Distinct VIP interneurons in the cingulate cortex encode anxiogenic and social stimuli

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ABSTRACT

A hallmark of higher-order cortical regions is their functional heterogeneity, but it is not well understood how these areas encode such diverse information. The anterior cingulate cortex (ACC), for example, is important in both emotional regulation and social cognition. Previous work shows activation of the ACC to anxiety-related and social stimuli, but it is unknown how subpopulations or microcircuits within the ACC simultaneously encode these distinct stimuli. One type of inhibitory interneuron, which is positive for vasoactive intestinal peptide (VIP), is known to alter the activity

40   of many cells in local cortical microcircuits, but it is unknown whether the activity of VIP cells in

41   the ACC (VIP^{ACC}) encodes anxiety-related or social information. Using in vivo calcium imaging

42   and miniscopes in freely behaving mice to monitor VIP^{ACC} activity, we identified distinct, non-

43   overlapping subpopulations of VIP^{ACC} that preferentially activated to either anxiogenic, anxiolytic,

44   social, or non-social stimuli. We determined that stimulus-selective cells encode the animal's

45   behavioral states and VIP interneuron clusters may co-activate, improving this encoding. Finally,

46   we used trans-synaptic tracing to show that VIP^{ACC} receive widespread inputs from regions

47   implicated in emotional regulation and social cognition. These findings demonstrate not only that

48   the ACC is not homogeneous in its function, but also that there is marked functional heterogeneity

49   even within disinhibitory interneuron populations. This work contributes to our understanding of

50   how the cortex encodes information across diverse contexts and provides insight into the complexity

51   of neural processes involved in anxiety and social behavior.

52

53   **INTRODUCTION**

54

55   Cortical subregions are often implicated in a variety of behavioral functions, but it is not well

56   understood how these areas encode such diverse information. The anterior cingulate cortex (ACC)

57   is necessary for emotional processing and social cognition, but how it encodes stimuli relevant to

58   both processes is unknown (1-6). In humans, ACC activity increases when healthy subjects

59   perform social tasks and is higher in anxiety disorder patients who demonstrate increased symptom

60   severity (7). In rodents and non-human primates, ACC inhibition via chemical lesions or genetic

61   manipulations impairs social behaviors (8-10). In addition, stimulating activity or silencing a

62   cytoskeletal protein in the ACC alters anxiety-like behaviors (11, 12). Recent studies in rodents

63 have provided insight into the ACC's importance in emotional and social behaviors by monitoring

64 either single unit or bulk activity of this region (6, 8, 11, 13-16). However, these data do not parse

65 out the roles of different neural subtypes or microcircuits within the ACC. It remains unknown

66 how neuronal representations of diverse stimuli are embedded within ACC subcircuits.

67   Inhibitory neurons that are positive for vasoactive intestinal peptide (VIP) inhibit other

68 inhibitory cells, thereby driving excitatory pyramidal cell (Pyr) activity (17-20). VIP interneurons,

69 therefore, are in a unique position to modulate local activity (21, 22). They also receive long-range

70 inputs from other brain regions, which may allow them to coordinate the activity of the ACC with

71 other brain regions to respond to diverse stimuli (18, 20, 23). In the hippocampus, VIP interneurons

72 form functional clusters that are differently modulated by behavioral states, but this has not been

73 studied in higher-order cortical regions like the ACC (24). Acetylcholine and serotonin can

74 modulate functionally distinct groups of VIP interneurons and may differentially engage them

75 during diverse behaviors (25-27). Previous work shows that cortical VIP cells exhibit diverse

76 molecular, morphological, and electrophysiological properties. However, it is unknown whether

77 VIP cells have heterogeneous functions in the ACC and whether disinhibitory circuits are involved

78 in ACC processing of diverse stimuli.

79   We used miniscopes to perform in vivo single-cell resolution calcium imaging of VIP

80 interneurons in the ACC (VIP^{ACC}) to investigate their functional heterogeneity while mice

81 performed tasks to assay anxiety-like behaviors, general sociability and social novelty. We

82 identified distinct VIP^{ACC} subgroups that reliably encoded behavioral states by preferentially

83 activating to anxiety-related, social, or non-social stimuli. In addition, we found that averaging

84 selective cell activity made this coding more reliable. These results suggest that the improvement

85 in coding was mediated through co-activation of these inhibitory subpopulations. When the same

86 neurons were monitored across anxiety-related and social tasks, we determined that the majority

87 of VIP^{ACC} that were engaged during these tasks were highly selective, activating to only one

88 specific stimulus. Lastly, using rabies trans-synaptic mapping, we showed that VIP^{ACC} receive

89 inputs from brain regions implicated in emotional regulation and social behavior. Our data show

90 that VIP^{ACC} are functionally heterogeneous and that non-overlapping subgroups of VIP^{ACC} activate

91 preferentially, providing a cellular substrate for encoding different types of stimuli.

92

93 **MATERIALS AND METHODS**

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95 **Animals**

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97 Animals were grouped housed in a 12-hr light/dark schedule vivarium with food and water ad

98 libitum. Experimental mice were male postnatal day 60-120 VIP-Cre mice (Vip-IRES-cre,

99 #010908, The Jackson Laboratory, Bar Harbor, Maine (28)). Stimulus mice for social interaction

100 were littermates (male VIP-Cre) or novel (age matched male CD-1 IGS, strain code: 022, Charles

101 River Laboratories, Wilmington, Massachusetts). All procedures were approved by the

102 Institutional Animal Care and Use Committee (IACUC) at Boston University and practices were

103 consistent with the Guide for the Care and Use of Laboratory Animals and the Animal Welfare

104 Act.

105

106 **Surgeries**

107

108   Surgeries were performed using aseptic surgical techniques with autoclaved instruments. Animals

109   were weighed and anesthesia was induced in a chamber with an isoflurane–oxygen mixture (4%

110   [v/v]). Anesthesia was maintained throughout the procedure via mask inhalation of an isoflurane–

111   oxygen mixture (Henry Schein, Melville, New York, 1-1.5% [v/v]). Animals were kept on a

112   heating pad (T Pump, Gaymar Industries Inc., Orchard Park, New York) for the duration of the

113   surgeries and for 30-min recovery periods before being returned to their home cages. Animals

114   were injected with buprenorphine (3.25 mg/kg; SC, Patterson Veterinary, Greeley, Colorado),

115   meloxicam (5 mg/kg; SC, Covetrus, Dublin, Ohio), and dexamethasone (Henry Schein, 2.5 mg/kg;

116   SC) and the fur on the top of the head was removed. Animals were head-fixed using a stereotax

117   (Kopf Instruments, Tujunga, California). The surgical area was sterilized with 10% povidone-

118   iodine and 70% isopropyl alcohol (CVS, Woonsocket, Rhode Island) and local anesthetic was

119   applied (lidocaine 1% and epinephrine 1:100,000; SC, Henry Schein). After surgeries, post-

120   operative analgesics were administered for 2 days, twice per day (buprenorphine 0.01 mg/kg; SC

121   and meloxicam 5 mg/kg; SC).

122   Viral injections: After the preparations above, an incision was made in the skin along the

123   midline of the skull. A craniotomy was made over the injection site using a pneumatic dental drill

124   (eBay, Inc., San Jose, California). Using a stereotax and the Nanoject II (Drummond Scientific,

125   Broomall, Pennsylvania), a pulled-glass pipette (BF150-117-10; tip size ~3-15 μm, Sutter

126   Instrument Co., Novato, California) was lowered into the ACC (AP: +0.90 mm, ML: -0.30 mm,

127   DV: -1.00 mm) and virus was injected. After pipette removal, the skin was sutured with non-

128   absorbable sutures (AD Surgical, Sunnyvale, California).

129   To monitor VIP^{ACC} activity, we injected 460 nl of an adeno-associated virus (AAV) that

130   expresses GCaMP6f, a fluorescent Ca^{2+} indicator, in a Cre-dependent manner (AAV9-CAG-Flex-

131   GCaMP6f.SV40, titer: 5.23 x 10^{13} GC/ml, packaged by the University of Pennsylvania Vector

132   Core, Philadelphia, Pennsylvania).

133   For trans-synaptic tracing of monosynaptic inputs to VIP^{ACC}, we first injected 128 nl of a

134   helper AAV that expresses target proteins under the human synapsin-1 promoter: AAV2/1-synP-

135   Flex-splitTVA-EGFP-B19G (AAV-TVA-Glyco) (29) (titer: 0.98 x 10^{12} GC/ml, University of

136   North Carolina Viral Core, Chapel Hill, North Carolina). This AAV contained genes to express

137   EGFP, the avian sarcoma/leukosis virus subtype A receptor (TVA, which confers infection

138   capability to rabies virus pseudotyped with the avian sarcoma leucosis virus glycoprotein (EnvA)),

139   and the rabies virus glycoprotein (G) (29, 30) (which is necessary for trans-synaptic transport of

140   glycoprotein gene-deleted (ΔG) rabies virus (RV)) (23). These three genes were in frame and

141   separated by porcine teschovirus self-cleaving 2A elements (29). After allowing the virus to

142   express for one month (31), the skin was re-incised, a new craniotomy was drilled, and 128 nl of

143   ΔG EnvA pseudotyped RV with mCherry was injected (RV*dG*, titer: 1.5 x 10^9 GC/ml, Boston's

144   Children Hospital Viral Core, Boston, Massachusetts). There is no cognate receptor for EnvA in

145   the mouse, so RV*dG* only infects TVA-expressing cells. Together, Glyco, TVA, and RV*dG* allow

146   for retrograde monosynaptic tracing only from Cre-expressing cells. In Cre-negative mice (N = 6),

147   we performed these injections and saw no labeled cells, suggesting a lack of leaky expression (data

148   not shown).

149   <u>GRIN lens implants</u>: To image neuronal activity with a miniaturized microscope

150   (miniscope), a gradient-index (GRIN) lens was implanted in the ACC at least 2 weeks after viral

151   injection (to allow for maximal GCaMP6f expression). After the preparations above, the scalp was

152   re-incised and a 1 mm diameter craniotomy was drilled, centered around the viral injection. Three

153   screws (Fine Science Tools Inc., North Vancouver, Canada) were inserted into the skull and a layer

154   of super glue (cyanoacrylate, Krazy glue, High Point, North Carolina) was applied to ensure the

155   lens and dental cement adhered strongly. Dura over the ACC and a small region of the secondary

156   motor cortex were aspirated using a blunted 18G needle (BD, Franklin Lakes, NJ) coupled to a

157   vacuum line. A GRIN lens (Table S1) attached to a stereotax via custom 3D-printed implant

158   assembly (Fig S1) was lowered into the ACC at a 20° angle (AP: +0.90 mm, ML: -0.12 mm, DV:

159   -0.13 mm), to improve access to the ACC and minimize the risk of puncturing the midline

160   vasculature. Lenses were adhered to the skull with optical glue (Norland Products Inc., Cranbury,

161   NJ) and dental cement (Ortho-Jet™ Liquid, Black, Lang Dental, Wheeling, Illinois). An antibiotic

162   was administered via the water supply (Biomox, 0.75mgl/ml, Henry Schein) for 10 days after

163   surgery.

164

165   **3D-printed miniscopes and baseplating**

166

167   3 weeks after lens implant surgeries (to allow for recovery and optimal GCaMP6f expression),

168   animals were anaesthetized, as described above, and GCaMP6f expression was assessed by

169   imaging the fluorescent signal using miniscopes. When GCaMP6f-positive neurons were visible,

170   baseplates were attached to the skulls with dental cement using a custom 3D printed baseplating

171   assembly (Fig S1E-F). The miniscopes used were modified from two existing designs (Ghosh et

172   al. 2011 (32), Liberti et al. 2017 (33)) to allow it to detach from the baseplate (Fig S1), which made

173   it possible to co-housed the animals without risking miniscope damage. Miniscopes were attached

174   to baseplates at a 15-20 degree angle relative to the midline to align with the GRIN lenses (Fig

175   S1B). Miniscope models are available at https://github.com/CruzMartinLab. Commercially

176   available parts are listed in Table S1. Custom parts were 3D printed (Form 3 Printer, Black Resin

177   FLGPBK03, Formlabs, Somerville, Massachusetts) and assembled in-house (Fig S1).

178

179   **Perfusions and Histology**

180

181   Animals were perfused to visualize viral injections and lens placements. Mice were injected with

182   an overdose of sodium pentobarbital (250 mg/kg; IP, Vortech Pharmaceuticals Ltd., Dearborn,

183   Michigan) and transcardially perfused with phosphate buffered saline (PBS) followed by 4%

184   paraformaldehyde in PBS (PFA). Brains were extracted, stored in PFA for 24 hr at 4°C, and then

185   transferred to a 30% (w/v) sucrose solution for 48 hr at 4°C. Tissue was sectioned at 50-100 μm

186   using a freezing sliding microtome (SM2000, Leica Biosystems, Buffalo Grove, Illinois). Sections

187   were mounted onto slides (Globe Scientific Inc., Mahwah, New Jersey) using Fluoromount-G

188   mounting medium with DAPI (Thermo Fisher Scientific, Waltham, Massachusetts) to visualize

189   nuclei.

190

191   **Imaging**

192

193   Sections were imaged using an upright wide-field microscope (Nikon Eclipse Ni, Nikon

194   Instruments Inc. Melville, New York) controlled by NisElements (Nikon Instruments Inc., 4.20).

195   Images were acquired using a Plan Fluor 4X (NA 0.13) or 10X (NA 0.3) objective with standard

196   Nikon HQ filter cubes for DAPI, EGFP/GCaMP, and mCherry. Images were analyzed as TIFFs in

197   ImageJ (National Institute of Health, Bethesda, Maryland) and compared to the Allen Mouse Brain

198   Atlas (34) (brain-map.org/api/index.html) to identify brain regions.

199

Behavioral assays and in vivo Ca^{2+} imaging

201

202 Before any behavioral testing, mice were handled for 10 min for 3 days to acclimate them to the

203 experimenter. For anxiety-related assays, mice were not exposed to the arenas prior to testing, but

204 for the social task, implanted mice were acclimated to the arena with empty cups for 10 min per

205 day for 2 days prior to the task. This ensured that, during the social behavioral tasks, mice were

206 familiar with the empty cups. In addition, stimulus mice were acclimated to being housed in cups

207 for 10 min on each of these days. Arenas were custom made from acrylic and HDPE (McMaster-

208 Carr, Elmhurst, Illinois) and were cleaned with 70% ethanol between trials and animals. Behavior

209 was recorded (C270 Webcam, Logitech, Lausanne, Switzerland) at 30 frames/s under overhead

210 lighting (20 lux). After miniscopes were attached, animals were given 10 min to rest in an empty

211 chamber before we started the experiments. Ca^{2+} videos were acquired at 20 frames/s using a

212 Miniscope Data Acquisition PCB and Data Acquisition Software ((35)Table S1). We acquired

213 images with a field of view of 720 x 480 pixels (approximately 800 μm × 600 μm). Excitation

214 LED power was adjusted to optimize imaging for each animal with a maximum output of 1 mW.

215 To avoid bleaching GCaMP6f, no trials were longer than 10 min.

216 Elevated zero maze (EZM): To assay anxiety-like behavior, we used the EZM, an elevated

217 circular arena with two open arms and two closed arms (track diameter = 50 cm, track width = 5

218 cm, wall height for closed arms = 40 cm, height of track = 61 cm). Open arms are considered

219 anxiogenic and closed are anxiolytic. Mice were initially placed in the closed arm and recorded

220 for 10 min (36).

221   Social task: The arena contained two mesh wire cups – one on each end of the arena (50 x

222   25 x 30.5 cm length-width-height). On Day 1 (Sociability), one mesh wire cup housed a littermate

223   and the other was empty. On Days 2 and 3 (Social Novelty), one cup housed a littermate and the

224   other housed an entirely novel male CD-1. Each day the experimental mouse was placed in the

225   center of the arena and given 10 min to explore the arena and cups. The side of the arena with each

226   stimulus was randomized.

227   Open field (OF): To assay anxiety-like behavior and locomotion, the OF was used. The

228   center of the arena is considered anxiogenic and the periphery is anxiolytic. Mice were placed in

229   the center of a custom-made acrylic arena ($50 \times 50 \times 30$ cm length-width-height) and allowed to

230   explore for 10 min.

231

232   **Behavioral analysis**

233

234   Behavior was analyzed using DeepLabCut (37), an open-source program that uses machine

235   learning to track body parts in behavioral videos, as described by Comer et al. (38). To assess

236   accuracy, videos labeled by the software were inspected by a trained observer and custom

237   MATLAB (MathWorks, Natick, Massachusetts) scripts were used to verify that DeepLabCut

238   located each body part at least 95% of the total time the animal was tracked. For EZM and OF, we

239   tracked the centroid of the mouse's body to determine velocity, distance traveled, and when the

240   mouse was in each zone of the arena. For the OF, we divided the arena into 25 squares (10 cm x

241   10 cm each) and defined the periphery as the outermost 16 and the remaining 9 as the center. For

242   the social tasks, we tracked the head to determine close proximity to either cup. Binary behavior

243 matrices (vectorized behavior) indicating the location of the animal were created from

244 DeepLabCut using custom MATLAB scripts.

245

246 **Ca^{2+} imaging analysis**

247

248 Ca^{2+} imaging data were processed using CaImAn (39) written in Python

249 (https://www.python.org/). Ca^{2+} recordings underwent piecewise rigid motion correction using

250 patches of 48 x 48 pixels with 24 x 24 pixel overlap. Following image stabilization, cell detection

251 and extraction of df/f traces were performed with a merging threshold of activity correlation greater

252 than 0.7 between nearby cells, and a 2.5 minimum threshold for the signal to noise ratio. df/f traces

253 and spatial information were exported and saved in .mat format using SciPy (40). All subsequent

254 analysis was performed using custom MATLAB and Python functions. Raw Ca^{2+} traces were z-

255 scored using the mean baseline df/f and sigma from the entire time series for each trial. Z-scored

256 traces report deltaf/f (df/f) values in units of standard deviation (SD). Due to the different

257 acquisition frequencies of behavioral and Ca^{2+} imaging videos, data were aligned using timestamps

258 from acquisition and custom MATLAB scripts. Gaps in neural data due to dropped frames (less

259 than 1% of the total frames) were filled with averaged z-scored df/f values from surrounding

260 frames. Starts and ends of behavioral epochs were matched to Ca^{2+} data timestamps to isolate

261 neural activity during select behaviors.

262   Calcium activity: For all figures, Ca^{2+} activity refers to the area under the curve. To

263 quantify this value, we used z-scored df/f traces and took their integral using the MATLAB trapz

264 function. For all figures, excluding Fig S2I-J, area under the curve was calculated for 5 s intervals,

265 which was chosen based on average transient length. When calculating the average area under the

266   curve across velocities (Fig S2I-J), the intervals were 1 s. These integral values were calculated

267   for each cell to get the Ca^{2+} activity values. When average Ca^{2+} activity is reported, that refers to

268   the average area under the curve for all cells for each mouse.

269   Single cell ROC analysis: Responses of individual cells during different behavioral

270   conditions were assessed within each behavioral trial using receiver operating characteristic (ROC)

271   analysis, as previously described (41). The ROC curve demonstrates how well a single neuron's

272   activity matches an animal's behavioral state, which can be quantified by calculating the area under

273   the ROC curve (auROC) (41, 42). For each neuron in each behavioral condition, an ROC curve

274   was generated using the true positive rate (TPR) and false positive rate (FPR) values for that cell

275   and behavioral state. TPR and FPR were calculated across multiple binary thresholds applied to z-

276   scored df/f traces of each cell, ranging from the minimum to maximum values of the Ca^{2+} signal.

277   For each threshold, binarized df/f traces were compared to the binary behavioral vectors, which

278   used binary values to indicate an animal's presence or absence in a specific zone of the arena. TPR

279   and FPR were then plotted against each other to create each ROC curve and auROC was calculated.

280   To classify cells as stimulus-selective or neutral, we determined whether the cell's auROC

281   value for a given stimulus was high enough to suggest it preferentially activated to a stimulus. To

282   do this while accounting for any random alignment in our data, we calculated 1000 null values for

283   each cell by applying circular permutations of randomized lengths to the Ca^{2+} data and calculating

284   auROC for each of these randomized versions of the data. Cells were considered selective for a

285   certain stimulus if the auROC of that cell was at least 2 SD greater than the mean of the null

286   distribution (auROC > 97.5th percentile). Otherwise, cells were considered neutral. For Fig 3, to

287   calculate the auROC of "super cells", we averaged the z-scored df/f traces of all selective cells and

288   re-calculated auROC for that averaged data.

289   <u>auROC analysis across tasks</u>: For Fig 4A-D, to determine if the same cells were responsive

290   to stimuli in different tasks, we registered cells across EZM and Sociability using CaImAn (39)

291   (127 registered VIP^{ACC} from all 6 animals). For each cell and behavioral condition, auROC was

292   calculated and cells were considered selective for multiple conditions (as described above) if they

293   were selective for different stimuli across these tasks.

294   <u>auROC analysis validation</u>: To validate the auROC analysis (Fig S5), we looked for

295   consistent activity changes in the cells we identified as selective. We calculated auROC values

296   using the first half of the EZM or Sociability data, rather than the entire dataset, to identify selective

297   cells (Fig S5A). Next, using these classifications of selectivity, we assessed Ca^{2+} activity from the

298   second half of the data under the cell's preferred and non-preferred conditions (Fig S5A). The

299   preferred condition was the one that cell was selective for, whereas non-preferred was the other

300   context or stimulus in that task.

301   <u>Activity heatmap</u>: For Fig S4D-E, the activity heatmap was plotted to visualize the average

302   cell activity in 5x5 pixel spatial bins across the OF arena. Z-scored df/f traces from individual cells

303   were normalized to their maximum value and matched with DeepLabCut centroid coordinates at

304   the closest timestamp.

305

306   **Analysis of rabies tracing data**

307

308   For retrograde mapping experiments, brains were scanned to identify signal from starter and

309   retrogradely labeled input neurons. Cells were quantified using ImageJ. Starter cells were defined

310   as cells that were positive for both GFP (from AAV-TVA-Glyco) and mCherry (from EnvA-ΔG-

311   mCherry). We confirmed with DAPI that starter and retrogradely-labeled cells had a nucleus. Each

312 animal had a different number of starter cells, so to normalize our data, we divided the number of

313 retrogradely-labeled neurons in each region by the number of starter cells for that mouse (Inputs

314 per starter cell). The number and location of labeled neurons in a given region was independently

315 confirmed by 3 trained scientists. After quantifying all cells, input brain regions were divided into

316 quartiles by number of input neurons. Only brain regions in the top two quartiles were graphed

317 and included in Fig 4. We did not include RVdG inputs at the site of the ACC injection since

318 leakage in TVA expression could lead to Cre-independent local labeling (43).

319

320 **Statistical analysis**

321

322 Statistical analyses were performed using Graph Pad Prism 8.0 (GraphPad Software Inc., San

323 Diego, California). For figure preparation, CorelDRAW Graphics Suite X8 (Corel Corporation,

324 Ottawa, Canada) and ImageJ were used. The threshold for significance was set to $\alpha = 0.05$ and

325 *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Data are presented as mean $\pm$ SEM, unless

326 otherwise noted. t-tests and ANOVAs followed by appropriate post tests were used and are

327 specified in the figure legends. For Fig S3A-B, Fig S4K-L, and Fig S6A-D, frequency distributions

328 were fitted with Gaussians and percentages of selective cells are represented in pie charts. For all

329 behavioral experiments, N = 6 implanted mice for Ca^{2+} imaging and N = 5 control mice that

330 underwent no surgeries. For EZM, n = 345 cells, for Sociability, n = 310 cells, for Social Novelty

331 Day 2, n = 350 cells, Day 3, n = 232 cells, and OF, n = 273 cells. For tracing experiments, N = 3

332 mice with n = 691 starter cells and 10107 retrogradely-labeled input cells.

333

334 **RESULTS**

335

Distinct VIP^{ACC} interneurons preferentially encode anxiogenic or anxiolytic contexts

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338   To determine whether VIP^{ACC} are a heterogeneous population, we imaged their activity as mice

339   performed behavioral tasks. To do this, we injected AAV9-flex-GCaMP6f and implanted GRIN

340   lenses into the ACC of VIP-Cre mice (Fig 1A and B). Post-mortem histology showed that

341   injections and lenses were successfully targeted to the ACC (Fig 1B), allowing monitoring of

342   VIP^{ACC} with single-cell resolution during behavioral assays (Fig S2A, Fig 1C). 3D-printed

343   miniscopes that detach from baseplates were adapted from two previous designs (Ghosh et al. 2011

344   (32), Liberti et al. 2017 (33)), assembled in-house, and used for Ca^{2+} imaging (Fig S1).

345   The elevated zero maze (EZM) was used to quantify anxiety-like behavior – closed arms

346   are anxiolytic and open arms are anxiogenic (Fig 1, Fig S2). Implanted mice spent 65% less time

347   in the open arms than in the closed, similar to unimplanted controls (Fig S2E-F and N). We

348   monitored Ca^{2+} dynamics while animals navigated the EZM to determine whether exploration of

349   anxiogenic areas altered VIP^{ACC} activity. We hypothesized that individual neurons would activate

350   preferentially to either anxiolytic or anxiogenic zones. To test this, we isolated Ca^{2+} transients from

351   each VIP^{ACC} and performed ROC analysis ((41)Fig 1D). auROC values quantify how well each

352   cell's activity matches a behavioral state; values near 0.5 are expected for cells that do not encode

353   behavioral states and higher auROCs reflect better encoding of this information. For each neuron,

354   we calculated an auROC for the closed arms and an auROC for the open arms. We identified

355   individual VIP^{ACC} that demonstrated selectively increased activity in one arm type and classified

356   them as open-selective (21%), closed-selective (11%), or neutral (which activated equally in both

357   zones, 68%) (Fig 1C-F, Fig S3). The activity of individual selective cells better encoded anxiolytic

358   states, as compared to neutral cells (Fig 1D). Closed-selective cells showed increased activity

359   while animals were in the closed, as compared to the open arms (Fig 1E, top and G), whereas open-

360   selective cells showed the opposite effect (Fig 1E, bottom and H).

361   To determine whether selective VIP^{ACC} activity rapidly changes in anxiolytic or anxiogenic

362   contexts, we isolated trials when animals transitioned from one arm to the other (Fig 1J) and plotted

363   VIP^{ACC} activity. Heatmaps and Ca^{2+} activity showed robust differences during behavioral

364   transitions (Fig 1K-R). Closed-selective cells preferentially activated soon after animals entered

365   the closed arms (27% increase, Fig 1K and O) or prior to entering the open arms (45% increase,

366   Fig 1L and P). Open-selective cells were preferentially active soon after animals transitioned into

367   the open arms (22% increase, Fig 1M and Q) or before leaving the closed arms (44% increase, Fig

368   1N and R).

369   We verified that this analysis method was reliable by using half of our data to identify

370   selective cells and using those classifications to perform Ca^{2+} activity analysis on the remaining

371   half (Fig S5A). In this second half of the data, activity of selective cells increased when mice were

372   in the preferred context, relative to the non-preferred (23%, Fig S5B). Additionally, the majority

373   of auROC values were near 0.5 for both zones (Fig S3A-B), suggesting that most VIP^{ACC} were

374   neutral and did not preferentially activate to a specific context (Fig 1I, Fig S3C).

375   We next assessed whether these effects were context-specific, or they could also be

376   identified in a second task of anxiety-like behavior. Similar to our findings in the EZM, we found

377   that mice in the open field (OF) avoided the anxiogenic center zone (Fig S2C and D) and we

378   identified zone-specific cells that encoded the animal's behavioral state (Fig S4). In contrast to the

379   OF, in the EZM there was no relationship between the animal's velocity and VIP^{ACC} activity (Fig

380   S2I and J), which suggests that the activity differences in EZM reflect the animal's anxiogenic

381 state, rather than signals related to locomotion. These data suggest that our selectivity

382 classifications accurately reflect the relationship between VIP^{ACC} activity and anxiety-related

383 behaviors.

384   Despite differences in behavior and the activity of individual neurons, there was no

385 difference in the average activity of all VIP^{ACC} as mice explored different zones of the EZM or OF

386 (Fig S2B and G-H), which suggests that VIP^{ACC} do not uniformly activate in anxiogenic or

387 anxiolytic contexts. Additionally, the behavior of implanted, miniscope-mounted animals was

388 compared to control mice and no behavioral differences were found, suggesting that neither

389 surgeries nor implants induced changes in locomotion or anxiety-like behaviors (Fig S2K-N).

390 Overall, these results support our hypothesis that VIP^{ACC} are a heterogeneous population, where

391 subpopulations preferentially activate in either anxiolytic or anxiogenic contexts.

392

393 **Distinct subpopulations of VIP^{ACC} encode social and non-social stimuli**

394

395 We next investigated whether individual VIP^{ACC} were selective for social or non-social stimuli by

396 recording their activity as mice interacted with these stimuli over three days (Fig 2A and D). In

397 Sociability, mice explored a chamber with an empty mesh cup (which they had been acclimated

398 to) and a littermate in a mesh cup; in Social Novelty, the cups housed either a littermate or a novel

399 mouse. Implanted mice spent more time with their littermate than an empty cup (Fig 2B, 2.4 fold-

400 change), and more time with the novel mouse than the littermate (Fig 2C, 1.8 fold change),

401 suggesting an interest in socialization and a preference for novel social stimuli.

402   Using ROC analysis, we identified cup-, littermate-, and novel-mouse-selective cells and

403 demonstrated that they better encode behavior states than neutral cells (Fig 2D-F, Fig S6E-F). For

404   Sociability, cells were classified as cup-selective (15%), littermate-selective (17%), selective for

405   both stimuli (2%), or neutral (Fig 2D-G). For Social Novelty, cells were classified as littermate-

406   selective (11% Day 2, 20% Day 3), novel mouse-selective (24%, 12%), or neutral (64%, 68%, Fig

407   2D-G). Very few cells were selective for both stimuli (Fig 2G, 0-1% of cells, Littermate- and cup-

408   selective or littermate- and novel mouse-selective cells). Littermate-selective cells showed

409   increased activity while animals interacted with the littermate, as compared to the cup (36%

410   increase, Fig 2F, top and H) or novel mouse (33% increase, Fig 2J). Cup-selective VIP^{ACC} were

411   more active when animals interacted with the cup than with the littermate (40% increase, Fig 2F,

412   middle and I). Novel mouse-selective neurons demonstrated increased activity while mice

413   interacted with novel mice than with littermates (36% increase, Fig 2K). Similar to the OF and

414   EZM (Fig 1I, Fig S3 and Fig S4J-M), most cells were classified as neutral and their activity did

415   not change as mice interacted with various stimuli (Fig 2F, bottom and Fig S6A-F). We also

416   verified that this analysis method was reliable as described above (Fig S5A) and the Ca^{2+} activity

417   of selective cells increased when mice interacted with preferred stimuli (preferred vs. non-

418   preferred, 17%) (Fig S5C). Similar to the anxiety-related assays, we found no differences in the

419   average activity of all VIP^{ACC} as mice interacted with the different social or non-social stimuli (Fig

420   S6G-H). This suggests that there is no global change in the VIP^{ACC} activity during interactions

421   with other mice or objects. These data also suggest that, in addition to encoding for anxiogenic

422   and anxiolytic contexts, distinct subgroups of VIP^{ACC} encode for interactions with objects, other

423   mice, and social novelty.

424

425   **Population coding of selective VIP^{ACC} improves behavioral encoding**

426

427   Our analysis thus far demonstrated that individual VIP^{ACC} can reliably encode diverse stimuli. To

428   determine whether subsets of selective VIP^{ACC} encode behavioral states as a population to increase

429   the reliability of their information, we averaged the Ca^{2+} traces from all cells that were selective

430   for a specific stimulus to create "super cells" (Fig 3A-D). This process determines whether

431   selective cells are co-activating or whether they primarily activate asynchronously.

432   Unsynchronized Ca^{2+} transients in averaged traces would cancel each other out, resulting in

433   auROC values similar to individual selective cells. If cells co-activate, however, the auROC values

434   of averaged traces would be significantly higher than individual auROC values. We compared

435   super cell auROC values to those from all VIP^{ACC} (Fig 3A, obtained by averaging the auROC

436   values for all cells) and from the selective cells (obtained by averaging the individual auROC

437   values of selective cells). In the EZM, the auROC values for closed super cells increased by 34%

438   and 12% relative to all or closed-selective VIP^{ACC}, respectively (Fig 3B, E). Similarly, for open

439   super cells, we found increases of 42% and 19% in auROC values relative to all or open-selective

440   VIP^{ACC}, respectively (Fig 3B, F). We found this pattern in other behavioral tasks, as well. In

441   Sociability, littermate super cells exhibited a 24% and 11% increase in auROC values relative to

442   all or littermate-selective VIP^{ACC}, respectively (Fig 3C, G). In Social Novelty, auROC values

443   increased by 27% and 10% relative to all or littermate-selective VIP^{ACC}, respectively (Fig 3D, I).

444   Cup super cell auROCs increased by 29% and 13% relative to all or cup-selective VIP^{ACC},

445   respectively (Fig 3C, H), and novel mouse super cells exhibited 24% and 11% increases in auROC

446   values relative to all or novel-mouse-selective VIP^{ACC}, respectively (Fig 3D, J). Overall, these data

447   support previous findings and demonstrate that averaging the activity of stimulus-selective VIP^{ACC}

448   increases the accuracy of their coding, suggesting that these cells encode information as

449   disinhibitory subclusters.

450

VIP^{ACC} subpopulations are non-overlapping and are recruited during distinct behaviors

452

To determine whether diverse neuronal representations are embedded in particular VIP^{ACC} subpopulations, we monitored the same cells across tasks (Fig 4A and B). By registering our neural data, we identified the same 127 neurons across tasks (out of 655 total neurons) and tracked their activity in both EZM and Sociability (Fig 4A and B). We identified distinct subpopulations of VIP^{ACC} that showed stimulus selectivity in only one task and others that were selectively active in specific zones of each task (Fig 4C). 27% of registered VIP^{ACC} were selective in the EZM and neutral during Sociability (Fig 4C). Similarly, about 22% were selective in Sociability, but neutral in the EZM (Fig 4C). Out of all registered VIP^{ACC}, 17% were only open-selective, 9% only closed-selective, 16% only littermate-selective, and 6% only cup-selective. Taken together, these data show that about half of all registered VIP^{ACC} were only selective for one of these 4 stimuli, whereas only one sixth were selective for 2 or 3 stimuli (e.g., both when the mouse was in the open arm of the EZM and when it interacted with the littermate in Sociability) (Fig 4C). This demonstrates that, on average, more cells were selective for one stimulus than for two or three (Fig 4C-D). Unlike in the previous data without registration, where the majority of cells were neutral, only 36% of cells were neutral in both tasks (Fig 4C). These data suggest that, within the ACC, there are non-overlapping VIP interneuron subcircuits dedicated to processing particular stimuli.

469

Whole-brain mapping of inputs to VIP^{ACC}

471

472   To determine whether VIP^{ACC} receive projections from other brain areas involved in anxiety and

473   social behavior, we used rabies virus-mediated trans-synaptic mapping. This technique allowed us

474   to retrogradely label only neurons that synapse onto VIP^{ACC} (Fig 4E-J). First, we injected AAV-

475   TVA-Glyco-GFP, which expresses target proteins in a Cre-dependent manner, in VIP-Cre mice to

476   target VIP^{ACC}. Four weeks later, we injected RVdG, such that starter neurons expressed both EGFP

477   (pseudo colored magenta, also expressing TVA and ΔG) and mCherry (pseudo colored yellow,

478   infected by RVdG) and input cells only expressed mCherry (Fig 4E and F). We identified these

479   labeled cells, quantified their numbers in each brain region (Fig 4E and F), and normalized input

480   cells to starters. We found retrogradely-labeled neurons were most prominent in other regions of

481   the PFC (about 18% of all labeled regions), primary and associative areas (prim/asso), thalamic

482   nuclei, and the medial septal complex (MSC), suggesting that these regions were highly connected

483   to VIP^{ACC} (Fig 4F). We further partitioned the regions with the greatest number of retrogradely-

484   labeled neurons into subregions and determined that VIP^{ACC} received connections from the

485   contralateral (cl) ACC (77% of retrogradely-labeled PFC neurons, Fig 4H), prelimbic cortex (PrL,

486   12% of retrogradely-labeled PFC neurons, Fig 4H), retrosplenial cortex (RS, including RS granular

487   (RSG) and dysgranular (RSD), 86% of retrogradely-labeled prim/asso neurons, Fig 4I),

488   anteromedial thalamic nucleus (AM) (46% of retrogradely-labeled thalamic neurons, Fig 4J), and

489   lateral posterior thalamic nucleus medio rostral part (LPMR, 8% of retrogradely-labeled thalamic

490   neurons, Fig 4J). Our data showed that VIP^{ACC} receive long-range projections, not only from

491   regions implicated in emotional regulation and social cognitive behavior, but also areas important

492   for neuromodulation, memory formation, and motor actions.

493

494   **DISCUSSION**

495

Multimodal disinhibitory circuits in the ACC

497

Like many prefrontal cortical regions, the ACC is implicated in a wide range of behavioral functions. Our work supports past findings by connecting ACC activity with anxiety-related and social behavior and identifies a neural substrate for processing of multimodal stimuli. However, an important facet of our finding is that a diverse range of neuronal representations are encoded in subgroups of VIP^{ACC}, which points to the varied roles of the ACC.

Human imaging studies have shown that ACC activity increases as individuals perform social and emotional tasks, as compared to non-social tasks (44). Interestingly, this previous work demonstrates heterogeneous activation of different subregions within the ACC, where increased activity of the perigenual ACC and rostral ACC is implicated in social tasks, whereas dorsal ACC is selectively activated in non-social cognitive tasks (44). Our histological analysis revealed that lenses were located at the border of the dorsal and ventral subregions of the ACC (A24a and A24b subregions). Future research could monitor VIP cells in different ACC subregions to determine whether the selectivity distributions differ. Additionally, we found subgroups of VIP^{ACC} that encoded interactions with objects, which supports past findings that characterized neural correlates in the ACC for object and location recognition and memory consolidation of object/place associations (14, 15). Our work shows that approximately 70% of VIP^{ACC} were neutral in the anxiogenic and social behavioral tasks. Perhaps this high prevalence of neutral cells reflects the multimodal nature of the ACC, where these cells encode other kinds of stimuli. For example, previous work highlighted the role of the ACC in pain processing (45). Alternatively, cells that were classified as neutral may be equally activated by multiple stimuli. For example, neutral EZM

518　cells may preferentially activate to both anxiogenic and anxiolytic stimuli and be involved in

519　encoding both.

520　　　　When we averaged the activity of stimulus-selective VIP^{ACC}, the reliability of their coding

521　increased across diverse stimuli. Electrical coupling has been observed within inhibitory networks,

522　including VIP interneurons (22, 46). VIP cells can disinhibit members of their own population,

523　leading to increased co-activation of the population, which may allow these subnetworks to encode

524　stimuli as a population and amplify the population output (22).

525

526　**Whole brain mapping of long-range direct inputs to VIP^{ACC}**

527

528　Using rabies trans-synaptic mapping, we provided a brain-wide map of inputs to VIP^{ACC}.

529　Additionally, we demonstrated that VIP^{ACC} receive extensive connections from the AM and RS,

530　which are both known to project to the ACC (47-50). AM lesions are associated with memory

531　impairments (51-53), so AM-ACC projections may be important for social memory. In support of

532　this, mice lacking *Shank3* in the ACC show abnormal behavior in a social novelty task (8).

533　Additionally, memory formation leads to induction of immediate-early genes in the ACC (54). In

534　the auditory cortex, VIP cells facilitate learning about unexpected, salient events (55), suggesting

535　that inhibitory circuits in the ACC could mediate specific aspects of memory formation. Although

536　it remains to be tested, AM-VIP^{ACC} projections are positioned to mediate social learning and

537　memory. In rodents, AM-ACC projections regulate histaminergic itch-induced scratching

538　behavior (56), suggesting that, similar to sensory thalamocortical projections, this higher-order

539　network could be composed of thalamic pathways for parallel processing (31). The RS plays a role

540　in learning, memory, and navigation and is highly connected to the anterior thalamic nuclei and

541   hippocampus (51, 57-59). Spatial information conveyed to VIP^{ACC} from the RS could guide motor

542   actions during social behavior (58, 60). We also showed that VIP^{ACC} are highly connected to other

543   prefrontal cortex subregions and the contralateral ACC. Our results support human imaging studies

544   demonstrating that distinct ACC-PFC networks are involved in diverse aspects of emotional

545   processing (1) and monitor behavior to guide compensatory systems (61).

546   Our injections and lenses were located at the border between the A24a and A24b

547   subregions of the ACC (62, 63). Past research shows that A24 receives projections from the

548   amygdala, orbitofrontal cortex, thalamus, RS, and motor cortex and moderate projections from

549   hippocampus, hypothalamus, and autonomic brain nuclei (63-65). Although our data is mostly

550   consistent with these findings, a major difference is that we used trans-synaptic viral mapping in

551   a cell-type specific manner, while other studies used classic neuronal tracers to map connectivity

552   to all ACC neurons (63-65). Mapping experiments that include inputs to other interneuron

553   subtypes will provide a better framework to understand ACC function and the connectivity patterns

554   of inhibitory circuits of the ACC.

555

556   **Heterogeneous inhibitory subcircuits in the ACC**

557

558   VIP interneurons express a variety of neuromodulator receptors, which may allow long-range

559   projections to exert context-dependent disinhibition (19, 20, 55, 66-69). Our data showed that

560   subgroups of VIP^{ACC} are engaged by particular stimuli. Through the actions of neuromodulators,

561   VIP interneurons in the ACC may recruit subpopulations of Pyr that are behaviorally relevant or

562   encode specific information.

563   We demonstrated that a subgroup of VIP^{ACC} are engaged by social novelty. Locus

564   coeruleus (LC) neurons, the primary source of norepinephrine in the forebrain, are recruited by

565   novel stimuli, so this neuromodulator may be important in social novelty (70-73). Additionally,

566   mice exhibit elevated arousal when exposed to novel or social stimuli, which is abolished upon

567   lesioning of the ACC or LC (74). This suggests that noradrenergic modulation of the ACC may be

568   a substrate for attention and novelty (74). In the cortex, norepinephrine differentially regulates the

569   activity of interneurons (75, 76), but it is unknown whether recruitment of VIP^{ACC} by novel social

570   stimuli is dependent on particular neuromodulators. We show that a small number of VIP^{ACC} are

571   selective to multiple stimuli or contexts. It is possible that through the actions of neuromodulators,

572   distinct groups of VIP^{ACC} could overlap in their activity, allowing for the co-activation of

573   segregated Pyr subgroups (77). This cellular mechanism could enable ACC networks to bind

574   multiple streams of information to guide behavioral actions or monitor optimal performance (60,

575   61, 78). In the barrel cortex, the majority of VIP interneurons are located in layer 2/3, but those in

576   other cortical layers exhibit different dendritic and axonal morphology (79). Due to the large field

577   of view in our Ca^{2+} imaging experiments and variability in lens placement, it was not possible to

578   correlate the activity of VIP to different cortical layers.

579   Using in vivo imaging with cellular resolution in freely moving mice, we showed that

580   VIP^{ACC} are functionally heterogeneous, where distinct subcircuits encode for diverse stimuli. ROC

581   analysis identified stimulus-selective VIP^{ACC} that encoded anxiety-like behaviors or interactions

582   with mice and objects, even though there was no difference in overall VIP^{ACC} activity. Averaging

583   the activity of selective VIP^{ACC} enhanced their ability to encode for specific stimuli. We also

584   determined that most VIP^{ACC} were either selective for only one stimulus or were neutral. Lastly,

585   we used trans-synaptic mapping to provide a map of VIP^{ACC} connectivity and show that VIP^{ACC}

586 receive inputs from regions implicated in emotional regulation, social cognition, and memory

587 formation. To our knowledge, these data provide the first evidence of functional heterogeneity of

588 VIP^{ACC} in vivo and show that population coding of selective VIP^{ACC} may encode stimulus-specific

589 information, which provides a framework for how the ACC encodes information across diverse

590 behavioral states.

591

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593

608

CONTRIBUTIONS

Connor Johnson: Conceptualization: formulated ideas for data analysis (equal), Software: code writing and analysis for behavioral and calcium imaging data, calcium activity analysis with CaImAn (lead). Formal Analysis: analysis of calcium data (equal). Investigation: surgeries, histology, episcope imaging, cell counting (equal). Validation: validation of calcium analysis strategy (lead). Writing – Original Draft: part of methods (support). Writing – Review & Editing: provided edits (equal). Visualization: figure generation (support).

Lisa N. Kretsge: Conceptualization: formulated composition and goals of the paper (equal), Software: behavioral analysis with DeepLabCut (equal). Formal Analysis: histological analysis, analysis of trans-synaptic tracing data, statistical analyses (equal). Investigation: behavioral experiments, histology, episcope imaging (equal), Writing – Original Draft: wrote initial draft (excluding part of methods and discussion) (lead). Writing – Review & Editing: incorporated edits (lead). Visualization: figure generation (equal).

William W. Yen: Methodology: miniscope adaptation design and construction, developed protocols for GRIN lens implant surgeries (lead). Investigation: surgeries and baseplating, behavioral experiments with miniscopes (lead). Writing – Review & Editing: provided edits (support). Visualization: figure generation (support).

Balaji Sriram: Conceptualization: provided ideas for analysis (support). Investigation: set up DeepLabCut with our data (support). Supervision: oversight of some analysis projects (equal).

Jessica C. Jimenez: Methodology: taught our lab to perform surgeries and use miniscopes (equal). Writing – Review & Editing: provided edits (equal).

631 **Tushare J. Jinadasa:** Formal Analysis: quantification of trans-synaptic tracing data (equal).

632 Investigation: surgeries, histology, episcope imaging, cell counting (equal). Writing – Review &

633 Editing: provided edits (support). Visualization: figure generation (support).

634 **Alexandra O'Connor:** Software: code writing and analysis of the output of DeepLabCut data

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636 **Ruichen Sky Liu:** Software: code writing for calcium imaging analysis (support). Writing –

637 Original Draft: part of methods (support). Visualization: activity heatmaps figure generation

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639 **Thanh P. H. Nguyen:** Methodology: miniscope adaptation design and construction (equal).

640 **Eun Seon Cho:** Investigation: annotation for behavioral analysis in DeepLabCut (support).

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643 **Berta Escude Velasco:** Investigation: cell counting (support). Writing – Review & Editing:

644 provided edits (support).

645 **Frances S. Hausmann:** Investigation: perfusions and histology (support).

646 **Alberto Cruz-Martín:** Conceptualization: formulated composition, goals, and scope of the paper

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648 Original Draft. Writing – Review & Editing: editing and feedback throughout (lead).

649 Visualization: figure design and generation (lead). Supervision: mentorship and oversight of the

650 project (lead). Project Administration: management and coordination (lead). Funding acquisition

651 (lead).

652

653 **CONFLICT OF INTEREST**

654

655   The authors declare no conflicts of interest in this work.

656

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850

851   **FIGURE LEGENDS**

852

853 **Figure 1. Distinct VIP^{ACC} interneurons preferentially encode anxiogenic or anxiolytic**

854 **contexts in the EZM.** (**A**) Experimental timeline. (**B**) Image (4x objective) showing location of

855 the GRIN lens and GCaMP6f expression. Blue: DAPI, green: VIP^{ACC} expressing GCaMP6f.

856 Dotted white overlay, brain regions. Yellow arrowheads, GRIN lens location in the ACC. **Left**:

857 left hemisphere. **Right**: zoomed image of yellow boxed region. Scale bar = 1mm (left panel) or

858 400 μm (right panel). (**C**) VIP^{ACC} expressing GCaMP6f (white) in vivo while mouse navigates the

859 open and closed arms of the EZM. Pink circles: open-selective, green circles: closed-selective.

860 Neutral cells are not circled. Scale bar = 100 μm. (**D**) ROC curves demonstrating how well cells

861 encode behavioral states for open-selective (pink, auROC = 0.75), closed-selective (green, auROC

862 = 0.66), and neutral (gray, auROC = 0.51) VIP^{ACC}. (**E**) Ca^{2+} transients: closed-selective (**top**),

863 open-selective (**bottom**). Shaded areas indicate the location of the mouse: open (pink) or closed

864 (green) arms. Scale bar = 25 s and 2 SD. (**F**) 21% of VIP^{ACC} were classified as open-selective,

865 11% as closed-selective, and 68% as neutral. (**G-I**) Ca^{2+} activity of selective VIP^{ACC} per mouse.

866 (**G**) Closed-selective. Closed vs. open. **p=0.0016. (**H**) Open-selective. Open vs. closed.

867 ***p=0.0002. (**I**) Neutral cells in the EZM. Closed vs. open. p=0.5239. (**J**) Movement of a mouse

868 from an open to a closed arm (**left**) or a closed to an open arm (**right**) of the EZM. (**K-L**) Activity

869 of closed-selective cells from 10 s prior to 10 s after entering either a closed (**K**) or an open (**L**)

870 arm. (**M-N**) Activity of open-selective from 10 s prior to 10 s after entering either an open (**M**) or

871 closed (**N**) arm. (**O-R**) Average of selective-cell Ca^{2+} activity per mouse. (**O**) Closed-selective

872 entering closed arm. -5 vs. +5 s. **p=0.0046. (**P**) Closed-selective entering open arm. -5 vs. +5 s.

873 **p=0.0056. (**Q**) Open-selective entering open arm. -5 vs. +5 s. ***p=0.0004. (**R**) Open-selective

874 entering closed arm. -5 vs. +5 s. **p=0.0015. N = 6 mice, n = 345 cells. All statistics performed

875 with Paired t-test. For all figures, Ca^{2+} activity refers to the area under the curve (integral df/f). All

876   ROC curves, traces, and images are representative. Each replicate in G-I and O-R represents one

877   mouse. C: closed arm, O: open arm, Closed-sel: closed selective, Open-sel: open-selective, df/f:

878   Deltaf/f.

879

880   **Figure 2. Distinct subpopulations of VIP^{ACC} encode social and non-social stimuli. (A)**

881   Behavioral paradigm. **Left**: Sociability (Day 1). **Right**: Social Novelty (Days 2/3). Littermate zone

882   (purple). Empty cup zone (pink). Novel mouse zone (orange). Neutral zone (white). (**B**)

883   Sociability. Interaction Time (%). Cup vs. littermate. *p=0.0199. (**C**) Social Novelty. Interaction

884   Time (%). Littermate vs. novel mouse. *p=0.0141. (**D**) Images of VIP^{ACC} expressing GCaMP6f

885   (white) in vivo during Sociability (**left**) or Social Novelty (**right**). Pink circles: cup-selective,

886   purple circles: littermate-selective, orange circles: novel mouse-selective. Neutral cells are not

887   circled. Scale bar = 100 μm. (**E**) ROC curves for littermate-selective (purple, auROC = 0.72),

888   novel-mouse-selective (orange, auROC = 0.67), cup-selective (pink, auROC = 0.69), and neutral

889   (gray, auROC = 0.51) VIP^{ACC}. (**F**) Ca^{2+} transients: littermate-selective (**top**), cup-selective

890   (**middle**), neutral cell (**bottom**). Shaded areas indicate location of mouse: littermate (purple), cup

891   (pink), neutral (white) zones. Scale bars = 25 s and 1 SD. (**G**) In Sociability, 17% of VIP^{ACC} were

892   classified as cup-selective, 15% as littermate-selective, 2% as selective for both cup and littermate,

893   and 66% as neutral. In Social Novelty, VIP^{ACC} were classified as littermate-selective (11% Day 2,

894   20% Day 3), novel-mouse-selective (24% Day 2, 12% Day 3), selective for both littermate and

895   novel (1% Day 2, 0% Day 3), and neutral (64% Day 2, 68% Day 3). (**H-K**) Average Ca^{2+} activity

896   of selective cells per mouse. (**H**) Littermate-selective. Sociability. Littermate vs. cup. *p=0.0132.

897   (**I**) Empty cup-selective. Sociability. Cup vs. littermate. **p=0.0017. (**J**) Littermate-selective.

898   Social Novelty. Littermate vs. novel mouse. ****p<0.0001. (**K**) Novel-mouse-selective. Social

899   Novelty. Littermate vs. novel mouse. ****p<0.0001. N = 6 mice, n = 310 cells for Sociability, n =

900   350 cells for Social Novelty Day 2, and n = 232 cells for Day 3. All ROC curves, traces, and

901   images are representative. Each replicate in B-C and H-K represents one mouse. All statistics

902   performed with Paired t-test. Lit: littermate, Nov: novel mouse, Lit-sel: littermate-selective cell,

903   Cup-sel: cup-selective cell. Social nov: Social Novelty, df/f: Deltaf/f.

904

905   **Figure 3. Population coding of selective VIP^{ACC} improves behavioral encoding.** (**A**) ROC

906   curves of open- (pink solid, auROC = 0.86), closed- (green, auROC = 0.70), littermate- (purple,

907   auROC = 0.74), empty cup- (pink dotted, auROC = 0.70), and novel-mouse-selective (orange,

908   auROC = 0.79) super cells. (**B-D**) Ca^{2+} transients of each type of super cell for the EZM (**B**) and

909   social interaction tasks (**C-D**). Scale bars = 10 s and 1 SD. Shaded areas represent location of the

910   mouse. Closed arm (**B**, green), open arm (**B**, pink), littermate (**C-D**, purple), empty cup (**C**, pink),

911   novel mouse (**D**, orange) or neutral (**C-D**, white) zones. Ca^{2+} signals overlayed. (**E-J**) auROC of

912   all cells (All), selective cells, and super cells (Super) per mouse in the EZM (**E-F**) and social

913   interaction tasks (**G-J**). In each task, selective cells had higher auROC than the whole population

914   and super cells had higher auROC values than selective cells. (**E-F**) This pattern was consistent in

915   the EZM for both closed- (**E**, All vs. closed: ****p<0.0001. All vs. super: ****p<0.0001. Closed

916   vs. super: ****p<0.0001) and open-selective cells (**F**, All vs. open: ***p=0.0009. All vs. super:

917   **p=0.0047. Open vs. super: *p=0.0370). (**G-H**) In Sociability, this pattern was consistent for

918   littermate- (**G,** All vs. littermate: **p=0.0022. All vs. super: **p=0.0011. Littermate vs. super:

919   *p=0.0326) and cup-selective (**H,** All vs. cup: **p=0.0026. All vs. super: ***p=0.0003. Cup vs.

920   super: **p=0.0010). (**I-J**) In Social Novelty, this pattern was consistent for littermate- (**I**, All vs.

921   littermate: ***p=0.0005. All vs. super: ***p=0.0008. Littermate vs. super: **p=0.0073.) and

922   novel-mouse-selective (**J**, All vs. novel: **p=0.0059. All vs. super: ****p<0.0001. Novel vs.

923   super: ***p=0.0007). ****p<0.0001. N = 6 mice, n = 345 cells for EZM, n = 310 cells for

924   Sociability, n = 350 cells for Social Novelty Day 2, n = 232 cells and Day 3. All ROC curves and

925   traces are representative. Each replicate in E-J represents one mouse. All statistics performed with

926   Repeated measures ANOVA with Tukey's post test. Lit: littermate, Soc: social.

927

928   **Figure 4. VIP^{ACC} subpopulations are non-overlapping and are recruited during distinct**

929   **behaviors.** (**A-B**) VIP^{ACC} were registered across tasks. Purple circles: registered cells monitored

930   during both EZM (**A**) and Sociability (**B**). (**A-B**) **Left**: Scale bar = 100 μm. **Right**: zoomed versions

931   of yellow outlined regions in left panels. Scale bar = 40 μm. (**C**) Venn diagram demonstrating cell

932   selectivity in the EZM and Sociability. Each number indicates what percentage of the registered

933   cells belong to each selectivity category. There is a gap between open and closed because these

934   classifications are mutually exclusive – by definition a cell cannot be selective for both. (**D**)

935   Percentage of cells selective for one, two, or three stimuli. Dotted line, one phase decay, half-life

936   of 0.81. (**E**) Image of starter VIP^{ACC} expressing both EGFP (pseudo colored magenta, also

937   expressing TVA and ΔG) and mCherry (pseudo colored yellow, infected by RV*dG)*, and

938   retrogradely-labeled input cells only expressing mCherry. Coronal section. **Left**: overlay of both

939   fluorophores and DAPI (cyan). Scale bar = 50 μm. **Right**: zoomed in superficial layers showing

940   overlap (starter cells, white arrows). Scale bar = 15 μm. (**F**) Images of retrogradely-labeled input

941   neurons (white) in the orbitofrontal (Orb) cortex (Ctx), anterior thalamic nuclei (Am Thal or AM),

942   retrosplenial (RS) cortex, and diagonal band of Broca (Diag Band, part of the medial septal

943   complex, MSC). **Left** panels: left hemisphere. Scale bar = 1.5 mm. **Right** panels: zoomed views

944   of the retrogradely-labeled brain regions (yellow arrowheads in left panel). Scale bar = 100 μm.

945   (**G-J**) Regions that are the most highly connected to VIP^{ACC}. Rabies trans-synaptic mapping

946   revealed that the prefrontal Ctx (PFC), primary and association Ctx (Prim/asso), thalamic regions,

947   and MSC are highly connected to VIP^{ACC}. (**H-J**) Highly connected areas (PFC, **H**, Prim/asso, **I**,

948   thalamic, **J**) divided into subregions. Error bars represent SEM. N = 6 mice for Ca^{2+} imaging, n =

949   345 cells for EZM, n = 310 cells for Sociability. For tracing experiments, N = 3 mice with n = 691

950   starter cells and 10107 input cells. All images are representative. Pref Stim: preferred stimuli,

951   Hypo: hypothalamus, Amyg: amygdala, cl: contralateral, PrL: prelimbic Ctx, VO: ventral orbital

952   Ctx, MO: medial orbital Ctx, LO: lateral orbital Ctx, RSG: restrosplenial granular Ctx, RSD:

953   retrosplenial dysgranular Ctx, V2MM: secondary visual Ctx mediomedial area, S1BF: primary

954   somatosensory Ctx barrel field, MPtA: medial parietal association Ctx, S1: primary somatosensory

955   Ctx, V1: primary visual Ctx, LPMR: lateral posterior thalamic nucleus medio rostral part, PF:

956   parafascicular thalamic nucleus, MDL: mediodorsal thalamic nucleus lateral part, LDVL:

957   laterodorsal thalamic nucleus ventrolateral part, CL: centrolateral thalamic nucleus, VA: ventral

958   anterior thalamic nucleus, AD: anterodorsal thalamic nucleus.

959

960   **AVAILABILITY OF DATA AND MATERIALS**

961

962   Data Availability: All relevant data are within the paper, and underlying data are available at

963   https://github.com/CruzMartinLab. Custom-written routines for behavioral tracking, calcium

964   imaging analysis, and miniscope models and tools are available at

965   https://github.com/CruzMartinLab. For further information, please contact the corresponding

966   author.

967

Figure 1

Figure 2

A Lit / Nov — Sociability / Social novelty — Cup / Lit

B Sociability — Interaction Time (%) — Cup, Lit — *

C Social novelty — Lit, Nov — *

D Sociability / Social novelty — Lit/Cup/Novel

E ROC Curve — TP Rate vs FP Rate — Lit, Cup, Novel, Neutral

F VIP^ACC Activity — Lit-Sel (Sociability) — Cup-Sel (Sociability) — Neutral (Sociability)

G Lit, Cup, Neutral, Novel, Lit/Cup, Lit/Novel

Sociability: 66%, 17%, 15%, 2%

Social novelty: 11%, 24%, 1%, 64%; 20%, 12%, 68%

H Lit (Sociability) — Ca²⁺ activity (integral df/f) — Cup, Lit — *

I Cup (Sociability) — Lit, Cup — **

J Lit (Social nov) — Nov, Lit — ****

K Novel (Social nov) — Lit, Nov — ****

Figure 3

A Super Cells

ROC Curve

TP Rate vs FP Rate

- Open
- Closed
- Lit
- Cup
- Novel

B VIP^ACC Activity

EZM

Closed Super Cell

Open Super Cell

C Sociability

Lit Super Cell

Cup Super Cell

D Social novelty

Lit Super Cell

Novel Super Cell

E Elevated Zero Maze

auROC: All, Closed, Super

F

auROC: All, Open, Super

G Sociability

auROC: All, Lit, Super

H

auROC: All, Cup, Super

I Social Novelty

auROC: All, Lit, Super

J

auROC: All, Novel, Super

Figure 4

A EZM

B Sociability

C

Open — 17.32

Closed — 9.45

Littermate — 15.75

Cup — 6.30

1.57

3.73

0

4.72

0.79

2.36

7.87

D

Cells (%) vs # of Pref Stim

E VIP-ACC

L1

L2/3

EGFP

mCherry

Merge

F Inputs

Orb Ctx

Am Thal

RS Ctx

Diag Band

G

Inputs per starter cell

PFC, Prim / Asso Ctx, Thalamic, MSC, Motor Ctx, Hypo, Amyg, Claustrum

H PFC

Inputs per starter cell

cl ACC, PrL, VO, MO, LO, cl PrL

I Primary and Asso

Inputs per starter cell

RSG, RSD, cl RSG, V2MM, S1BF, MPtA, S1, V1

J Thalamic

Inputs per starter cell

AM, LPMR, PF, MDL, LDVL, CL, VA, AD