward inhibition on the PC principal cells, with the feedforward inhibition shaping the stimulus
 296 receptive fields of the PC (Large et al., 2016; Stokes and Isaacson, 2010; Suzuki and Bekkers, 2012).
 297 However, there is still no clear consensus on how these two types of neurons in the PC are connected
 298 by their concurrent inputs. In addition, we also found that the excitatory Vglut2+ neurons and
 299 inhibitory Gad2+ neurons in both the APC and PPC share approximately similar input in proportion
 300 from most input sources. This is similar to many tracing results from other brain areas, that showed
 301 different types of neurons within the same brain regions and received approximately similar inputs
 302 from the whole-brain areas (Ahrlund-Richter et al., 2019; Cai et al., 2019; Zhang et al., 2017). It
 303 should be noted that different subtypes of PC neurons may be distinct in their cell morphology, layer
 304 distribution, neural circuit and neural response characteristics (Diodato et al., 2016; Large et al.,
 305 2016; Suzuki and Bekkers, 2006, 2011). In our studies, we were just concerned with the input
 306 connectivity of two types of PC neurons, the excitatory Vglut2+ neurons and inhibitory Gad2+
 307 neurons, however, it still needs to be determined if all types of PC neurons share similar input
 308 patterns, although different PC subdivisions show distinct features in input patterns.

309 **Input Patterns to Distinct Subdivisions of the PC**

310 The PC is one key cortical region in the brain responsible for olfactory information processing. Our
 311 results revealed that the olfactory system contributes dominant inputs to both the APC and PPC,
 312 regardless of excitatory or inhibitory neurons. The MOB and AON are two main olfactory inputs to
 313 the PC, and also key nodes in the bottom-up olfactory information transfer process (Shipley and
 314 Ennis, 1996). Our results showed that, for both types of neurons, the APC receives stronger
 315 innervation from both the MOB and AON compared to the PPC, suggesting that the APC may be
 316 innervated more heavily by peripheral olfactory inputs. Our results are consistent with previous
 317 tracing studies using traditional tracers, for instance, mitral/tufted cells in the MOB send denser
 318 axons to the APC than to the PPC (Igarashi et al., 2012), and the APC was innervated heavily by the
 319 AON (Kowianski et al., 1999). Similar conclusions were also drawn in some electrophysiology
 320 studies, for instance, it has been established that the neurons in the APC show more robust odor
 321 responses and are increased in the phase of respiration than the neurons in the PPC (Litaudon et al.,
 322 2003). In addition, we also note that, the APC and PPC are heavily innervated by each other,
 323 especially the PPC receives dominant olfactory inputs from the APC and more limbic inputs,
 324 implying that the PPC may receive more associational inputs. A previous study demonstrated that by
 325 using the GABA(B) receptor agonist to attenuate PC associational inputs, pattern separation of
 326 within-category odors is interfered with in the PPC (Bao et al., 2016), meaning that the neural
 327 activities in the PC, especially the PPC, may strongly be affected by their associational connections.
 328 Our tracing results together with those of previous studies indicate that the APC may be inclined to
 329 integrate olfactory gestalts from the AON to generate odor perception and is more sensitive to
 330 predator or food-related odors (Morrow et al., 2000). It also receives heavy direct peripheral olfactory
 331 inputs from the MOB and AOB; whereas the PPC may be more suitable to encode highly integrated
 332 and plastic olfactory information, as it strongly depends on the associational network. Besides, it is
 333 noteworthy that, although the PC is traditionally defined as a part of the main olfactory pathway, our
 334 results showed that the PC receives a considerable amount of inputs from the AOB and COApm,
 335 which are the major parts of the accessory olfactory system. The anatomical connection between the
 336 AOB and PC showed that the AOB sends sparse axons to the APC (Gutierrez-Castellanos et al.,
 337 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; Vismar 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 (Vismar 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.

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

407 **Contralateral Inputs to the PC**

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

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

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

443

444 **Figure Legends**

445 **Figure 1.** Experimental procedures for cell-type-specific retrograde monosynaptic tracing of different
 446 PC subregions. (A) Recombinant AAV strains and rabies virus. (B) Experimental design. (C, D)
 447 Representative images of coronal brain sections containing the injection sites and the magnifications
 448 of the starter cells in the Vglut2-cre mice (C, APC^{Vglut2+} tracing group; D, PPC^{Vglut2+} tracing group).
 449 The starter cells were indicated by co-expressing the TVA-GFP and EnvA-dsRed. Scale bar: 200 μ m.

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

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

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

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

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

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

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

482

483 **AUTHOR CONTRIBUTIONS**

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

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