Control of innate olfactory valence by segregated cortical amygdala circuits

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SUMMARY

Animals perform innate behaviors that are stereotyped responses to specific evolutionarily relevant stimuli in the absence of prior learning or experience. These behaviors can be reduced to an axis of valence, whereby specific odors evoke approach or avoidance. The cortical amygdala (plCoA) mediates innate attraction and aversion to odor. However, little is known about how this brain area gives rise to behaviors of opposing motivational valence. Here, we sought to define the circuit features of plCoA that give rise to innate olfactory behaviors of valence. We characterized the physiology, gene expression, and projections of this structure, identifying a divergent, topographic organization that selectively controls innate attraction and avoidance to odor. First, we examined odor-evoked responses in these areas and found sparse encoding of odor identity, but not valence. We next considered a topographic organization and found that optogenetic stimulation of the anterior and posterior domains of plCoA elicits attraction and avoidance, respectively, suggesting a functional axis for valence. Using single cell and spatial RNA sequencing, we identified the molecular cell types in plCoA, revealing an anteroposterior gradient in cell types, whereby anterior glutamatergic neurons preferentially express *Slc17a6* and posterior neurons express *Slc17a7*. Activation of these respective cell types recapitulates appetitive and aversive valence behaviors, and chemogenetic inhibition reveals partial necessity for valence responses to innate appetitive or aversive odors. Finally, we identified topographically organized circuits defined by projections, whereby anterior neurons preferentially project to medial amygdala, and posterior neurons preferentially project to nucleus accumbens, which are respectively sufficient and necessary for innate negative and positive olfactory valence. Together, these data advance our understanding of how the olfactory system generates stereotypic, hardwired attraction and avoidance, and supports a model whereby distinct, topographically distributed plCoA populations direct innate olfactory valence responses by signaling to divergent valence-specific targets, linking upstream olfactory identity to downstream valence behaviors, through a population code. This represents a novel circuit motif in which valence encoding is represented not by the firing properties of individual neurons, but by population level identity encoding that is routed through divergent targets to mediate distinct valence.

INTRODUCTION

Innate behaviors are ubiquitous across the animal kingdom, allowing specific sensory stimuli to yield stereotypical behavioral responses even in the absence of learning or past experience. These behaviors include feeding, fighting, fleeing, and mating, among others, and many can be simplified onto an axis of positive or negative valence representing approach and avoidance. Innate behaviors are the result of evolutionary selection, guiding initial behaviors that can be updated by future experiences. Given that innate behaviors are genetically hardwired, it is thought they should be mediated by simple circuits with specified connections between layers of the nervous system. Innate behaviors are common across sensory modalities but are especially prominent in olfaction, whereby diverse chemical signals, critical to survival and reproduction, must be quickly and robustly detected and processed, in the absence of prior experience. For instance, predator odors represent a potentially imminent threat and necessitate a quick, decisive, aversive response.[1] Conversely, innately appetitive odors represent potentially rewarding stimuli like food or heterospecifics, inducing attraction.[2–4] These odors comprise a small subset of perceptible chemical space, and the detection of specific odorants is both species-specific and under genetic control.[5–7]

Valence is a fundamental perceptual feature of olfaction. Motivational valence can be defined as seeking or avoiding specific stimuli, and it is observed across sensory stimuli in both innate and learned responses. Multiple circuit motifs have been proposed to mediate such valence responses.[8] In the simplest form, labeled line motifs segregate information from sensation to action throughout the nervous system. This has been observed in the taste and somatosensory systems, as well as hints of labeled lines in the olfactory system, where individual glomeruli are necessary and sufficient for innate responses consistent with this model.[9–11] Many circuits are organized along divergent path motifs, where a region receives the same sensory input but instead acts akin to a switchboard, processing and sorting its output to distinct downstream targets to convey positive or negative signals. This motif is most associated with the BLA and associated learning processes.[8,12] Still other circuits contain opposing components motifs, in which opposing inputs target a single effector region to control the balance of one target. It remains unclear which, if any, of these generalized circuit motifs are present in the olfactory system.

Olfactory sensation begins with olfactory sensory neurons (OSNs) in the olfactory epithelium (OE) that express a single receptor, projecting to spatially stereotyped glomeruli in the olfactory bulb (OB). Postsynaptic mitral/tufted cells within the OB project in parallel to third-order olfactory areas, including the posterolateral cortical amygdala (plCoA). Unlike in other third-order olfactory areas, such as piriform cortex, projections from individual glomeruli from the OB to plCoA are spatially restricted and stereotyped, consistent with genetically hardwired circuits.[13–16] Past work has demonstrated that plCoA is necessary and sufficient for innate olfactory responses, with spatially ordered labeling of responsive neurons via immediate early gene labeling.[3] However, other work suggests that there is no spatial organization to odor responses or valence encoding in plCoA.[17] Thus, a further investigation of plCoA organization is necessary to understand how this structure controls innate olfactory responses.

73 Distinct brain areas employ distinct coding strategies to represent information. Neuronal
74 ensembles within all other major olfactory regions observed thus far, such as the anterior
75 olfactory nucleus, OB, OE, olfactory tubercle (OT), piriform cortex (PIR), and tenia tecta all
76 generally perform sparse population encoding of odor identity, despite the major differences in
77 neuronal composition, organization, and function between the six regions.[12,17–30] On the other
78 hand, ensembles within amygdala subnuclei, most notably the basolateral amygdala (BLA),
79 instead tend to represent the valence of stimuli instead, with considerable heterogeneity based
80 on a given population's projection target, molecular identity, and topography.[12,18,19] A complete
81 investigation of the encoding properties and organization of plCoA is needed to determine how
82 innate olfactory valence emerges from this region.

83 To identify the plCoA circuitry that underlies innate valence, we investigated multiple
84 intersecting scales of organization, from single cell transcriptomes and spatial gene expression,
85 to circuit mapping, manipulation, and physiology. First, we used 2-photon calcium imaging to
86 find that plCoA ensembles encode odor identity, but not valence. Next, we identify a functional
87 gradient in plCoA where activation of anterior and posterior neurons drives responses of
88 opposing valence. Next, we characterized the cell types within plCoA, identifying novel,
89 molecularly defined populations specific to each domain of plCoA, which are respectively
90 sufficient and partially necessary for innate olfactory valence. Further, we perform
91 comprehensive projection mapping to identify downstream projection targets of plCoA,
92 identifying projections to the medial amygdala (MeA) and nucleus accumbens (NAc) that are
93 enriched based on molecular and topographic identity. Finally, manipulations of neuronal
94 ensembles projecting to these targets are sufficient and necessary to control innate olfactory
95 valence responses. Together, these findings identify a novel topographically distributed circuit
96 from plCoA to MeA and NAc that controls innate olfactory aversion and attraction, respectively,
97 consistent with a hybrid model mixing features of labeled lines and divergent path circuit motifs.

98 **RESULTS**

99 **Population Encoding of Odor Identity in plCoA**

100 To better understand how plCoA circuitry mediates innate olfactory valence, we first
101 decided to examine the relationship between its spatial organization and odor-evoked activity.
102 Prior analysis of immediate early gene expression following minutes-long odor exposure
103 suggests that activity in the anterior and posterior domains of plCoA could respectively mediate
104 innate aversive and appetitive odor responses.[3] However, *in vivo* electrophysiology with high-
105 density electrode arrays found no evidence for spatial organization or valence encoding in
106 plCoA.[17] These two studies propose contradictory models of plCoA encoding properties that are
107 both plausible, given that the former is common in the extended amygdala and insular cortex,
108 and the latter is ubiquitous across olfactory regions.[22,31,32] We speculated that technical
109 differences could be responsible for these opposing findings. Immediate early gene labeling has
110 extremely low temporal resolution and likely requires a high amount of neural activity to activate
111 gene expression. On the other hand, the recording sites in the latter study appear biased
112 towards the middle of plCoA, and odor was given for 2 seconds in interleaved trials, whereas
113 olfactory valence behavior evolves on a minutes-long timescale.[3,33] We wondered if the
114 apparent contradictions in these studies could be resolved by applying a longer odor delivery
115 protocol that both matches the timescale of behavior and balances spatial and temporal
116 resolution.

117 Therefore, we developed an approach to image neural activity in plCoA with a modified
118 odor delivery schedule, expressing GCamP8s targeted towards either the anterior or posterior
119 subsection of plCoA and implanting a gradient-index relay (GRIN) lens above to allow *in vivo*
120 imaging of calcium transients via head-fixed two-photon microscopy (**Figure 1A-C, S1A-B**). We
121 then examined calcium responses in these mice during a long odor exposure, where odors were
122 presented repeatedly in 20-trial blocks for 5 seconds each in counterbalanced order (**Figure**
123 **1D**). We chose two odorants of each innate valence: the appetitive odors 2-phenylethanol (2PE)
124 and peanut oil (PEA), the neutral odors heptanol (HEP) and isoamyl acetate (IAA), and the
125 aversive odors trimethylthiazoline (TMT) and 4-methylthiazoline (4MT).[3,17] In total, we recorded
126 Ca^{2+} signals from 345 neurons across 13 mice.

127 First, we pooled anterior and posterior plCoA neurons together and performed hierarchical
128 clustering of their trial-averaged responses to the 6 odors to categorize odor responses in an
129 unbiased manner (**Figure 1E**). Consistent with the previous *in vivo* electrophysiology study, we
130 found that the majority of plCoA neurons did not seem to selectively respond to odors of one
131 valence group (**Figure 1E-F**). Across mice, a majority of plCoA neurons did not reliably respond
132 to any of the six odors, and activity was sparse: only 34.5% of plCoA neurons responded to 1 of
133 the 6 odors and 10% to 2 odors, while a much smaller portion of plCoA neurons responded to 3
134 or more odors (**Figure 1G**). Further, we found no significant relationship between the valence of
135 odor and proportion of responsive neurons, and no difference in the proportion responsive to the
136 different odors across anterior and posterior plCoA, suggesting a lack of bias in responsiveness
137 to aversive or appetitive odors across the anterior-posterior axis (**Figure 1H, S1C**).

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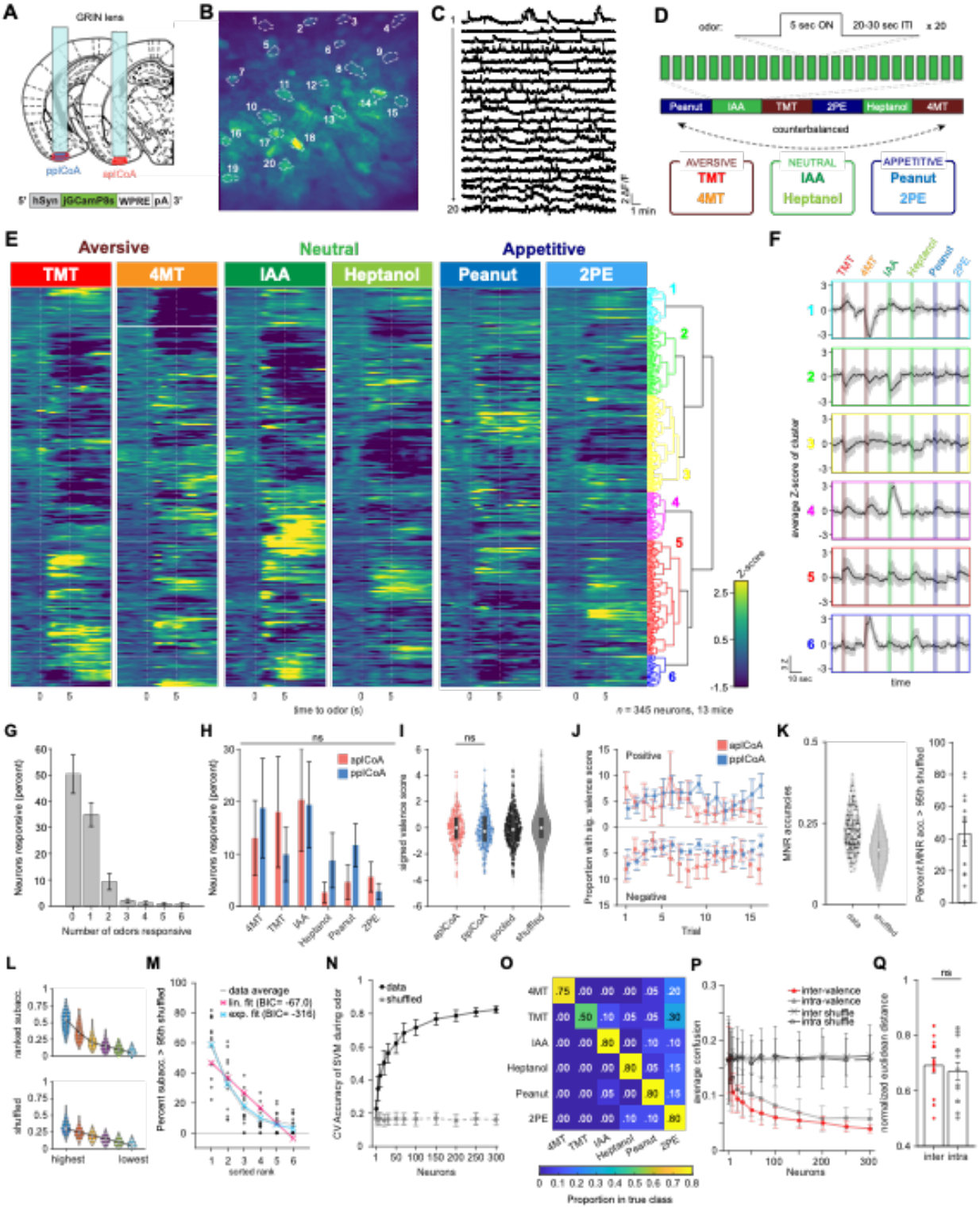
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GRIN lens
ppl CoA
apl CoA
5' hSyn jGCaMP8s WPRE pA 3'
B
C
1 min
D
odor:
5 sec ON
20-30 sec ITI
x 20
Peanut
IAA
TMT
2PE
Heptanol
4MT
counterbalanced
AVERSIVE
TMT
4MT
NEUTRAL
IAA
Heptanol
APPETITIVE
Peanut
2PE
E
Aversive
Neutral
Appetitive
TMT
4MT
IAA
Heptanol
Peanut
2PE
time to odor (s)
n = 345 neurons, 13 mice
Z-score
F
average Z-score of cluster
time
G
Neurons responsive (percent)
Number of odors responsive
H
ns
apl CoA
ppl CoA
Neurons responsive (percent)
4MT
TMT
IAA
Heptanol
Peanut
2PE
I
ns
signed valence score
apl CoA
ppl CoA
pooled
shuffled
J
Positive
Negative
apl CoA
ppl CoA
Proportion with sig. valence score
Trial
K
MNR accuracies
data
shuffled
Percent MNR acc. > 95th shuffled
L
ranked subacc.
shuffled
highest
lowest
M
data average
lin. fit (BIC= -67.0)
exp. fit (BIC= -316)
Percent subacc. > 95th shuffled
sorted rank
N
data
shuffled
CV Accuracy of SVM during odor
Neurons
O
4MT .75 .00 .00 .00 .05 .20
TMT .00 .50 .10 .05 .05 .30
IAA .00 .00 .80 .00 .10 .10
Heptanol .00 .00 .00 .80 .05 .15
Peanut .00 .00 .05 .00 .80 .15
2PE .00 .00 .00 .10 .10 .80
Proportion in true class
P
inter-valence
intra-valence
inter shuffle
intra shuffle
average confusion
Neurons
Q
ns
normalized euclidean distance
inter
intra

139

Figure 1. The plCoA encodes innately-valenced odor identity using a population code.

(A) Schematic representation of virus injection and GRIN lens implantation into aplCoA or pplCoA for two-photon microscopy.
(B-C) Representative images (**B**) and traces (**C**) of fluorescence changes in individual neurons over an approximately 20-minute period.
(D) Schematic of odor exposure paradigm. Each trial presented 5 seconds of odor followed by a variable inter-trial interval (20-30s). Odors were present in blocks of 20 trials per odor, with 2 counterbalanced block schedules (1 & 2). Six odors were used: the appetitive odors 2-phenylethanol (2PE) and peanut oil (PEA), the neutral odors heptanol (HEP) and isoamyl acetate (IAA), and the aversive odors trimethylthiazoline (TMT) and 4-methylthiazoline (4MT).
(E) Heatmap of trial-averaged and Z-scored odor-evoked activity over time from pooled plCoA neurons. Responses are grouped by hierarchical clustering, with the dendrogram (right). Odor delivery marked by vertical red lines.
(F) Average of trial-averaged and Z-scored odor-evoked activity for each cluster concatenated. The order of color-coded blocks corresponds to the order of clusters in (**E**).
(G) Proportion of neurons responsive to different numbers of odors. Bars represent the mean across 13 animals and the error bars show SEM.
(H) Proportion responsive to each odor for aplCoA (red) or pplCoA (blue).
(I) Valence scores of individual neurons. White circles show the median of each distribution, whereas the gray rectangle shows the 25th-75th percentile range.
(J) Proportion of neurons with significant valence scores calculated as a function of trial number. Calculated with a 10-trial moving window. Top half shows those with significant positive valence scores, the bottom half shows those with significant negative valence scores.
(K) Left, MNR accuracies for all pooled plCoA neurons (data) and a control distribution where the training labels are shuffled (shuffled) in a violin plot. Right, proportion of neurons in each animal that have MNR accuracy greater than the 95th percentile of the shuffled MNRs.
(**L**) Ranked sub-accuracies for single-neuron MNR classifiers in violin plots for those trained on real (top) or shuffled (bottom) data. Black lines connect the median across each rank.
(**M**) Scatter plot of percentage of sub-accuracies above the 95th percentile of the shuffled controls for each animal plotted against the rank of the sub-accuracy. Red and blue lines show the linear and exponential fit, respectively, and a gray dashed line connects data averages.
(**N**) Cross-validated average accuracies of multinomial SVM's plotted as a function of the number of neurons used for training during the odor period. Circles represent the mean across 100 iterations of random sampling of neurons and error bars show the standard deviation.
(**O**) An example confusion matrix for a multinomial SVM trained with 200 neurons.
(P) Comparison of inter-valence and intra-valence confusion across number of neurons used in training the classifiers. Filled circles show the average of the data across 100 iterations, open circles show shuffled controls.
(Q) The normalized average distance between odor pairs that have different valence (inter) or same valence (intra).
Across panels, ns, not significant. Additional specific details of statistical tests can be found in Supplemental Table 1.

We next attempted to quantify valence encoding by calculating a valence score for each neuron by subtracting the average integrated aversive odor response from the average integrated appetitive odor response and dividing this resulting difference in valence by the integrated odor response ($V = \frac{\Sigma_{appetitive} - \Sigma_{aversive}}{\Sigma_{total}}$). Although the valence score was more negatively skewed amongst anterior neurons than posterior ones, there was no significant difference in their distributions, failing to support a model in which anterior and posterior plCoA neurons broadly encode opposing innate olfactory valence (**Figure 1I**). Given that the innate aversive and appetitive behaviors evolve over time in freely moving animals, we wanted to assess if odor responses changed between early and late trials. Using a moving window of 5 trials, we examined if the proportion of neurons with significant aversive or appetitive valence scores differed over time in anterior or posterior plCoA but found no significant interaction between time and imaging location (**Figure 1J**).

We further investigated odor encoding at the single neuron level using multinomial regression (MNR) 6-odor classifiers trained on single neuron data and found that they perform only marginally better than chance, indicating that the majority of single plCoA neurons do not broadly encode discriminatory information about odor identity (**Figure 1K**). Across mice, only 43.1% have overall classification rates above the 95th percentile of shuffled controls, indicating that the majority of single plCoA neurons do not broadly encode discriminatory information about odor identity. Interestingly, when comparing the ranked sub-accuracies for single odors compared to shuffled controls, we found the discrepancy between data and shuffled controls to be accentuated in the 2 highest performing sub-accuracies (**Figure 1L**). Quantifying the proportion of neurons with sub-accuracies higher than the 95th percentile of the shuffled across biological replicates, we found that the decrease of sub-accuracies down the rank falls exponentially, rather than linearly (**Figure 1M**). This, paired with the observation that most plCoA neurons respond to one or none of the six odors tested, indicates that individual plCoA neurons are sparsely responsive and carry little information about identity or valence.

Surprisingly, we saw no evidence of valence encoding at the single neuron level in either the anterior or posterior plCoA. Instead, the plCoA neurons appear to primarily encode odor identity in a sparse manner much like the other higher-order olfactory areas, known to use a population code. Thus, we next considered identity and valence encoding at the population level. In contrast to the poor single neuron classification, SVMs trained on population-level data vastly outperform the shuffled data, indicating good encoding of identity at the population level (**Figure 1N**). We further used a confusion matrix to ask whether training the classifier with a given odor could accurately predict the identity of another odor. In the matrix each column corresponds to a predicted class and each column corresponds to the actual class (**Figure 1O**). If the population level activity is similar between odor pairs, we expect the confusion to be higher than less similar pairs. The output of a confusion matrix yields a proportion of true class labels, whereby a high proportion indicates good prediction. We found no difference between the confusion rates for intra-valence classification or inter-valence classification, suggesting a lack of valence encoding at the population level (**Figure 1P**). The similarity between inter-valence and intra-valence confusion was mirrored when quantifying the range-normalized pairwise Euclidean distance across biological replicates (**Figure 1Q, S1D**). Thus, plCoA

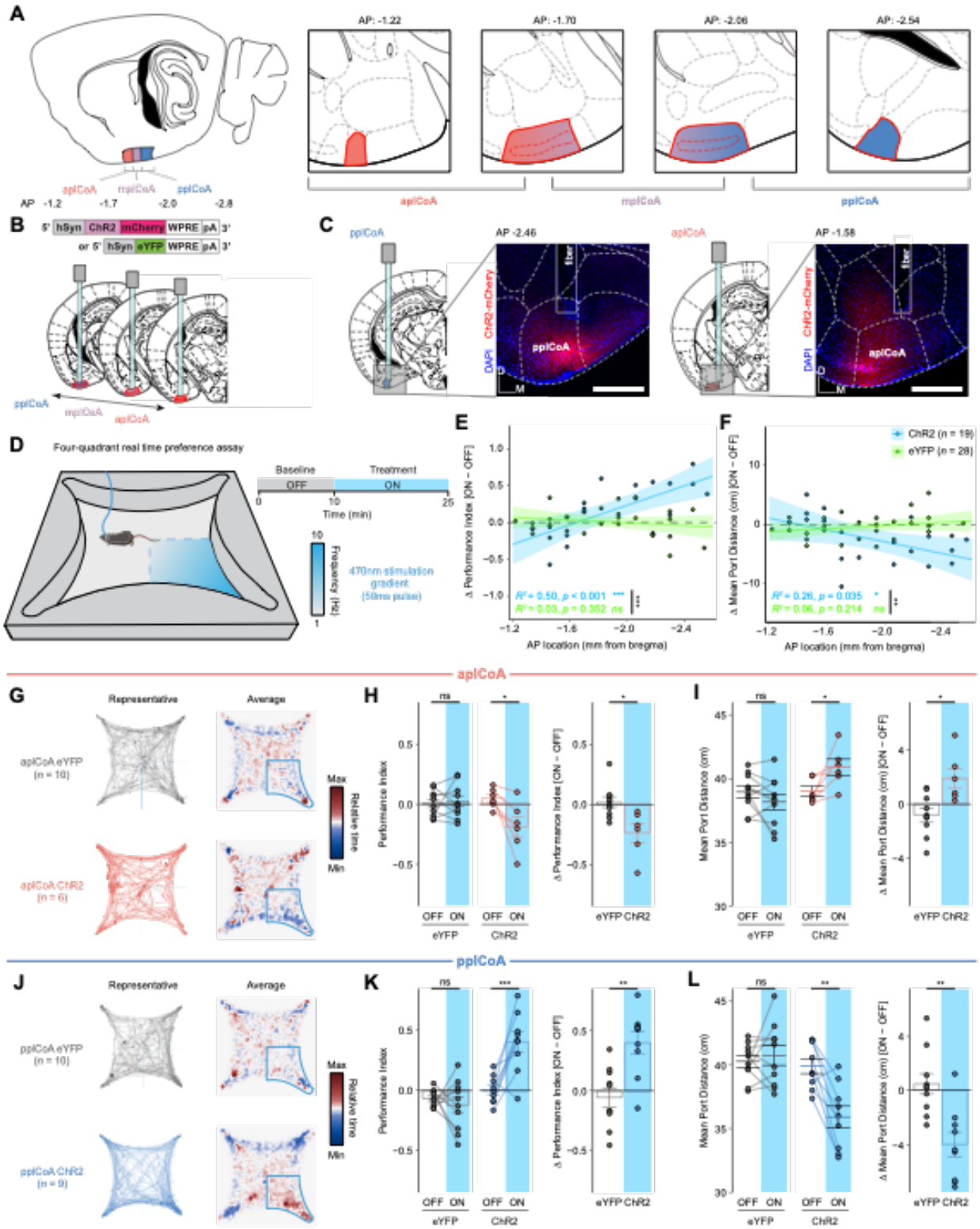
224 appears to encode odor identity in a population code like other higher order olfactory regions,
225 with no apparent encoding of valence.

226 **A Functional Axis for Valence in plCoA**

227 Since we did not observe clear evidence for valence encoding, we considered other
228 organizational principles that could support behaviors of opposing valence. Spatial organization
229 for valence processing has been previously observed in the insular cortex and BLA, whereby
230 discrete subsections of the region contain neurons wired to preferentially signal positive or
231 negative valence.[18,19,34,35] Given plCoA's spatially-ordered afferent projections from OB, we next
232 hypothesized that plCoA circuitry could still be organized along the anterior-posterior (AP) axis
233 to generate innate olfactory valence. If true, it follows that activation of small ensembles of
234 neurons along the AP axis should generate behavioral responses along a corresponding axis of
235 valence. We tested this prediction by expressing channelrhodopsin (ChR2) in subsets of
236 neurons at different positions along this axis and photostimulating them during behavioral
237 testing in the previously established four-quadrant open field arena.[3]

238 Based on cytoarchitecture, we more formally parcellated plCoA into three domains: the
239 anterior plCoA (aplCoA), a two-layered region on the ventral surface lateral to the anterior
240 cortical amygdala, the posterior plCoA (pplCoA), a three-layered region on the ventrolateral
241 surface lateral to the posteromedial cortical amygdala, and a middle transition zone (mplCoA)
242 between them (**Figure 2A**).[3,36] To determine the potential relationship between position on the
243 anterior-posterior axis of plCoA and evoked valence, we performed optogenetic stimulation at
244 points along this entire axis, expressing channelrhodopsin (ChR2) and implanting fibers into
245 each zone (**Figures 2B-C, S2A**). Valence using the four-quadrant open field assay, where mice
246 freely explored a chamber with or without stimulation, and valence was scored with a
247 performance index measuring quadrant occupancy relative to chance, as well as the mean
248 distance to the corner port. Mice experience a 10 minute baseline period followed by 15 minutes
249 of closed loop optogenetic (470nm, increasing up to a maximum of 10 Hz as the mouse
250 proceeds closer to the corner port) stimulation in one quadrant (**Figure 2D**).[3]

251 Throughout the trial period, we observed a negative linear relationship between the
252 anterior-posterior position of the photostimulation site and the evoked change in performance
253 index and distance to the corner port in ChR2, but not eYFP-infected mice, where evoked
254 valence shifted from positive to negative as stimulation became more anterior. (**Figure 2E-F**).
255 We grouped these responses to determine whether these bidirectional responses were specific
256 to the identified plCoA zones. We found that photostimulation in aplCoA significantly reduced
257 time spent in the 'on' quadrant and increased the average distance to the corner port during the
258 treatment period, indicating activation of aplCoA neurons is aversive and leads to avoidance of
259 the quadrant paired with stimulation (**Figure 2G-I**). We also found the opposite was true in
260 pplCoA, where stimulation in that zone instead increased the time in the 'on' quadrant and
261 decreased average distance to the corner port, indicating pplCoA neuron activation instead is
262 appetitive and leads to attraction to the stimulation quadrant (**Figure 2J-L**).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AP: -1.70
AP: -2.06
AP: -2.54
aplCoA
mplCoA
pplCoA
AP -1.2 -1.7 -2.0 -2.8
B
5' hSyn ChR2 mCherry WPRE pA 3'
or 5' hSyn eYFP WPRE pA 3'
C
AP -2.46
AP -1.58
ChR2-mCherry
DAPI
fiber
D
Four-quadrant real time preference assay
Baseline
Treatment
OFF
ON
Time (min)
Frequency (Hz)
470nm stimulation gradient (50ms pulse)
E
Δ Performance Index [ON – OFF]
AP location (mm from bregma)
F
ChR2 (n = 19)
eYFP (n = 28)
Δ Mean Port Distance (cm) [ON – OFF]
AP location (mm from bregma)
aplCoA
G
Representative
Average
aplCoA eYFP (n = 10)
aplCoA ChR2 (n = 6)
Max
Min
Relative time
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Performance Index
Δ Performance Index [ON – OFF]
OFF ON
eYFP
ChR2
I
Mean Port Distance (cm)
Δ Mean Port Distance (cm) [ON – OFF]
pplCoA
J
pplCoA eYFP (n = 10)
pplCoA ChR2 (n = 9)
K
L

263

Fig. 2. The plCoA has topographic organization capable of driving approach and avoidance behaviors.

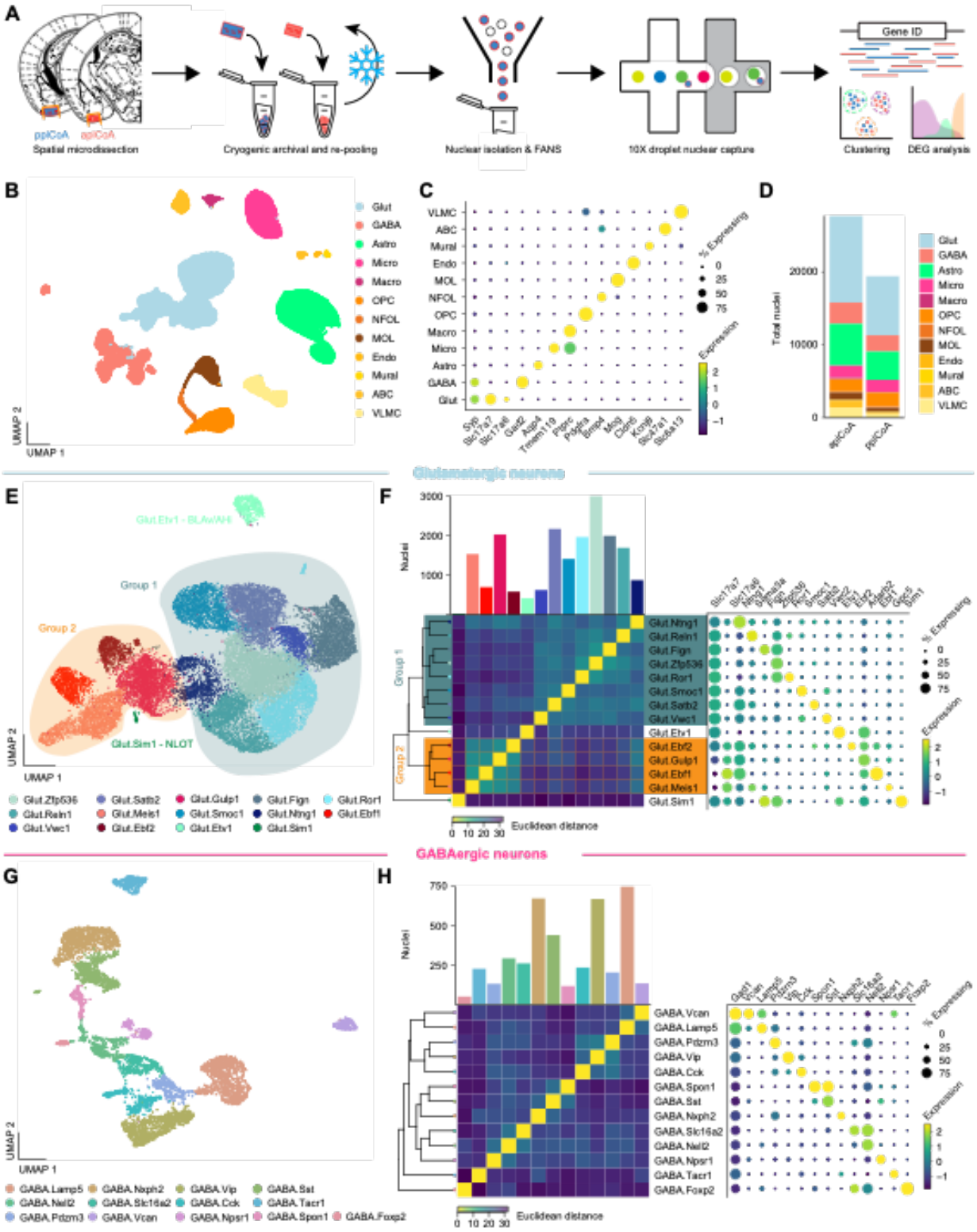
(A) Schematic of plCoA domains divided into anterior (aplCoA), middle (mplCoA), and posterior (pplCoA) regions based on histology, positioning, and gradients observed in past observations.[3,15]
(B) Strategy to activate anterior-posterior topographical ensembles via optogenetics.
(**C**) Representative histology and fiber/virus placement for aplCoA and pplCoA ChR2 animals.
(**D**) Schematic of four-quadrant open field behavioral assay with closed-loop photostimulation.
(E-F) Linear-fit of change in performance index (**E**) or mean port distance (**F**) as a function of anterior-posterior position along plCoA for optical stimulation.
(G) Paths traveled during the stimulus period for a representative mouse (left) and baseline-normalized collective heatmaps (right) from both the ChR2- and eYFP-infected groups with aplCoA-localized fiber implants. Lower right stimulus quadrant indicated in blue.
(H-I) Mean effect of Photostimulation of aplCoA neurons on time spent in stimulated quadrant (performance index) (**K**) and distance from the corner (port distance) (**I**).
(J) Paths traveled during the treatment period for a representative mouse (left) and baseline-normalized collective heatmaps (right) from both the ChR2- and eYFP-infected groups with pplCoA-localized fiber implants. Lower right stimulus quadrant marked in blue.
(**K-L**) Mean effect of photostimulation of pplCoA neurons infected with ChR2, but not eYFP, is sufficient to increase time spent in the stimulation quadrant (**K**) and reduce its average distance from the stimulation port during the stimulation period (**L**).
Abbreviations: aplCoA, anterior zone of posterolateral cortical amygdala; mplCoA, middle zone of posterolateral cortical amygdala; pplCoA, posterior zone of posterolateral cortical amygdala.
Across panels, ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Specific details of statistical tests can be found in Supplemental Table 1.

289 We further examined the effects of anterior-posterior plCoA stimulation on other
290 behaviors to determine whether these effects were specific to valence, or if they extended to
291 other affective or motor phenomena. Using the elevated plus maze, we found no change in
292 anxiety based on open arm time or entries, across both the length of plCoA or within either
293 aplCoA or pplCoA (**Figure S3A-C, S3E-F, S3H-I**). In the open field test, we similarly found no
294 changes to thigmotaxis, based on time spent in corners of the open field, or exploration, based
295 on time spent in the center of the open field (**Figure S3K-M, S3EO-P, S3R-S**). Further,
296 locomotion remained constant during stimulation across both assays and the entirety of plCoA
297 (**Figure S3D, S3G, S3J, S3N, S3Q, S3T**). Together, these data indicate that the effects of
298 plCoA neuron activation across the entire anterior-posterior axis are specific to valence, with
299 few other behavioral effects. Overall, we find activation of plCoA neurons is sufficient to drive
300 valence behaviors in a topographically organized and bidirectional manner, where aplCoA
301 drives aversion and pplCoA drives attraction.

302 **Molecular Diversity of Transcriptomic plCoA Cell Types Along the Anteroposterior Axis**

303 Having identified a functional axis sufficient to produce valence behaviors (**Figure 2**) that
304 does not appear to encode valence (**Figure 1**), we next considered if the plCoA could be
305 topographically organized by molecular cell type to support behaviors of opposing valence.
306 Thus, we sought to determine if there is an axis of molecular cell types along the anteroposterior
307 domains of plCoA. To investigate this phenomenon, we performed single-nucleus RNA
308 sequencing (snRNA-seq) to determine the cell type composition and its relationship to the
309 anterior-posterior axis of plCoA.[37] To simultaneously profile these cell types and identify
310 domain-specific patterns, we separately extracted tissue samples from aplCoA and pplCoA by
311 microdissection, verified accurate dissection by histology, and pooled qualifying samples from
312 the selected plCoA domain for each sequencing run (**Figure 3A, S4A-D**). We also confirmed
313 there were few region- or batch-specific differences in sequencing depth or nuclear quality
314 markers (**Figure S4E-M**). Clustering of sequenced nuclei by gene expression allowed us to
315 initially identify all major canonical neuronal and glial cell types in plCoA based on known
316 marker genes identified in past scRNA-seq studies (**Figure 3B-C**).[38,39] Neurons in plCoA are
317 80% glutamatergic, while 20% of neurons are GABAergic (**Figure 3D**). We also identified large
318 numbers of vascular leptomeningeal cells (VLMCs) and arachnoid barrier cells (ABCs), two
319 fibroblast-like meningeal cell types that interface with vasculature and form a barrier between
320 the brain and CSF, likely due to meningeal presence on the cortical surface during extraction.[39–
321 41]

322 We further characterized the heterogeneity of glutamatergic and GABAergic neurons
323 within plCoA by re-processing and subclustering both major neuronal cell types. Within
324 glutamatergic neurons, we identified 14 distinct subtypes by gene expression, with largely
325 continuous variation between glutamatergic subtypes (**Figure 3E**). However, when examining
326 the relationships between these subtypes, we identified two broader groups of glutamatergic
327 neurons via hierarchical clustering, where subtypes within each group displayed a lower
328 Euclidean distance from one another in high-dimensional gene expression space (**Figure 3F**).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apCoA
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Gene ID
Clustering
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GABA
Astro
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Macro
OPC
NFOL
MOL
Endo
Mural
ABC
VLMC
UMAP 1
UMAP 2
C
% Expressing
Expression
D
Total nuclei
apCoA
ppCoA
Glutamatergic neurons
E
Glut.Etv1 - BLA/AHi
Group 1
Group 2
Glut.Sim1 - NLOT
F
Nuclei
Euclidean distance
GABAergic neurons
G
H

329

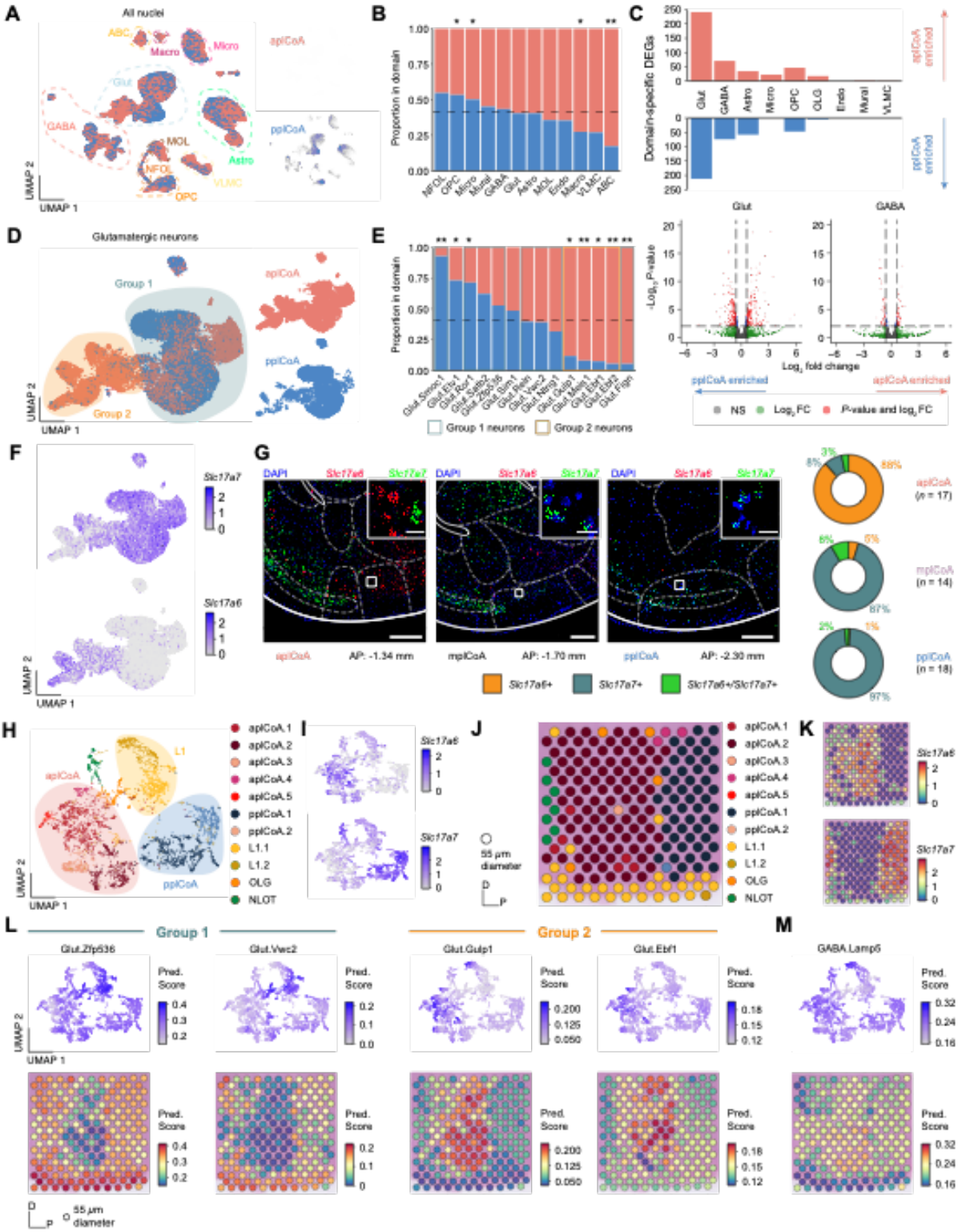
330 **Fig. 3. Transcriptomic heterogeneity of plCoA molecular cell types.**

331 **(A)** Schematic of freeze and-re-pool strategy for snRNA-seq.
332 **(B)** Two-dimensional UMAP (n = 47,132 nuclei, see also **Figure S4.3**), colored by broad cellular
333 identity assigned by graph-based clustering of neuronal and non-neuronal nuclei.
334 (**C**) Cell-type-specific expression of canonical marker genes indicating broad cellular identity in
335 the brain. Dot size is proportional to percentage of nuclei expressing the marker, with color
336 scale representing normalized expression level.
337 (**D**) Total proportion of cells of each identified type in each domain of plCoA.
338 **(E)** Two-dimensional UMAP of glutamatergic neurons, colored by molecular cell type.
339 **(F)** Clustered heatmap showing Euclidean distance between averages of each subtype
340 positioned based on hierarchical clustering (left), and dot plot of marker genes for all
341 glutamatergic subtypes (right).
342 **(G)** Two-dimensional UMAP of GABAergic neurons, colored by molecular cell type, like in (**E**).
343 **(H)** Clustered heatmap showing Euclidean distance between averages of each subtype
344 positioned based on hierarchical clustering (left), and dot plot of marker genes for all GABAergic
345 subtypes (right), like in (**F**).

346 Each of these two broader groups had a marker for every type within either group, where the
347 larger Group 1 of glutamatergic neurons express *Slc17a7 (VGLUT1)*, and the smaller Group 2
348 expresses *Slc17a6 (VGLUT2)*. Within each of these glutamatergic groups, most observed
349 marker genes are non-canonical in the amygdala and cortex, suggesting unique glutamatergic
350 ensembles or patterns of gene expression within glutamatergic neurons in plCoA compared to
351 other regions previously described. Two subtypes did fall outside of either broad glutamatergic
352 group, varying in a more discrete manner than most plCoA glutamatergic neuron subtypes.
353 Interestingly, examination of data from the Allen ISH Atlas for their respective marker genes
354 *Etv1* and *Sim1* showed these two groups fall into adjacent regions outside of plCoA, where
355 Glut.Etv1 neurons localize to the posterior basomedial amygdala and Glut.Sim1 neurons
356 localize to the nucleus of the lateral olfactory tract (**Figure S5A-B**). However, gene expression
357 patterns for GABAergic neurons displayed an opposing form of heterogeneity, where subtypes
358 are far more discrete, without broad groups linking related subtypes (**Figure 3G**). Marker genes
359 for GABAergic neurons are also more canonical than those in glutamatergic neurons, whereby
360 most GABAergic neurons in plCoA have interneuron-like identities, expressing canonical marker
361 genes such as *Vip, Sst,* and *Cck* (**Figure 3H**).

362 We hypothesized that differences in these populations' abundance could potentially be
363 responsible for the difference observed between different plCoA domains, and thus examined
364 potential domain-specific enrichment of certain cell types within plCoA. Visualization of these
365 nuclei showed little clear region-specific structure in dimension-reduced space for any major cell
366 types (**Figure 4A**). This lack of structure was broadly confirmed quantitatively, where a few low-
367 abundance glial cell types showed significant domain-specific enrichment, but the high-
368 abundance major cell types did not (**Figure 4A-B**). When looking to other regions, however,
369 heterogeneity is usually more pronounced between subsets of the major cell types, instead of
370 by the quantities of the major cell types themselves.[42,43] We then examined abundance of
371 differentially expressed genes (DEGs) between plCoA domains for each major cell type. Here,
372 we found that both major neuronal cell types had more abundant DEGs than all major glial cell
373 types (**Figure 4C**). Glutamatergic neurons DEGs exceeded all other major cell types by a factor
374 of 4, making it likely that differences between the anterior and posterior domains are most likely
375 to be observed via variation in glutamatergic neurons.

376 Upon examination of domain-specific variation in plCoA glutamatergic neurons, we
377 initially observed a greater degree of domain-specific clustering in dimension-reduced space
378 (**Figure 4D**). Glutamatergic neuron subtypes correspondingly displayed domain-specific
379 enrichment, where more than half of glutamatergic neuron subtypes were significantly enriched
380 in either the anterior or posterior plCoA domain (**Figure 4E**). Upon closer examination, we found
381 that every glutamatergic subtype in the *Slc17a6*-expressing Group 1 was enriched in anterior
382 plCoA, while *Slc17a7*-expressing Group 2 subtypes are evenly distributed across fields or
383 biased towards the posterior, with one exception, Glut.Fign, which likely derives from the
384 aplCoA-adjacent cortex-amygdala transition area CxA, based on Allen ISH data of *Fign*
385 expression (**Figures 4F, S5A**). In contrast to glutamatergic neurons, we did not observe
386 significant plCoA domain-specific variation for any GABAergic neuron subtypes (**Figure S5D-E**).
387 We also found additional heterogeneity within glial cell types, including additional subtypes in


A
All nuclei
ABC
Macro
Micro
Glut
GABA
MOL
NFOL
OPC
VLMC
Astro
apICoA
ppICoA
UMAP 1
UMAP 2
B
Proportion in domain
NFOL
OPC
Micro
Mural
GABA
Glut
Astro
MOL
Endo
Macro
VLMC
ABC
C
Domain-specific DEGs
Glut
GABA
Astro
Micro
OPC
OLG
Endo
Mural
VLMC
apICoA enriched
ppICoA enriched
D
Glutamatergic neurons
Group 1
Group 2
apICoA
ppICoA
E
Proportion in domain
Group 1 neurons
Group 2 neurons
Glut
GABA
-Log10 P-value
Log2 fold change
ppICoA enriched
apICoA enriched
NS
Log2 FC
P-value and log2 FC
F
Slc17a7
Slc17a6
G
DAPI
Slc17a6
Slc17a7
apICoA
AP: -1.34 mm
mpICoA
AP: -1.70 mm
ppICoA
AP: -2.30 mm
Slc17a6+
Slc17a7+
Slc17a6+/Slc17a7+
apICoA (n = 17)
mpICoA (n = 14)
ppICoA (n = 18)
H
L1
apICoA
ppICoA
apICoA.1
apICoA.2
apICoA.3
apICoA.4
apICoA.5
ppICoA.1
ppICoA.2
L1.1
L1.2
OLG
NLOT
I
J
55 µm diameter
K
L
Group 1
Group 2
Glut.Zfp536
Glut.Vwc2
Glut.Gulp1
Glut.Ebf1
Pred. Score
M
GABA.Lamp5
55 µm diameter

388

Fig. 4. Glutamatergic neurons subtypes in plCoA are spatially distributed along an anteroposterior molecular gradient.

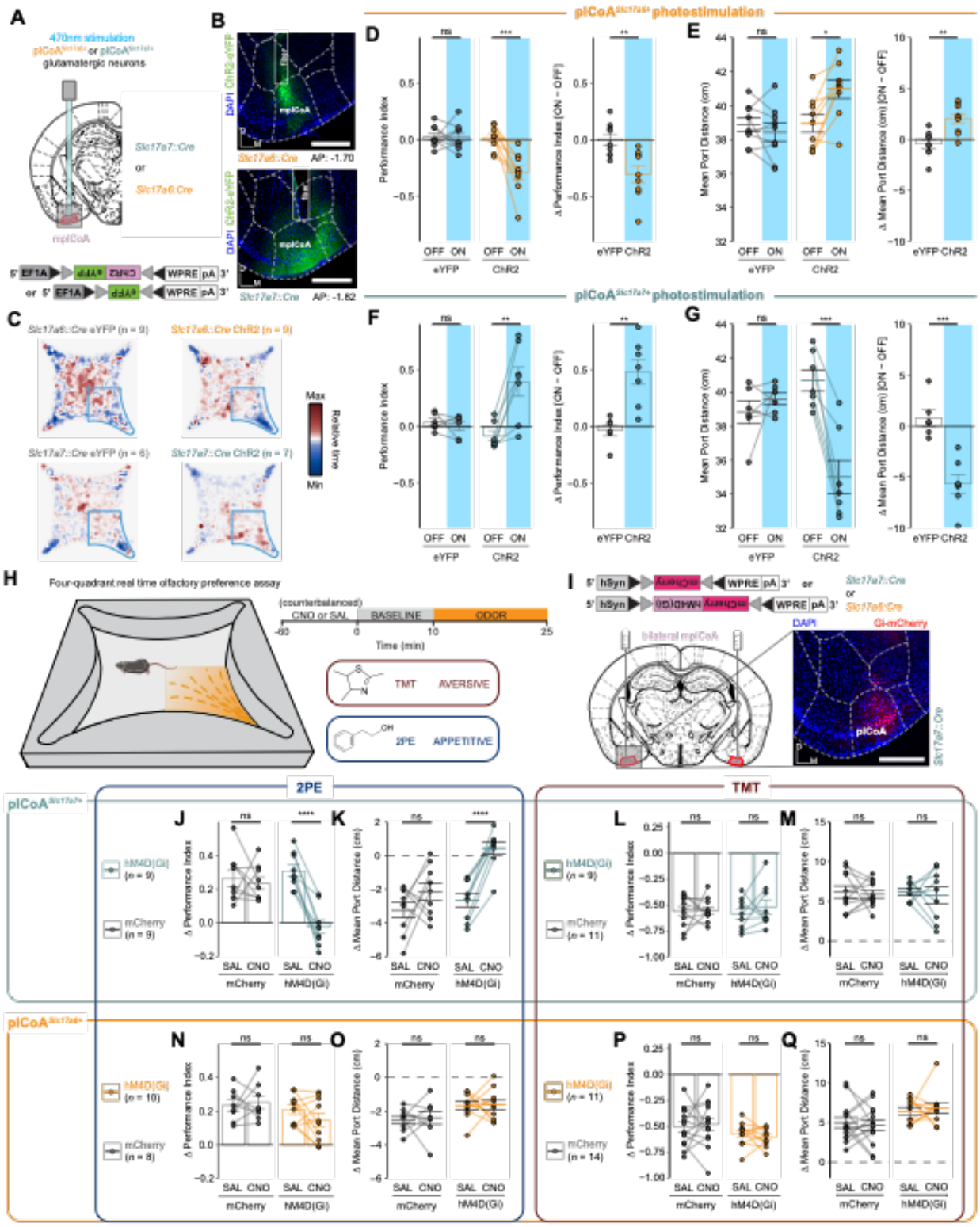
(**A**) UMAP of all plCoA nuclei colored by zone of origin, with dotted outlines and labels denoting the major cell types.
(**B**) Relative proportion of nuclei from each domain within each broad identity class. Dotted line indicates chance level for all plCoA nuclei.
(**C**) Top, abundance of domain-specific DEGs for each major cell type, either enriched in aplCoA nuclei (top) or pplCoA nuclei (bottom). Bottom, volcano plots for domain-specific DEGs for glutamatergic (left) and GABAergic neurons (right), the two cell types with the greatest degree of domain specific gene expression, where negative log-fold changes indicate enrichment in pplCoA and positive log-fold changes indicate enrichment in aplCoA.
(**D**) UMAP of plCoA glutamatergic neurons colored by domain of origin, with dotted outlines and labels denoting the subtypes on the graph. Groups of glutamatergic neuron types identified previously via Euclidean distance and hierarchical clustering are overlaid on top of the neuron types of interest.
(**E**) Relative proportion of molecular subtypes from each domain within glutamatergic neurons, where relevant subtypes are outlined according to their glutamatergic neuron group. Dotted line indicates chance level for plCoA glutamatergic neuron nuclei.
(**F**) UMAP of all glutamatergic neuron nuclei, colored by expression levels of *Slc17a6* (top) or *Slc17a7* (bottom).
(**G**) Left, representative images of *in situ* RNAscope labeling of *Slc17a6* RNA (red) and *Slc17a7* RNA (green) across plCoA domains. Right, proportions of glutamatergic neurons expressing *Slc17a6, Slc17a7,* or both. Scale bars, 500 μm (main image), 50 μm (inset).
(**H**) UMAP of all plCoA-overlapping Visium capture spots, colored by cluster. Broad spatial position of groups of clusters are overlaid on top of the capture spots of interest.
(**I**) UMAP of all plCoA-overlapping Visium capture spots, colored by expression levels of *Slc17a6* (top) or *Slc17a7* (bottom).
(**J**) Representative plCoA-overlapping region of one section on a Visium slide capture area, with capture spots colored by cluster.
(**K**) Representative plCoA-overlapping region of one section on a Visium slide capture area, with capture spots colored by expression levels of *Slc17a6* (top) or *Slc17a7* (bottom).
(**L**) Prediction scores for representative glutamatergic neuron subtypes within Group 1 (left) and Group 2 (right), shown on a UMAP of all plCoA-overlapping capture spots across all sections (top) and on a representative plCoA-overlapping region of one section (bottom).
(**M**) Prediction scores for a representative GABAergic neuron subtype, shown on a UMAP of all plCoA-overlapping capture spots across all sections (top) and on a representative plCoA-overlapping region of one section (bottom).
Across panels: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Additional specific details of statistical tests can be found in Supplemental Table 1.

428 astrocytes and VLMCs (**Figure S5G-J, O-R**). We also observed high-dimensional domain-
429 specific divergence in gene expression for OPCs and astrocytes corresponding to domain-
430 specific DEG differences, though they did not correspond to any observable differences in
431 subtype enrichment (**Figure S5F, K-N**).

432 To confirm these findings and validate our snRNA-seq data, we directly examined spatial
433 RNA expression patterns. First, we used RNAscope labelling to examine the expression of
434 *Slc17a6* and *Slc17a7 in situ* in the plCoA, quantifying the number of nuclei expressing these
435 genes. We found that anterior plCoA had a much greater proportion of RNAscope-labelled
436 *Slc17a6*+ nuclei (88%) than *Slc17a7*+ nuclei (11%) compared to the rest of plCoA, where
437 *Slc17a7*+ neurons predominate; posterior plCoA nuclei were almost entirely *Slc17a7*+ (97%)
438 expressing (**Figure 4G**). It is noteworthy that these numbers were generally consistent with
439 those in our sequencing data. Next, we analyzed spatial gene expression in the plCoA from an
440 existing Visium spatial transcriptomics dataset that contained sagittal sections bisecting the
441 plCoA along the midline (Romero et al., in preparation; **Figure S5S**). We asked if the domain-
442 specific molecular cell type composition can be recovered directly from spatial information,
443 without depending on inference from dissection histology. All sections used were of similarly
444 high quality and did not display any clearly observable batch effects, with all but one having
445 more than 100 spots covering the plCoA (**Figure S5T-W**). When clustering directly on spatial
446 data, we observed significant heterogeneity separating into three broad groups (**Figure 4H**).
447 Like in scRNA-seq, we found highly specific expression of *Slc17a6* and *Slc17a7* to two of the
448 three broad spot groups (**Figure 4I**). When examining the spatial configuration of these groups,
449 we found the *Slc17a6*-expressing group of clusters was in aplCoA, while the *Slc17a7*-
450 expressing group was in pplCoA**,** with the third intermediate group corresponding to layer 1
451 (**Figure 4J-K**). When computationally projecting transcriptomic cell type identities onto spatial
452 data, we observed that Group 1 glutamatergic neuron types would project onto pplCoA spots
453 and Group 2 glutamatergic neuron types would project onto aplCoA spots, while negligible
454 anteroposterior bias could be observed when projecting GABAergic neuron types onto plCoA
455 spots (**Figure 4L-M**). Overall, these data demonstrate that plCoA contains an extremely diverse
456 population of numerous glutamatergic and GABAergic neuron subtypes, whereas glutamatergic
457 neuron subtypes vary significantly along the anteroposterior axis, such that aplCoA-enriched
458 subtypes express *Slc17a6* and pplCoA-enriched subtypes express *Slc17a7.*

459 **Molecularly Defined plCoA Glutamatergic Neuron Populations Are Sufficient to Drive**
460 **Valence Behavior**

461 Given this spatial distribution bias of plCoA$^{Slc17a6+}$ neurons into aplCoA and plCoA$^{Slc17a7+}$
462 neurons into pplCoA, we further hypothesized that these glutamatergic neuron subtypes could
463 be responsible for the opposing valence responses observed during topographic plCoA
464 stimulation (**Figure 2**). If distinct molecular cell types mediate opposing valence, the
465 topography-independent activation should elicit opposing responses. To investigate this, we
466 expressed ChR2 in a non-spatially-biased, cell type-specific manner using a Cre-dependent
467 viral construct in *Slc17a6::Cre* and *Slc17a7::Cre* transgenic mice, targeting AAV-DIO-hSyn-
468 ChR2 into mplCoA and implanting optic fibers above the injection site (**Figures 5A-B, S2B**).[44,45]
469 Using the prior four-quadrant open field task, we found that photostimulation of plCoA$^{Slc17a6+}$


A
470nm stimulation
glutamatergic neurons
Slc17a7::Cre
or
Slc17a6:Cre
mpICoA
B
ChR2-eYFP
DAPI
mpICoA
Slc17a6::Cre
AP: -1.70
Slc17a7::Cre
AP: -1.82
EF1A
WPRE
pA
C
Slc17a6::Cre eYFP (n = 9)
Slc17a6::Cre ChR2 (n = 9)
Slc17a7::Cre eYFP (n = 6)
Slc17a7::Cre ChR2 (n = 7)
Max
Min
Relative time
D
E
F
G
Performance Index
Δ Performance Index (ON – OFF)
Mean Port Distance (cm)
Δ Mean Port Distance (cm) (ON – OFF)
OFF ON
eYFP
ChR2
H
Four-quadrant real time olfactory preference assay
(counterbalanced)
CNO or SAL
BASELINE
ODOR
Time (min)
TMT
AVERSIVE
2PE
APPETITIVE
I
hSyn
mCherry
hM4D(Gi)
WPRE
pA
Slc17a7::Cre
Slc17a6::Cre
bilateral mpICoA
DAPI
Gi-mCherry
pICoA
J
K
L
M
N
O
P
Q
2PE
TMT
hM4D(Gi) (n = 9)
mCherry (n = 9)
hM4D(Gi) (n = 10)
mCherry (n = 8)
hM4D(Gi) (n = 11)
mCherry (n = 11)
hM4D(Gi) (n = 11)
mCherry (n = 14)
Δ Performance Index
Δ Mean Port Distance (cm)
SAL CNO
mCherry
hM4D(Gi)

470

Fig. 5. Glutamatergic plCoA$^{Slc17a6+}$ and plCoA$^{Slc17a7+}$ neurons contribute to innate olfactory behaviors of opposing valence.

(**A**) Schematic for selective photostimulation of distinct glutamatergic cell type. *Slc17a6::Cre* and *Slc17a7::Cre* animals were injected with Cre-dependent viral vectors into mplCoA with a fiber optic implant placed just above the injection site.
(**B**) Representative histology from ChR2 viral injection and fiber implantation site in an *Slc17a6::Cre* animal (top) and an *Slc17a7::Cre* animal (bottom).
(**C**) Baseline-normalized collective heatmaps from both the ChR2- and eYFP-infected groups in *Slc17a6::Cre* and *Slc17a7::Cre* animals with plCoA-localized fiber implants. Lower right stimulus quadrant marked in blue.
(D-G) Effect of photostimulation of plCoA$^{Slc17a6+}$ neurons (**D-E**) or plCoA$^{Slc17a7+}$ neurons (**F-G**) on time spent in the stimulation quadrant (**D, F**) and distance from the corner (**E, G**).
(**H**) Behavioral paradigm to assess innate valence responses to odor. Left, schematic of four-quadrant open field behavioral assay for spatially-specific odor delivery. Upper right, within-trial timeline. Lower right, odors delivered and their associated innate valence.[3]
(**I**) Schematic of strategy for selective chemoinhibition of molecularly defined glutamatergic plCoA neurons.
(**J-M**) Effect of chemoinhibition of plCoA $^{Slc17a7+}$ neurons on time spent in the odor quadrant (**J, L)** or decrease in mean port distance (**K, M**) in response to 2PE (**J-K**) or TMT (**L-M**)**.**
(**N-Q**) Effect of chemoinhibition of plCoA $^{Slc17a6+}$ neurons on time spent in the odor quadrant (**N, P)** or decrease in mean port distance (**O, Q**) in response to 2PE (**N-O**) or TMT (**P-Q**)**.**
Across panels, ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.
Additional specific details of statistical tests can be found in Supplemental Table 1.

494 neurons significantly reduced time spent in the 'on' quadrant and increased the average
495 distance to the corner port during the treatment period , indicating activation of plCoA$^{Slc17a6+}$
496 neurons is aversive and leads to avoidance of the quadrant when paired with stimulation
497 (**Figure 5C-E**). In contrast, photostimulation of the plCoA$^{Slc17a7+}$ neurons instead increased the
498 time in the 'on' quadrant and decreased average distance to the corner port, indicating
499 plCoA$^{Slc17a7+}$ neuron activation instead is appetitive and leads to attraction to the stimulation
500 quadrant (**Figure 5C,F-G**). These data indicate that the divergent domain-specific valence
501 effects of plCoA activity are likely due to the divergent molecularly defined neuronal ensembles
502 predominant in each topographical field of plCoA.

503 Next, we sought to determine whether these two glutamatergic populations are
504 respectively required for innate olfactory aversion and/or attraction. We used the above
505 transgenic mouse lines to drive expression of a viral Cre-dependent hM4D(Gi) construct to
506 selectively inhibit these neurons' activity via chemogenetics (**Figure 5I, Figure S6A-C**).[46] We
507 administered clozapine-N-oxide (CNO) or a vehicle control and used the four-quadrant open
508 field assay to deliver odorants in a quadrant-specific manner to assess their behavioral
509 responses to the innately-attractive 2PE or the innately-aversive TMT to determine the
510 difference in the magnitude of temporally-counterbalanced valence responses when the
511 respective populations are chemogenetically silenced.[3]

512 We observed that while both transgenic mouse lines preserved the baseline attraction
513 and aversion to both 2PE and TMT following administration of the vehicle control, silencing
514 plCoA$^{Slc17a6+}$ neurons did not affect the response to either odorant (**Figure 5J-Q**). However,
515 silencing plCoA$^{Slc17a7+}$ neurons abolished the positive valence response to 2PE, without having
516 any effect on TMT-evoked aversion (**Figure 5J-Q**). In other words, neither group of plCoA
517 glutamatergic neurons are necessary for TMT aversion, but plCoA$^{Slc17a7+}$ neurons are required
518 for expression of 2PE attraction behaviors. Further, silencing of either population did not lead to
519 any broader non-olfactory behavior effects as measured by the EPM and OFT assays, including
520 anxiety, exploration, and motility, showing the effects of silencing these neurons are likely
521 limited to valence and/or olfaction alone, instead of exploratory or defensive behaviors (**Figure
522 S5C-N**). The necessity of plCoA$^{Slc17a7+}$ neurons for 2PE attraction, combined with their ability to
523 drive positive valence responses indicates that these of plCoA$^{Slc17a7+}$ neurons control innate
524 olfactory attraction, but plCoA$^{Slc17a6+}$ neurons do not completely control innate olfactory aversion,
525 instead proving sufficient but not necessary.

526 **A Topographic Organization of plCoA Defined by Limbic Projection Targets**

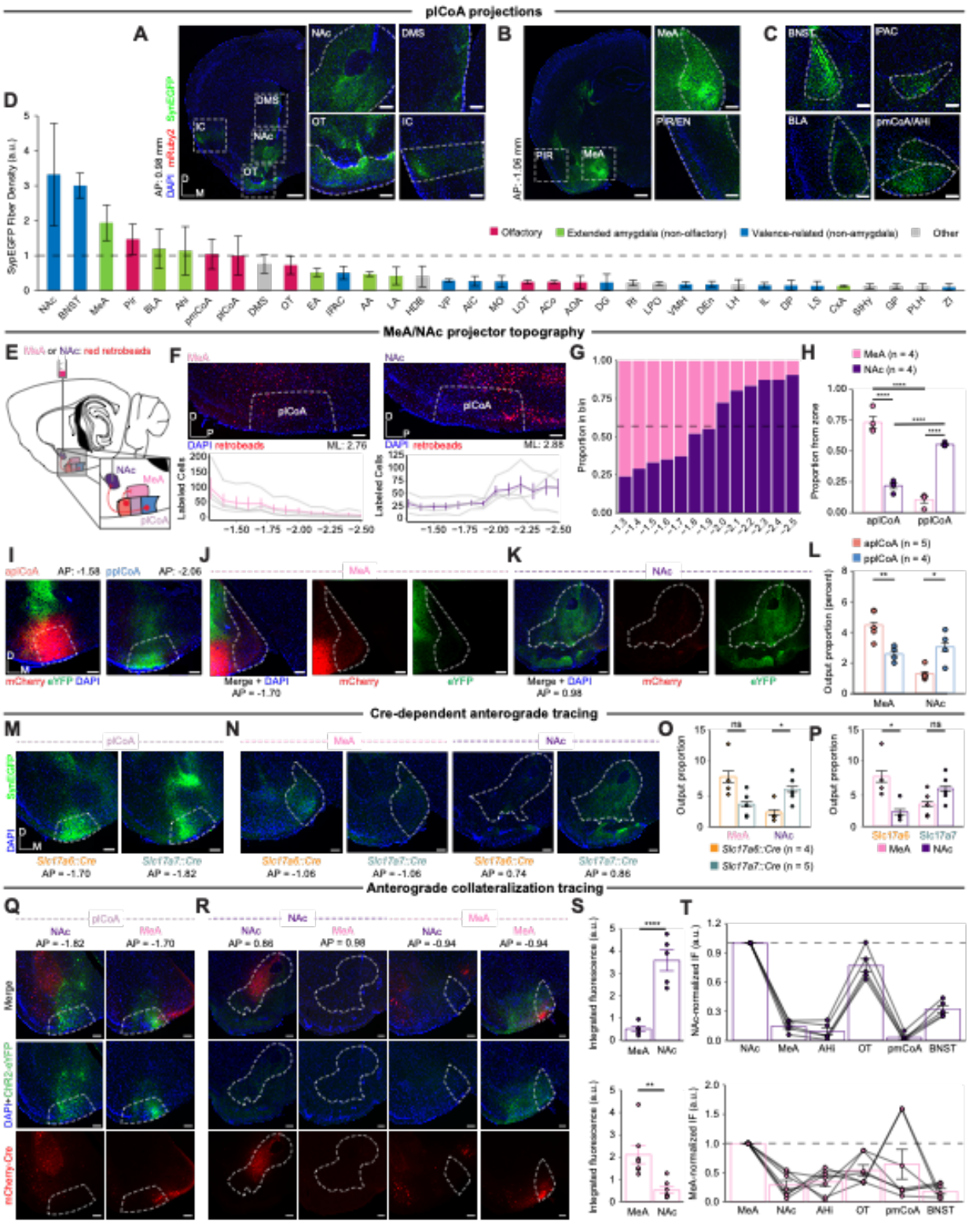
527 While comprehensively characterizing the circuit features of plCoA, we noted that the
528 anatomical connectivity of the plCoA has not yet been defined in the mouse brain. Further, we
529 posited that the differences in necessity of plCoA cell types could be due to divergent
530 downstream connections instead of divergent molecular features, which may partially, but not
531 completely overlap. Thus, we next sought to identify distinct downstream outputs of plCoA that
532 could explain the bidirectional valence effects of its topography. We first characterized the
533 downstream outputs of plCoA by co-injecting the anterograde viral tracer AAV-DIO-hSyn-
534 mRuby2-T2A-synaptophysin-EGFP and a constitutive AAV-Cre virus into mplCoA to label

535 presynaptic terminals with EGFP (**Figure S7A-B**). We observed a high amount of terminal
536 fluorescence within plCoA itself, suggesting the presence of recurrent connections within the
537 region (**Figure S6B**). In addition, we observed long-range projections to a diverse set of
538 regions, including surrounding extended amygdala subregions, such as MeA and the amygdalo-
539 hippocampal transition area (AHi), regions controlling valence and emotion, like the NAc and the
540 bed nucleus of the stria terminalis (BNST), and regions involved in olfactory processing,
541 primarily PIR and OT (**Figure 6A-D**).

542 Among these outputs, we hypothesized that the NAc and the MeA could be responsible
543 for the behavioral divergence between anterior and posterior plCoA, given their known
544 involvement in reward expectation and aversion, respectively.[47,48] We sought to confirm these
545 differences using retrograde tracing from MeA or NAc, where red retrobeads were injected into
546 MeA or NAc and labeled neurons were quantified along the anterior-posterior axis (**Figure 6E**).
547 For both downstream targets, we observed opposing gradients of retrobead projector labeling
548 throughout the entire plCoA anteroposterior axis (**Figure 6F**). MeA-projecting neurons are
549 enriched in aplCoA, and NAc-projecting neurons are enriched in pplCoA, with each having a
550 frequency of around chance level in mplCoA (**Figure 6G**). Further, the majority of labeled
551 plCoA-MeA projection neurons were in aplCoA, while the majority of labeled plCoA-NAc
552 projection neurons were in pplCoA (**Figure 6H**). To further confirm the spatial bias in projection
553 targets, we performed anterograde tracing from the aplCoA and pplCoA by injecting viruses
554 expressing either eYFP or mCherry into either domain of plCoA in a counterbalanced manner
555 (**Figure S6D-E**). Anterograde projection strength from aplCoA and pplCoA revealed that
556 projections to MeA were most dense from aplCoA, and projections to NAc were most dense
557 from pplCoA (**Figure 6I-K**). Proportionally, aplCoA sent a significantly higher proportion of its
558 projections to MeA than pplCoA, whereas pplCoA sent a significantly higher proportion of its
559 projections to NAc (**Figure 6L**).

560 We reasoned that the topographical valence sufficiency we observed could be explained
561 by cell type-specific divergence in projection target, whereby the topographical biases in
562 downstream targets are recapitulated by their underlying molecular cell type. To determine the
563 relationship between cell types and projection targets, we injected Cre-dependent eYFP into
564 mplCoA in *Slc17a6::Cre* and *Slc17a7::Cre* transgenic mice, targeting the middle to ensure
565 differences result from cell type, instead of simply redundant topography (**Figure S7F-G)**.
566 Interestingly, the relationship was not as simple as one cell type, one primary projection target.
567 Instead, both cell types project to both structures in different proportions. The plCoA$^{Slc17a6+}$
568 neurons primarily project to MeA with a significant bias for that target over NAc, while
569 plCoA$^{Slc17a7+}$ neurons project to both MeA and NAc, with a statistically insignificant bias toward
570 NAc (**Figure 6M,P**). These findings demonstrate that NAc primarily receives projections
571 plCoA$^{Slc17a7+}$ neurons, whereas the MeA receives input from both populations. Moreover,
572 projection of both cell types to the MeA may explain why neither population was selectively
573 required for innate aversion (**Figure 5J-Q**).

574 Given that both cell types project to both MeA and NAc, we sought to determine the
575 extent of collateralization in neurons composing the two pathways. To test whether plCoA-MeA
576 and plCoA-NAc projection neurons also project to multiple or overlapping downstream targets,


pICoA projections
A
B
C
D
SypEGFP Fiber Density (a.u.)
Olfactory
Extended amygdala (non-olfactory)
Valence-related (non-amygdala)
Other
MeA/NAc projector topography
E
F
G
H
Proportion in bin
Proportion from zone
aplCoA
pplCoA
I
J
K
L
Output proportion (percent)
Cre-dependent anterograde tracing
M
N
O
P
Output proportion
Anterograde collateralization tracing
Q
R
S
T
Integrated fluorescence (a.u.)
NAc-normalized IF (a.u.)
MeA-normalized IF (a.u.)

577

578 **Fig. 6. Projections to MeA and NAc from plCoA are Topographically Organized**

579 (**A**) Right, whole-hemisphere view at AP = 0.98 mm from bregma. Scale bar, 500 µm. Left,
580 Magnified images of the areas highlighted inside white dashed lines. Scale bar, 200 µm.
581 (**B**) Right, whole-hemisphere view at AP = -1.06 mm from bregma. Scale bar, 500 µm. Left,
582 Magnified images of the areas highlighted inside white dashed lines. Scale bar, 200 µm.
583 (**C**) Other plCoA projections not found in cross-sections of the brain found in **(A)** and **(B)**. Scale
584 bar, 200 µm.
585 (**D**) Magnitude of anterograde synaptophysin-eYFP fluorescence in primary downstream targets
586 of plCoA projection neurons ordered by total output strength, colored based on each region's
587 function.
588 (**E**) Schematic for topographic retrograde mapping strategy from MeA and NAc into plCoA. Red
589 retrobeads are injected into MeA or NAc and topographical projection bias is examined along
590 the anterior-posterior axis.
591 (**F**) Representative images (top) for injection into MeA (left) or NAc (right) and number of
592 neurons labeled along the anterior-posterior axis as distance (mm) from bregma (bottom). Gray
593 lines denote individual replicates, where colored lines indicate mean ± s.e.m.
594 (**G**) Proportion of retrobead-labeled neurons projecting to MeA or NAc for each 100 µm segment
595 as a function of distance from bregma. Dashed line indicates overall balance of all retrobead-
596 labeled neurons across entire plCoA.
597 (**H**) Proportion of retrobead-labeled neurons from either target within each plCoA zone. MeA-
598 labeled neurons are significantly enriched in aplCoA compared to NAc-labeled neurons, while
599 NAc-labeled neurons are significantly enriched in pplCoA compared to those labeled from MeA.
600 (**I**) Representative histological images for the injection sites in aplCoA (left) and pplCoA (right)
601 from a representative animal. Scale bar, 200 µm.
602 (**J**) Representative histological images for MeA from the animal in (**J**). Scale bar, 200 µm.
603 (**K**)**.** Representative histological images for NAc from the animal in (**J**). Scale bar, 200 µm.
604 (**L**) Output strength as a proportion of total fluorescence from aplCoA and pplCoA to MeA and
605 NAc.
606 (**M**) Representative histological images for the injection site in plCoA from a representative
607 *Slc17a7::Cre* and *Slc17a6::Cre* animal. Scale bar, 200 µm.
608 (**N**) Representative histological images from MeA and NAc from a representative animal of
609 either genotype. Scale bar, 200 µm.
610 (**O**) Left, output strength as a proportion of total fluorescence from plCoA$^{Slc17a6+}$ and plCoA$^{Slc17a7+}$
611 neurons to MeA and NAc. Right, comparison of same data, but by target region within genotype.
612 (**P**) Same data as **(O)**, but by target region within genotype.
613 (**Q-V**) Mapping collateral projections from NAc- and MeA projecting neurons.
614 (**Q**) Representative histological images for the injection site in plCoA from a representative
615 animal receiving retrograde virus into MeA or NAc. Scale bar, 200 µm.
616 (**R**) Representative histological images of NAc and MeA retro-Cre targeting (red) and outputs
617 (green).
618 (**S**) Comparison of absolute integrated fluorescence intensities in MeA and NAc when retroAAV
619 was injected into NAc (top) or MeA (bottom).

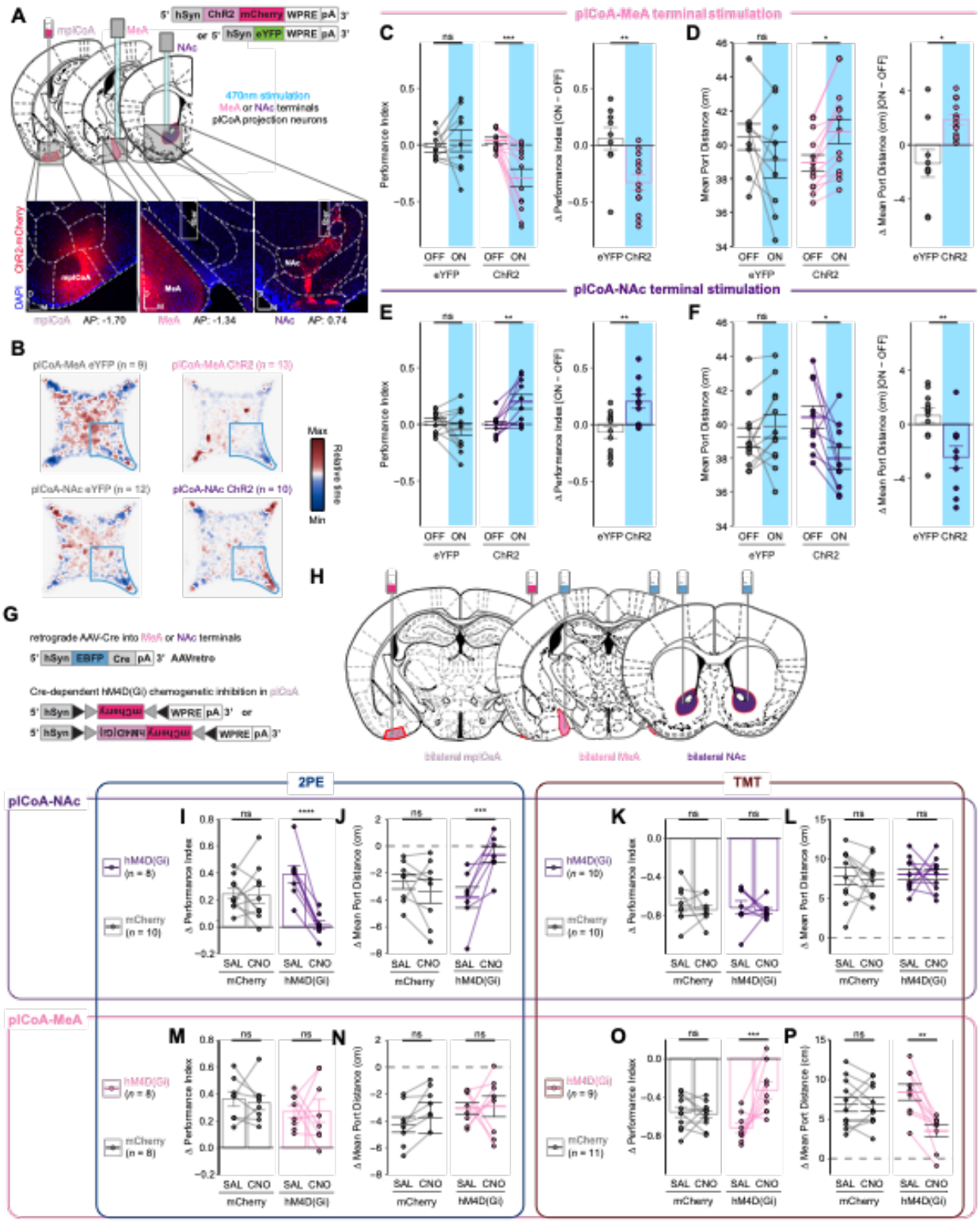
620 (**T**) Quantification of fluorescence in selected downstream brain regions from plCoA originating
621 from plCoA-NAc neurons proportional to eYFP fluorescence in NAc (top) or MeA (bottom).
622 Abbreviations: NAc, nucleus accumbens; BNST, bed nucleus of stria terminalis; MeA, medial
623 amygdala; Pir, piriform cortex; BLA, basolateral amygdala; Ahi, amygdalo-hippocampal
624 transition area; pmCoA, posteromedial cortical amygdala; Str, striatum; OT, olfactory tubercle;
625 EA, extended amygdala; IPAC, inferior peduncle of the anterior commissure; AA, anterior
626 amygdala; LA, lateral amygdala; HDB, horizontal limb of the diagonal band; VP, ventral
627 pallidum; AIC, anterior insular cortex; mfb, medial forebrain bundle; MO, medial orbitofrontal
628 cortex; LOT, lateral olfactory tract; ACo, anterior cortical amygdala; AOA, anterior olfactory area;
629 DG, dentate gyrus; Rt, reticular nucleus; LPO, lateral preoptic area; VMH, ventromedial
630 hypothalamus; DEn, dorsal endopiriform claustrum; LH, lateral hypothalamus; IL, infralimbic
631 cortex; DP, dorsal peduncular cortex; LS, lateral septum; CxA, cortex-amygdala transition area;
632 sox, supraoptic decussation; StHy, striohypothalamic nucleus; GP, globus pallidus; PLH,
633 perirhinal cortex; ZI, zona incerta.
634 Across panels, ns, not significant; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. Additional specific
635 details of statistical tests can be found in Supplemental Table 1.

636 we employed a combination of retrograde Cre and Cre-dependent anterograde tracer viral
637 vectors. A retroAAV-hSyn-Cre-mCherry virus was into either MeA or NAc, and AAV-DIO-ChR2-
638 eYFP was injected into plCoA to label outputs of MeA- or NAc-projecting neurons (**Figure 6Q-R,**
639 **S7H-I**). We focused on MeA and NAc, as well as the ancillary primary downstream targets
640 implicated in valence or olfaction.[25,49–51] We found different collateralization patterns for both
641 populations, where NAc-projecting neurons did not collateralize to MeA, but very strongly
642 collateralized to OT. In contrast, MeA-projecting neurons minimally collateralized to NAc and
643 most strongly collateralized to pmCoA (**Figure 6S-T**). Notably, neither projection of interest
644 significantly collateralized to the other. These data indicate that plCoA-MeA and plCoA-NAc
645 projection neurons are non-overlapping, spatially biased populations that output to different
646 downstream subnetworks.

647 **Projections from plCoA NAc to MeA and Respectively Control Innate Olfactory Attraction**
648 **and Aversion to Odor**

649 The topographic separation of MeA- and NAc-projecting neurons are consistent with a
650 model of divergence valence that could support the observed topographic divergence in valence
651 behaviors. To investigate the behavioral contributions of these projections, we first determined
652 whether the neurons projecting to the MeA and NAc are able to drive behavior with optogenetic
653 stimulation. We expressed ChR2 in a non-spatially biased manner by injecting AAV-hSyn-ChR2
654 into mplCoA, and we placed an optic fiber above MeA or NAc for selective optogenetic
655 stimulation at plCoA axon terminals (**Figure S1C**). We found that photostimulation of the plCoA-
656 MeA circuit in the four-quadrant open field task significantly reduced time spent in the 'on'
657 quadrant and increased the average distance to the corner port during the treatment period,
658 indicating activation of the plCoA-MeA circuit is aversive and leads to avoidance of the quadrant
659 paired with stimulation (**Figure 7A-D**). The opposite was true for the plCoA-NAc projection,
660 where stimulation in that zone instead increased the time in the 'on' quadrant and decreased
661 average distance to the corner port, indicating activation of the plCoA-NAc circuit is instead
662 appetitive and leads to attraction to the stimulation quadrant (**Figure 7B,E-F**). We next asked if
663 the effects of stimulating these circuits affected other non-valence behaviors by testing the mice
664 in the EPM and OFT. Using the EPM, we found no change in anxiety based on open arm time
665 or entries when stimulating either projection to the MeA or NAc (**Figure S8A-C,E-F,H-I**).
666 Similarly, stimulation in the OFT did not cause any change to thigmotaxis, based on time spent
667 in corners of the open field, or exploration, based on time spent in the center of the open field
668 (**Figure S8K-M,O-P,R-S**). Further, locomotion remained constant during stimulation across
669 both assays (**Figure S8D, S8G, S8J, S8N, S8Q, S8T**). These data indicate that the divergent
670 projections from plCoA to the MeA and the NAc evoke valence-specific behaviors without
671 modulating anxiety.

672 Finally, we sought to determine whether plCoA-MeA or plCoA-NAc projections are
673 necessary for the expression of odor-evoked appetitive or aversive behaviors. To target these
674 projection neurons for chemogenetic silencing, we injected a retroAAV bearing an hSyn-EBFP-
675 Cre construct into MeA or NAc, along with an AAV in plCoA bearing a Cre-dependent hM4D(Gi)
676 construct (**Figure 7G,H**). We then tested the innate responses of these animals to 2PE or TMT


A
mpICoA
MeA
NAc
5' hSyn ChR2 mCherry WPRE pA 3'
or 5' hSyn eYFP WPRE pA 3'
470nm stimulation
MeA or NAc terminals
pICoA projection neurons
ChR2-mCherry
DAPI
mpICoA AP: -1.70
MeA AP: -1.34
NAc AP: 0.74
B
pICoA-MeA eYFP (n = 9)
pICoA-MeA ChR2 (n = 13)
pICoA-NAc eYFP (n = 12)
pICoA-NAc ChR2 (n = 10)
Max
Relative time
Min
pICoA-MeA terminal stimulation
C
D
Performance Index
Δ Performance Index (ON – OFF)
Mean Port Distance (cm)
Δ Mean Port Distance (cm) (ON – OFF)
OFF ON
eYFP
ChR2
pICoA-NAc terminal stimulation
E
F
G
retrograde AAV-Cre into MeA or NAc terminals
5' hSyn EBFP Cre pA 3' AAVretro
Cre-dependent hM4D(Gi) chemogenetic inhibition in pICoA
5' hSyn mCherry WPRE pA 3' or
5' hSyn mCherry hM4D(Gi) WPRE pA 3'
H
bilateral mpICoA
bilateral MeA
bilateral NAc
2PE
TMT
pICoA-NAc
pICoA-MeA
I
J
K
L
M
N
O
P
hM4D(Gi) (n = 8)
mCherry (n = 10)
hM4D(Gi) (n = 10)
hM4D(Gi) (n = 9)
mCherry (n = 8)
mCherry (n = 11)
Δ Performance Index
Δ Mean Port Distance (cm)
SAL CNO
mCherry
hM4D(Gi)

677

Fig. 7. Projections from plCoA to NAc and MeA control innate olfactory attraction and aversion.

(**A**) Schematic for optogenetic MeA terminal stimulation in plCoA neurons. Strategy to activate MeA-projecting plCoA neuron terminals via optogenetics (top) and representative histology from ChR2 viral injection and fiber implantation site (bottom).
(**B**) Baseline-normalized collective heatmaps from both the ChR2- and eYFP-infected plCoA groups with MeA- and NAc-localized fiber implants. Lower right stimulus quadrant marked in blue.
(C-D) Optogenetic MeA terminal stimulation of plCoA neurons infected with ChR2, but not eYFP, is sufficient to reduce time spent in the stimulation quadrant (**C**) and increase its average distance from the port (**D**) during the stimulation period.
(E-F) Optogenetic NAc terminal stimulation of plCoA neurons infected with ChR2, but not eYFP is sufficient to increase time spent in the stimulation quadrant (**E**) and decrease its average distance from the stimulation port (**F**) during the stimulation period.
(**G**) Viral strategy for selective retrograde chemoinhibition of projection-defined plCoA neurons.
(**H**) Schematic for selective retrograde chemoinhibition of projection-defined plCoA neurons.
(**I-L**) Chemoinhibition of NAc-projecting plCoA neurons significantly eliminates the 2PE-evoked increase in time spent in the odor quadrant (**I)** and decreases in mean port distance (**J).** The response to TMT is unaffected in time spent in odor quadrant (**K**) or port distance (**L**).
(**M-P**) Chemoinhibition of MeA-projecting plCoA neurons does not affect 2PE-evoked increase in time spent in the odor quadrant (**M)** or decrease in mean port distance (**N)** significantly decreases the TMT-evoked reduction in time spent in the odor quadrant (**O)** or increase in mean port distance (**P).**
Across panels, ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Additional specific details of statistical tests can be found in Supplemental Table 1.

703 in the four-quadrant assay following administration of CNO or a vehicle control, as above.
704 Inhibition of plCoA-NAc projection neurons abolished innate attraction to 2PE without having
705 any effect on aversion to TMT (**Figure 7I-L**). Conversely, inhibition of plCoA-MeA projection
706 neurons had no effect on innate 2PE attraction, but significantly decreased the aversion to TMT
707 (**Figure 7M-P**). Silencing these neurons had no effect in the EPM and OFT assays, indicating
708 the effects of silencing these neurons are limited to valence or olfaction, and not anxiety or
709 exploration (**Figure S8M-X**). Thus, plCoA-MeA projection neurons are necessary and sufficient
710 for innate olfactory aversion, whereas plCoA-NAc projection neurons are necessary and
711 sufficient for innate olfactory attraction.

712 **DISCUSSION**

713 **Topographic Organization of Valence in plCoA**

714 The neural circuits mediating innate valence responses to odor have not been fully
715 defined. Here, we have advanced our knowledge of the circuitry underlying innate olfactory
716 behaviors by defining its activity and organization within plCoA and further extending the innate
717 olfactory pathway from a third order olfactory brain area to limbic structures. We have identified
718 a novel functional axis for valence with the plCoA that is defined by histologically and
719 functionally distinct domains along the anteroposterior axis. We have characterized odor
720 encoding in plCoA, identifying a sparse population code for identity of an odor, but not its innate
721 valence. We have also determined the composition of molecular cell types in plCoA and
722 identified spatially biased populations enriched within each domain, which we find sufficient to
723 drive their respective domain-specific behaviors, though only partially necessary for their
724 functions in olfaction. Moreover, we identified the outputs of plCoA and quantitatively
725 characterized the relative strength of each, as well as how it relates to plCoA topography and
726 domain-specific molecular cell types, demonstrating that neurons projecting to the MeA and
727 NAc are topographically and molecularly biased. Finally, we demonstrate that neurons
728 projecting to the to the NAc and MeA are capable of driving approach and avoidance
729 responses, and loss of function experiments demonstrate that the neurons projecting to the NAc
730 or MeA are selectively involved in innate olfactory attraction and avoidance, respectively. Thus,
731 plCoA is composed of dissociable, spatially segregated ensembles for divergent valence,
732 defined by their downstream projection target.

733 Spatial organization for features of sensory stimuli is common in sensory cortex, and has
734 been previously proposed as fundamental to sensory processing.[52] Visual cortex is
735 topographically organized by retinotopic locations in space, somatosensory cortex contains a
736 map of the body, auditory cortex has a crude tonotopic organization by frequency, and gustatory
737 cortex is segregated by taste qualities.[34,35,53–55] However, topographic organization by
738 perceptual feature has not been observed in the olfactory system, which has been attributed to
739 the high dimensional nature of olfactory information.[56,57] Our findings identify a topographically
740 organized divergence motif for valence in plCoA, where activation of aplCoA evokes aversive
741 behavioral responses, while activation of pplCoA evokes attractive behavioral responses, with a
742 graded transition between the two domains. This is consistent with prior studies implicating a
743 spatial organization to inputs from the OB to plCoA, where glomerulus-specific anterograde
744 tracing from OB shows specific glomeruli send fibers to invariant, densely clustered,
745 anatomically distinct locations within plCoA, and retrograde tracing from plCoA shows that
746 upstream glomeruli are spatially biased within OB and closer examination reveals that OB input
747 can co-vary with plCoA anteroposterior position.[15] Interestingly, spatial segregation of divergent
748 features is present elsewhere in olfaction as well, although not by perceptual feature. Olfactory
749 sensory receptors display stereotyped spatial organization even within zones of the olfactory
750 epithelium, with a corresponding spatially-stereotyped glomerular topography in OB.[16,24,58–60]
751 Further, these topographic domains in OB have functional relevance, where region-specific OB
752 manipulations selectively alter different olfactory valence behaviors.[61–63] However, the piriform
753 cortex generally lacks apparent spatial organization or spatial patterning for odor reponses.[22] To

our knowledge, the topographical organization of valence observed here in plCoA represents one of the first descriptions of these spatial patterns occurring in central olfactory areas.

Topographic gradients for valence have been observed in other limbic regions, including the BLA for overall valence, the medial amygdala for innate social behaviors, *Drosophila* dopaminergic mushroom body neurons for general valence, and the gustatory insular cortex for taste valence.[19,34,35,64,65] The gustatory insular cortex is of particular interest, given that it also primarily encodes taste identity and hedonics in a spatially-nonspecific manner.[31,35,66–69] Our results extend this phenomenon of spatially segregated valence into both olfaction and into a less well-described amygdala nucleus, and suggest that it could serve as a potential common motif within the limbic system to organize motivational information, especially for innate behaviors, which require stereotyped neurocircuitry. We believe a model where aplCoA and pplCoA are parts of the same region with similar underlying composition, but with a gradual change in the factor that defines the valence output for a given part of the gradient would best explain the underlying gradient-like effect on positive and negative valence. We considered two such organizational principles underlying this topography: molecular cell types and projection targets. We found that broad divisions of cell types by Slc17a6 and Slc17a7 did not fully define valence, though more specific subtypes could be responsible. Our results demonstrate that the projection targets of plCoA neurons are a fundamental feature for imparting valence on this circuitry.

Molecular Cell Types in plCoA Segregate Topographically and Drive Behaviors of Divergent Valence

While examining the cell types composing plCoA, we noticed numerous notable, novel features. First, despite its small area (~1.04 mm^3 and ~170,000 cells), plCoA displays remarkable diversity, hosting dozens of distinct, robustly separable cell types.[70] The plCoA appears to have multiple domains positioned at the transition between highly dissimilar brain tissue types. Interestingly, the high-dimensional structure of molecular variation differs between glutamatergic and GABAergic neurons. Glutamatergic molecular variation within plCoA is continuous, with two broadly nested groups generally marked by either *Slc17a6* or *Slc17a7* along with one or more additional marker gene(s), though it should be noted that expression of the two broad glutamatergic markers is not necessarily mutually exclusive and a few low abundance “transition” cell types can express both. This leaves open the possibility that a more specific cell type could be necessary for aversion, given that this necessity for this behavior did not perfectly map onto either broad molecular cell type. In contrast, molecular variation in GABAergic neurons is far more discretized, with ensembles expressing one of a few well-characterized interneuron markers found throughout the brain, such as *Sst, Vip,* and *Pvalb*, among others. This is consistent with other studies in neocortex, hippocampus, and subiculum that find similar patterns of variation, where variation within glutamatergic neurons is more continuous than in GABAergic neurons.[42,43,71] In this way, we find the continuous gradient-like structure of valence in plCoA is recapitulated with gradient-like variation in glutamatergic neuron gene expression in plCoA.

794 In these molecular datasets, we further observed highly specific differences in cell type
795 enrichment within aplCoA and pplCoA within glutamatergic neurons, but not GABAergic
796 neurons or glia. Within plCoA, *Slc17a6*+ neurons are enriched in aplCoA and *Slc17a7*+ neurons
797 are enriched in pplCoA, though there is gradient-like intermingling of populations, especially
798 toward the middle of plCoA, and all glutamatergic neuron types are present, albeit with high
799 variability along the anteroposterior axis. This molecularly defined order suggests a
800 programmed organization, rather than stochastically distributed populations within the region,
801 especially given that its boundaries match the domains previously identified based on behavior
802 and histology.[72] This phenomenon also broadly matches observations in the neocortex,
803 hippocampus, and subiculum, where glutamatergic neurons across subdivisions molecularly
804 diverge to a greater degree than GABAergic neurons or glia, albeit across a correspondingly
805 greater area than within plCoA, which is generally accompanied by distinct morphological and
806 electrophysiological properties broadly corresponding to these transcriptomic
807 differences.[42,43,71,73–76] Spatial segregation of molecular cell types is also observed within deeper
808 brain regions including BLA, thalamus and habenula, and these molecular differences are also
809 accompanied by extended phenotypic differences.[19,77–80] Investigation of such properties held in
810 common and diverging within and between *Slc17a6*+ and *Slc17a7*+ glutamatergic neuron types
811 could also serve to further define the local neurocircuitry and information processing dynamics
812 within plCoA and along its anteroposterior axis.

813 Interestingly, few if any populations within plCoA are clearly separable from most of the
814 regions surrounding it (e.g. piriform cortex, basomedial amygdala, and MeA) based on primary
815 marker gene identity. Instead, plCoA ensembles seem to be defined by the interplay of all three
816 regions within the same tissue. The predominance of populations resembling different regions
817 does appear related to this anteroposterior organization, though, where *Slc17a6*+ neurons
818 predominate in both aplCoA and MeA, and more specific marker genes like *Meis2* are
819 expressed in both regions as well.[81] Conversely, *Slc17a7*+ neurons predominate in both pplCoA
820 and piriform cortex, and the major marker genes like *Satb2* are similarly expressed in both
821 regions.[82] Such phenomena also cohere with more general characterizations made in whole-
822 brain molecular taxonomies, which divide plCoA along its axis, grouping aplCoA with MeA and
823 pplCoA with paleocortex.[83–85] It would be misleading to characterize plCoA populations as mere
824 extensions of surrounding populations into an adjacent region, though. *Slc17a7*+ neurons from
825 the cortex-amygdala transition zone are also present in the dataset and are continuously
826 separable from *Slc17a7*+ plCoA neurons based on the expression of marker genes like *Fign*.
827 Instead, plCoA may itself be a transition region, given that such a relationship with its
828 neighboring regions is very similar to that of the amygdalostriatal transition area, one of the only
829 transition regions to undergo high-resolution molecular profiling.[86] Given such commonalities
830 between these two putatively dissimilar regions, molecular characterization of additional
831 transition regions could potentially uncover similar organizational motifs, especially if compared
832 with adjacent regions of interest, and allow for a much more in-depth exploration and
833 characterization of the boundaries and transitions between proximally located, distantly related
834 brain regions.

835 These broad molecular groups of glutamatergic cell types themselves do not completely
836 explain valence in the plCoA. While aplCoA-enriched *Slc17a6*+ neurons are sufficient to drive

837 aversion and pplCoA-enriched *Slc17a7*+ neurons are sufficient to drive attraction, as would be
838 predicted from the valence responses evoked from each anterior-posterior domain enrichments,
839 it might be expected that these populations would also be necessary for the respective odor-
840 evoked valence. However, only *Slc17a7*+ neurons are necessary for 2PE attraction, whereas
841 the *Slc17a6*+ neurons were not required for aversion to TMT. Given that the plCoA is necessary
842 for TMT aversion, it is unlikely that such a difference is due to additional redundant function
843 within other regions for TMT aversion.[3] Instead, although *Slc17a6*+ neurons likely contribute to
844 the valence behavior, other populations within the region not expressing the marker gene could
845 also contribute to the behavioral response. Thus, although these two broad glutamatergic
846 groups can drive innate responses of valence, the *Slc17a6*+ population doesn't fully represent
847 the population that control aversive responses, which is supported by other findings presented
848 here showing that both *Slc17a6*+ and *Slc17a7*+ neurons project to MeA, while the projection to
849 NAc is almost entirely composed of *Slc17a7*+ neurons. Regarding more specific molecular cell
850 types, it could be possible that only a subset of *Slc17a7*+ neurons are required for 2PE
851 attraction. Similarly, the neurons required for TMT aversion could be marked by a gene
852 orthogonal to the observed *Slc17a6*/*Slc17a7* gradient, and genetic access to olfactory aversion
853 could potentially be established by investigating these more sparsely expressed marker genes.
854 In both cases, though, further investigation into these other cell types would enhance our
855 understanding of both plCoA and innate olfactory valence and allow more precise manipulations
856 in the future.

857 **Downstream Projection Targets of plCoA Divergently Control Innate Olfactory Valence**

858 In this work, we perform the first characterization of plCoA's downstream outputs in the
859 mouse brain. These outputs are dominated by regions generally involved in valence and
860 emotion, such as the NAc, BNST, MeA, BLA, and other amygdalar nuclei, or olfactory areas,
861 such as the pmCoA, OT, and PIR. These outputs are consistent with a role for plCoA in
862 motivational valence for odor. The plCoA also appears to form numerous intra-regional
863 connections, where a significant proportion of synapses formed with other neurons are within
864 the region itself. This raises the possibility that plCoA is not simply a feedforward relay but
865 performs local recurrent processing as well. Recurrent networks in other sensory systems
866 expand the dimensionality of encoding schemata and incorporate additional features to
867 generate mixed, continuously updating representations of relevant information.[87] This raises a
868 number of interesting questions regarding information processing within plCoA. For instance,
869 how does odor representation change based on differences in experience and internal state?
870 Further explorations of information transformation and encoding within plCoA will certainly
871 enrich our understanding of both this region and olfaction in general.

872 The NAc and MeA are interesting downstream targets given their pre-existing known
873 relationships to attraction and aversion, respectively. NAc has historically been critical to the
874 manifestation and processing of reward and motivated behaviors, though this view has been
875 expanded and made more subtle with a recent focus on action selection in general.[88] On the
876 other hand, MeA has been linked to defensive and stress-related behaviors at baseline and in
877 response to aversive stimuli, as well as chemosensation.[89] MeA has also been specifically
878 linked to olfactory aversion in past studies, as TMT has previously been shown to activate the

879 MeA, which is necessary for TMT-induced defensive behaviors, though the upstream circuits
880 and processing were not yet investigated.[48,90,91] These circuits are also notable regarding other
881 features of plCoA spatiomolecular organization, as the projections to the downstream regions of
882 interest are the two that diverge to the greatest extent between aplCoA and pplCoA, and
883 between plCoA$^{Slc17a6+}$ and plCoA$^{Slc17a7+}$ glutamatergic neurons. Given the relationship between
884 spatiomolecular patterning and the simple wiring and organizational rules used to structure
885 innate circuits it would be interesting to investigate the plCoA through the lenses of development
886 and genetic variation. These intersect in recent discussions of genetic bottlenecking, where the
887 genome encodes general rules for circuit organization and development that nevertheless yield
888 specific responses to specific stimuli.[92] Such networks have numerous theoretical advantages,
889 such as reduced information requirements and higher performance at criterion, providing a
890 conceptual basis for why spatiomolecularly stereotyped circuits yield innate behaviors, and why
891 these innate behaviors are adaptive in naturalistic settings.[92–94]

892 **Neuronal Activity in plCoA Encodes Odor Identity Via Sparse Population Code**

893 The plCoA receives spatially-ordered inputs and has a cell-type-specific topographical
894 organization with divergent outputs that mediate responses of opposing valence. This
895 anatomical organization is consistent with either labeled-line or divergent paths motifs, though
896 precise connectivity from OB has not yet been described. However, label line coding motif is not
897 apparent in neural activity, but instead there is a sparse population code for odor identity with no
898 apparent valence-specific responses, indicating that the plCoA cannot function as a pure
899 labeled-line relay. Moreover, the absence of valence encoding is distinct from that seen in other
900 divergent path motifs, suggesting the plCoA represents a different circuit model for valence.

901 We were surprised to find that despite a robust organization of plCoA cell types and
902 projections that support behaviors of valence, the neurons do not appear to encode valence at
903 the single neuron or population level. However, when put in the context of the olfactory system,
904 these results are less surprising. Sparse, distributed population encoding appears to be a
905 general feature of all olfactory regions observed thus far, regardless of their specific structure or
906 computational function within olfaction. In the OE, epithelium-resident OSNs expressing a single
907 receptor will bind multiple odorants and establish odor identity through a combinatorial code.[21,23]
908 In the OB, axon terminals from OSNs expressing the same receptor gather into specific
909 stereotyped glomeruli, where mitral/tufted cells (M/T) then transmit this information to third-order
910 olfactory regions, where both steps represent the odor identity via a sparse population code in a
911 spatially-distributed manner.[24,26,32,95] The primary olfactory cortex further represents odor identity
912 and chemical space similarly through sparse, spatially distributed combinatorial population
913 activity in a similar manner to its M/T inputs.[17,22,32,96] The OT, a striatal region primarily
914 composed of *Drd1*- or *Drd2*-expressing medium spiny neurons, also broadly represents odor
915 identity across both cell types, despite their opposing roles elsewhere in striatum, though a
916 secondary contribution by hedonic value is currently debated.[25,97–101] Although direct
917 comparisons of olfactory regions find some differences in features like sparsity, clustering, or
918 correlation structure, these are generally reported as differences in degree, not in
919 kind.[17,30,32,95,99,100] Given the remarkable similarity in encoding between these structurally and
920 functionally distinct regions, it logically follows that an olfactory region with amygdalar structure

would follow similar population encoding principles. Indeed, were plCoA to predominantly encode stimulus valence in a manner akin to other amygdalar regions, it would reflect a greater divergence from olfactory coding principles than what we observe. Instead, the olfactory encoding scheme employed by plCoA reveals a novel framework for valence encoding that is distinct from that in the extended amygdala.

How does innate valence arise from a population code imposed on stereotyped valence circuitry? One possibility is that the valence of an odor is defined by the proportion of NAc and MeA-projecting neurons activated, mixing features of divergent paths and opposing components motifs. In this model a given odor will activate some balance of NAc and MeA projecting neurons and the proportional balance determines the valence. If true, one should be able to predict the valence of the odor by recording from a large sample of these neurons, which is beyond the scope of this study. Another possibility is that activity could evolve over the time course of behavior such that a mixture of inputs from local circuitry and long-range circuits could function like attractor networks to shape output into one of a few convergent states. A third possibility is that valence may emerge in combination with behavior and internal state. In this model, the plCoA integrates sensory information with other state variables to shift the activity towards one output. If either of these latter two models is correct, recording activity over longer time scales in freely moving animals could provide insight into how activity correlates with behavior. Alternatively, valence may arise downstream of plCoA, but molecular mechanisms to support this model are currently undescribed, and such a model would be at odds with our finding of divergent valence-specific circuitry required for innate valence responses. Innate behavior is indeed dynamic and more complex than attraction and avoidance. These different models for valence encoding could serve to increase the flexibility of the system to adapt to changes in experience or internal state.

A Novel Circuit Motif for Valence

The widespread circuit model for valence encoding centers on divergence, in which information is routed to different pathways depending on the valence of the stimulus. The plCoA circuit we have identified is anatomically similar, but unique in its encoding properties. Most divergent circuits function to generalize stimuli, discarding information about stimulus identity to simplify to low dimensional valence signals from distinct neuronal populations. In contrast, the plCoA groups stimuli, using a high dimensional code of odor identity that is routed through divergent projections to mediate opposing valence responses. In this model, the valence of an odor could be determined based on the population dynamics and composition within a distributed, sparse population code that ultimately funnels information through divergent pathways corresponding to their innate valence. Such a model could serve to increase the flexibility of the system while retaining the ability to yield stereotyped responses.

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AUTHOR CONTRIBUTIONS

J.R.H. and C.M.R. conceived the project. J.R.H., C.L.C., D.L., M.B., and A.N.Z. performed surgeries and analyzed histological data. J.R.H., C.L.C., and A.N.Z. performed behavioral experiments, managed the colony, and analyzed behavioral data. J.R.H. and D.L. performed calcium imaging experiments and analyzed calcium imaging data. J.R.H. performed sequencing experiments, analyzed sequencing data, and prepared all figures. H.K.R. and P.A.D. designed and performed spatial transcriptomics experiments and provided data for analysis. F.M., M.E.L., and K.M.T. provided the CellProfiler processing pipeline, as well as intellectual, facilities, and logistic support and guidance. C.M.R. supervised the project. J.R.H., D.L., M.B., H.K.R., and C.M.R. wrote the manuscript. All authors edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Cory M. Root (cmroot@ucsd.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All snRNA-seq and Visium spatial sequencing data generated for this study is deposited on Gene Expression Omnibus (GEO) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. All other data reported in this paper will be shared by the lead contact upon request. The code used to analyze all data and generate all graphs can be found online at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

1002 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

1003 All procedures at the University of California, San Diego, and Columbia University were
1004 performed in accordance with Institutional Animal Care and Use Committee protocols in
1005 accordance with NIH guidelines. All mice were provided food and water ad libitum and
1006 maintained on a regular 12-hour reverse light/dark cycle at room temperature, with weight,
1007 health, and immune status monitored daily and verified to be within normal ranges. Mouse
1008 cages were changed regularly based on degree of soiling. All mice were group-housed with
1009 randomly assigned littermates prior to surgery, and single-housed after surgery. All animals
1010 were used in a single experiment each, except for a subset of mice who underwent 4-quad,
1011 EPM, and OFT experiments, who performed each test in no specific order.

1012 **Subject Details for Sequencing Experiments**

1013 ***Subject details for single-nucleus sequencing***

1014 All mice for snRNA-seq in the study were males on the wild-type C57BL/6J background
1015 (RRID:IMSR_JAX:000664) and received directly from Jackson Laboratories at 6 weeks of age
1016 and acclimated to the colony prior to experiments. Animals were single-housed and placed into
1017 sensory deprivation 24 hours prior to sacrifice to reduce artifactual immediate early gene
1018 expression. Sacrifice was performed at P60 ± 3 days (n = 5-10 mice per pool). Sample size was
1019 determined based on number of expected nuclei per region per mouse: estimates of expected
1020 nuclei were determined empirically, though nuclear recovery was approximately 20% of total
1021 based on cellular density estimates from the Blue Brain Cell Atlas.[70] 20,000 nuclei were targeted
1022 per combination of assay, condition, and region, which was determined using SCOPIT v1.1.4,
1023 allowing potential detection of at least 10 nuclei from 10 rare subpopulations at 0.1% frequency
1024 with 95% probability.[103] A total of 50 mice were used for this purpose.

1025 ***Subject details for spatial transcriptomics***

1026 In the separate study for spatial transcriptomics, APP23 (B6.Cg-Tg(Thy1-APP)3Somm/J;
1027 C57BL/6J background, RRID:IMSR_JAX:030504) non-transgenic (NTG) littermates control mice
1028 were housed in light-tight enclosures.[104] The mice were given ad libitum food and water access.
1029 This study used a total of 17 mice almost equally distributed across sex, of which sections from
1030 11 sagitally bisected the plCoA and were used in downstream analysis. No analysis of sex
1031 differences was performed due to inaccessibility of that information on a per-section basis.
1032 However, no such differences were apparent from per-section gene expression correlations
1033 reported in supplementary information.

1034 **Subject Details for Calcium Imaging, Tracing, and Activity Manipulations**

1035 ***Subject details for wild-type experiments***

1036 All mice for topographic and projection-defined manipulation and tracing experiments, as well as
1037 calcium imaging, were males on the wild-type C57BL/6J background and received directly from

1038 Jackson laboratories before 12 weeks of age. After surgery, mice incubated for at least 21 days
1039 if injected with virus or at least 7 days if injected with a retrograde tracer (e.g. retrobeads,
1040 cholera toxin B) to allow virus to express and tracers to travel in retrograde, respectively. All
1041 surgeries and downstream experiments were performed on mice at least 8 weeks of age.

1042 ***Subject details for transgenic experiments***

1043 We used VGlut2-IRES-Cre (Slc17a6tm2(cre)Lowl; C57BL/6J background,
1044 RRID:IMSR_JAX:028863; *Slc17a6::Cre*) and VGlut1-IRES2-Cre-D (Slc17a7tm1.1(cre)Hze;
1045 C57BL/6J background, RRID:IMSR_JAX:023527; *Slc17a7::Cre*) strain mice for molecularly
1046 defined optogenetic stimulation experiments and genotype-specific tracing.[44,45] These mice
1047 were bred on-site at UCSD and were genotyped in-house using genomic DNA from ear tissue
1048 amplified with the default primer sets listed by Jackson Laboratories. All mice used for
1049 experiments had a heterozygous genotype for the transgenic construct of interest. After surgery,
1050 mice incubated for at least 21 days to allow viral expression.

1051 **METHODS DETAILS**

1052 **Stereotactic surgery procedures**

1053 All surgeries were performed under aseptic conditions using a model 1900 digital small animal
1054 stereotaxic instrument (Kopf Instruments). Mice were initially anesthetized in a sealed box
1055 containing 5% gaseous isoflurane, and then deeply anesthetized using isoflurane (2.5% in
1056 1L/min of O_2) during surgeries (VetFlo, Kent Scientific Corporation). We immobilized and leveled
1057 the head in a stereotaxic apparatus (Kopf Instruments), removed fur from the scalp by shaving,
1058 applied eye lubricant (Optixcare), cleaned the incision site with 70% ethanol and betadine prior
1059 to incision, peeled off connective tissue, and dried the surface of the skull prior to craniotomy
1060 before proceeding with injections and implantations specific to certain experiments. All virus
1061 injections were performed at 2 nL/sec using a pulled glass pipette (Drummond) and a Nanoject
1062 III pressure injector (Drummond). To prevent backflow, the pipette was left in the brain for 15
1063 minutes after injection.

1064 ***Surgeries for calcium imaging***

1065 Surgeries for imaging experiments were performed in a manner similar to that previously
1066 described.[33] The skull was prepared with OptiBond™ XTR primer and adhesive (KaVo Kerr)
1067 prior to the craniotomy. After performing a craniotomy 1 mm in diameter centered around the
1068 virus injection site, a 27G blunt needle was used to aspirate ~2.5 mm below the brain surface.
1069 600 ul of AAV9-hSyn-FLEX-jGCaMP8s-WPRE (2.5×10^{13} gc/ml, Addgene) was diluted to ~$1 \times$
1070 10^{12} gc/ml gc/ml and injected into the left middle plCoA in two 300 ul boli, one in layer 2 and one
1071 400 um dorsal (+0.4 DV) in layer 3. Following the viral injection, a head-plate (Model 4,
1072 Neurotar) was secured to the mouse's skull using light-curing glue (Tetric Evoflow, Ivoclar
1073 Group). At least 30 minutes after viral injection, a 1 mm GRIN lens (NA, ~1.9 pitch, GrinTech)
1074 was sterilized with Peridox-RTU then slowly lowered at a rate of 500 um/min into the craniotomy
1075 until it was 200 um dorsal (+0.2 DV) to the injection coordinate. The lens was adhered to the

1076 surface of the skull using Tetric Evoflow. We then placed a hollow threaded post (AE825ES,
1077 Thorlabs) to act as a housing for the lens and adhered it using Tetric Evoflow. Any part of the
1078 skull that was still visible was covered using dental cement (Lang Dental). Finally, the housing
1079 was covered with a Nylon cap nut (94922A325, McMaster-Carr) screwed onto the thread post to
1080 protect the lens in between imaging. Animals were left on the heating pad until they fully
1081 recovered from anesthesia.

1082 ***Surgeries for optogenetic stimulation***

1083 For optogenetic topographic- or projection-specific stimulation experiments, we injected wild-
1084 type C57BL/6J mice between 2-4 months of age with 100 (if topographic) or 150 (if projection-
1085 specific) nL of either AAV5-hSyn-eYFP (3.3 × 10^{12} gc/ml, UNC Vector Core) or AAV5-hSyn-
1086 ChR2-mCherry-WPRE-PA (4.1 × 10^{12} gc/ml, UNC Vector Core). For Cre-dependent molecularly
1087 defined optogenetic stimulation, we injected 200 nl AAV5-EF1A-DIO-hChR2(H134R)-eYFP (5.5
1088 × 10^{12} gc/ml, UNC Vector Core) or AAV5-EF1A-DIO-eYFP (4.0 × 10^{12} gc/ml, UNC Vector Core)
1089 All such injections were left unilateral, into either aplCoA or pplCoA for topographic
1090 photostimulation, and into middle plCoA for projection- or genotype-specific photostimulation.
1091 For topographic and genotype-specific photostimulation animals, we implanted the fiber 300 um
1092 (+0.3 DV) directly above the injection site with all other coordinates remaining constant.
1093 Anterior-posterior axis positioning arose from stochastic variation in virus and fiber placement.
1094 For projection-specific photostimulation animals, we implanted the fiber 300 um (+0.3 DV)
1095 directly above either the MeA or NAc, holding all other coordinates for the two regions constant
1096 as described above. For all optogenetic stimulation experiments, we implanted a fiber optic
1097 cannula (2.5 mm ferrule outer diameter, 200 um core, 0.39 numerical aperture; RWD) 300 um
1098 above the targeted stimulation site. These fibers were affixed onto the skull using OptiBond XTR
1099 (Kerr) and stably secured with Tetric Evoflow (Ivoclar Vivadent) coated with cyanoacrylate
1100 (Toagosei). After surgery, we injected all mice with 0.04 mL Buprenorphine SR (Ethiqa XR,
1101 Fidelis) for pain management. All mice were given their own cage immediately after surgery and
1102 returned to the colony once ambulatory.

1103 ***Surgeries for chemogenetic inhibition***

1104 For all plCoA inhibition experiments, we bilaterally injected C57BL/6J mice between 2-4 months
1105 of age with 250 nL of either AAV2-hSyn-DIO-hM4D(Gi)-mCherry (7.1 × 10^{12} gc/ml, Addgene) or
1106 AAV2-hSyn-mCherry (1.8 × 10^{13} gc/ml, Addgene) virus. For projection-specific inhibition
1107 experiments, wild-type C57BL6/J mice were used, and AAVretro-hSyn-EBFP-Cre (1.5 × 10^{12}
1108 gc/ml, Addgene) either 50 µL were injected into MeA or 300 µL were injected into NAc. For
1109 genotype specific inhibition experiments, *Slc17a6::Cre* or *Slc17a7::Cre* mice were used. All
1110 injections were bilateral and targeted to middle plCoA.

1111 ***Surgeries for fluorescent tracing***

1112 For non-topographic anterograde tracing experiments, we bilaterally co-injected of mixed 50 nl
1113 AAVDJ-hSyn-FLEX-mRuby-T2A-SynEGFP (4.0 × 10^{12} gc/ml, Addgene, a gift from Byungkook
1114 Lim) and AAV5-EF1A-mCherry-IRES-Cre-WPRE (1.9 × 10^{12} gc/ml, UNC Vector Core) into
1115 middle plCoA (-1.8 AP, +/-2.9 ML, -5.95 DV).[105,106] For topographic anterograde tracing

experiments, we unilaterally injected 20 nl AAV8-hSyn-hChR2(H134R)-mCherry (2.1 × 10^{13} gc/ml, Salk GT3 Viral Vector Core) and AAV8-hSyn-hChR2(H134R)-eYFP (3.2 × 10^{13} gc/ml, Salk GT3 Viral Vector Core) into aplCoA (-1.4 AP, -2.8 ML, -5.95 DV) and pplCoA (-2.1 AP, -3.0 ML, -5.95 DV), counterbalancing region by fluorophore. For retrograde topographic tracing experiments, we unilaterally injected Red Retrobeads IX (Lumafluor) into either MeA (50 nl, -1.2 AP, -2.0 ML, -5.5 DV) or NAc (+1.1 AP, -1.35 ML, -4.5 DV), at volumes of 50 nl or 300 nl, respectively. For anterograde collateralization experiments, AAVretro-EF1A-IRES-Cre (1.3 × 10^{13} gc/ml, Addgene) into either MeA or NAc, and AAVDJ-EF1A-DIO-hChR2(H134R)-eYFP-WPRE-pA (4.03 × 10^{13} gc/ml, Salk GT3 Viral Vector Core) was injected into middle plCoA. For genotype-specific anterograde tracing experiments we injected 50 µl AAVDJ-Ef1a-DIO-ChR2(H134R)-eYFP-WPRE-pA into middle plCoA in Slc17a6::Cre or Slc17a7::Cre mice.

Calcium Imaging

Odor exposure

Odor exposure for imaging experiments was adapted from methods previously described elsewhere.[25] Odor was delivered to the mouse using a custom-built olfactometer. Compressed medical air was split into 2 gas-mass flow controllers (Aalborg). One flow controller directed a constant rate of 1.5 L/min to a hollowed out teflon cylinder. The other flow regulator was connected to a 3-way solenoid valve (The Lee Co.). Prior to odor delivery, the 3-way valve directs clean air at 0.5 L/min to the teflon cylinder. During odor delivery, the 3-way valve directs air to an odor manifold, which consists of an array of 2-way solenoid valves (The Lee Co.), each connected to a different odor bottle. The kinetics and consistency of odor delivery were characterized using a miniature Photoionization Detector (mPID) (Aurora Scientific). Depending on the trial type, the appropriate 2-way valve opens, directing 0.5 L/min of air flow through the odor bottle containing a kimwipe blotted with 20 ul of odorant, except for 2PE and peanut oil, which were undetectable to human perception and the mPID after repeated presentation, and were provided in excess to ensure consistent detectability after numerous exposures.

Prior to imaging, mice were habituated to the head fixation device (Neurotar) and treadmill for at least 3 days for at least 5 minutes per session beginning at least 10 weeks after surgery. The treadmill parts were 3D printed using an LCD printer (EPAX) from publicly available designs.[116] Walking behaviors were measured using a quadrature encoder (Broadcom). A video feed of the animal's face was also recorded using a camera (Basler) with an 8-50mm zoom lens (Arducam) at 20 Hz with infrared illumination (Lorex Technology). Animals were exposed to the following odors for 2 seconds: the appetitive odors 2-phenylethanol (Sigma-Aldrich) and peanut oil (Spectrum), the neutral odors 1-heptanol (Sigma-Aldrich) and isoamyl acetate (Sigma-Aldrich), and the aversive odors trimethylthiazoline (BioSRQ) and 4-methylthiazole (Sigma-Aldrich). Within a single contiguous exposure session, each of the 6 odors were provided 20 times with 12-18 seconds of inter-trial interval. Trials were organized into 6 blocks, each of which consisted of 20 trials of each of the 6 odors in counterbalanced order without any odors of similar innate valence adjacent to each other in the trial structure.

2-photon microscope data acquisition

1156 Ca^{2+} imaging data was acquired using an Olympus FV-MPE-RS Multiphoton microscope with
1157 Spectra Physics MaiTai HPDS laser, tuned to 920 nm with 100 fs pulse width at 80 MHz. Each
1158 128 × 128 pixel scan was acquired with a 20x air objective (LCPLN20XIR, Olympus), using a
1159 Galvo-Galvo scanner at 5 Hz. Stimulus delivery and behavioral measurements were controlled
1160 through a custom software written in LabVIEW (National Instruments) and operated through a
1161 DAQ (National Instruments). Each imaging session lasted up to 90 minutes and was
1162 synchronized with the stimulus delivery software through a TTL pulse. Animals were excluded
1163 from analysis if histology showed that either the GRIN lens or the jGCaMP8s virus was
1164 mistargeted or the motion during imaging was too severe for successful motion correction.

1165 **Behavioral Assays**

1166 Mice had been handled for 5 days prior to experiments and acclimated to the room for an hour
1167 prior to testing. All behavioral experiments were performed during the dark period of the
1168 light/dark cycle at least an hour away from the switch between the two photoperiods. Not all
1169 mice were run in all assays, as elevated plus maze and open field tests were added after a
1170 significant proportion of four-quadrant data was collected at targeted sample sizes and mice had
1171 already been sacrificed.

1172 For all optogenetic experiments, optical fibers (200 mm, 0.39 numerical aperture, Thorlabs)
1173 were epoxied to 2.5 mm stainless steel ferrules (Precision Fibre Products), and polished with a
1174 fiber optic polishing kit (Thorlabs) to achieve a minimum of 80% transmission. After surgical
1175 implantation, the ferrules protruding from the mouse's head were coupled to an ADR-800A 100
1176 mW 473 nm laser (LaserCentury) via custom-made patch cables with a single rotary joint (Doric
1177 Lenses) between the mouse and laser. Laser intensity was set to 5-8 mW at the end of the
1178 patch cable. For inhibition experiments, all mice were injected intraperitoneally 60 minutes prior
1179 to the beginning of the behavioral trial with either sterile PBS vehicle or with clozapine-N-oxide
1180 (CNO) dihydrochloride (Hello Bio) dissolved in sterile PBS for a dosage of 2 mg/kg.

1181 ***Four quadrant open field assay***

1182 The four-quadrant open-field task was performed as previously described.[3] In short, all
1183 behavioral assays took place in a four-quadrant open field chamber. Airflow was pumped into
1184 each quadrant via gas-mass flow controllers 150 mL/min (Cole-Parmer). Airflow exited the
1185 chamber via a 1-inch outlet in the center of the chamber's floor covered by steel mesh, and the
1186 outlet was connected a vacuum line with a gas-mass controller set to 750 ml/min. The chamber
1187 was housed in the dark and illuminated from below by infrared lighting. A Basler A601FM
1188 camera (Edmund Optics) mounted above the chamber recorded videos of behavioral trials at 4
1189 Hz, and custom software written in Labview (National Instruments) tracked the position of the
1190 mouse in real time for each frame. The symmetrical four-quadrant open field chamber was
1191 contained in a lightproof structure (0-10 lux) and illuminated by infrared lights, removing any
1192 potential spatial cues available to the animals with respect to the room or its surroundings. In
1193 optogenetic experiments, an additional 5 cm spacer was added to the chamber flush with the
1194 walls to raise their height for more naturalistic behavior, and an acrylic ceiling with a top with a

circular opening 30.5 cm in diameter was added to prevent escape while allowing the fiber optic cable to move freely.

In optogenetic experiments, the laser was pulsed with 50 ms bins at 10 Hz, and there was a steep gradient from 1 to 10 Hz along the perimeter of the quadrant, increasing as proximity to the corner of the quadrant decreased. Preliminary experiments in topographical stimulation animals identified no clear behavioral effects from the location of the stimulus quadrant itself (data not shown), and all other downstream stimulation experiments exclusively used the lower right quadrant for stimulation to simplify data analysis. The lasers were controlled by TTL modulation from custom Labview software synchronized to the video capture system.

For inhibition experiments, odor was applied by solenoid valves redirecting airflow through 100 mL glass bottles containing 1 μL of a pure odorant on a small piece of Kimwipes. Odorants used were either the previously-validated innately aversive 2,5-dihydro-2,4,5-trimethylthiazoline (BioSRQ) or the innately appetitive 2-phenylethanol (Sigma-Aldrich) on a small piece of Kimwipe.[3] All odors were presented in the lower-right quadrant, and all trials were spaced out with at least an hour between runs, during which vacuum was applied to the chamber. Odors and injection treatments were given in counterbalanced, independent order within experimental groups.

Open field test

The open field is a square arena illuminated to 100-150 lux by ambient lighting. Mice were habituated to the room for at least an hour prior to testing, but otherwise had no prior experience in the arena prior to exposure. Mice were placed in the center of a square arena (27.3 × 27.3 × 20.3 cm, Med Associates) with four transparent plexiglass walls. Overall locomotion, immobility, and time spent in corners and center regions of arena during each epoch was analyzed for each mouse. Immobility was defined as movement under 0.5 cm/s for a period of at least 1 s, while the center was defined as the middle 13.7 × 13.7 cm square in the center of the arena and the corners as the corner regions that do not overlap with the center square in either direction (25% of arena area for each region). For optogenetic experiments, mice were allowed to move freely throughout the arena for 25 min total, with 5-8 mW 473 nm light stimulation pulsed with 50 ms bins at 20 Hz, alternately delivered during the 5-10 min and 15-20 min epochs (OFF, ON, OFF, ON, OFF). For chemogenetic experiments, mice were allowed to freely move through the area for 10 min total.

Elevated plus maze

The arms of the elevated plus maze were 30.5 × 5.5 cm. The height of the closed arm walls was 15 cm. The maze was elevated 40 cm from the floor and was placed in the center of the behavior room away from other stimuli. Arms were illuminated to 0-10 lux, with infrared illumination. Mice were placed in the center of maze at the beginning of each trial. For optogenetic experiments, mice were allowed to move freely throughout the maze for 15 min total, with 5-8 mW 473 nm light stimulation pulsed with 50 ms bins at 20 Hz delivered during the 5-10 min epoch (OFF, ON, OFF). For chemogenetic experiments, mice were allowed to freely move through the area for 10 min total.

1235 **Histology**

1236 ***Non-RNAscope section preparation***

1237 All sacrifices were performed during the dark period of the light cycle. Animals were
1238 anesthetized prior to sacrifice via combined intraperitoneal injection of 150 mg/kg ketamine
1239 (Zetamine, Vet One) and 15 mg/kg xylazine (AnaSed, AMRI Rensselaer). Except for animals
1240 used in sequencing studies, animals were subject to transcardial perfusion with 10 mL of sterile
1241 phosphate-buffered saline (PBS), followed by 10 mL 4% paraformaldehyde (PFA) solution. The
1242 brain was then extracted from the animal and placed into a 4% (PFA) solution in PBS for at
1243 least 36 hours until it was sectioned on a VT1000S vibratome (Leica). For tissue extracted for
1244 non-RNAscope histology, mice were transcardially perfused with 20 ml phosphate buffered
1245 saline (PBS) followed by 20 ml 4% paraformaldehyde (PFA) in PBS. All brains were extracted
1246 and post-fixed for at least 24 hours in 4% PFA. For tissue extracted for RNAscope, mice under
1247 6 months of age were decapitated once unconscious and their brains were extracted into a
1248 square Peel-A-Way embedding mold (Polysciences) and embedded in OCT (Fisher), and then
1249 snap-frozen a dry-ice/isopentane slurry and stored at -80˚C until cryosectioning within a month
1250 of sacrifice.

1251 Tissue was mounted in 5% agarose and sectioned sagittally on a vibratome for retrograde
1252 experiments, or sectioned coronally without mounting for all other non-RNAscope experiments.
1253 These sections were cut at 50 µm and stored in PBS before processing. All connectomic
1254 quantitation was performed on samples using epifluorescence without immunolabeling to avoid
1255 potential bias due to non-stoichiometric antibody binding, while all others were immunolabeled
1256 for visualization of viral targeting accuracy and collection of representative images.
1257 Immunolabeling of eYFP and other GFP-derived fluorophores was performed using goat anti-
1258 GFP primary antibodies (Abcam) and Alexa Fluor 488-conjugated anti-goat secondary
1259 antibodies (Invitrogen), while immunolabeling of mCherry and other DsRed-derived fluorophores
1260 used rabbit anti-DsRed primary antibodies (Takara) and Alexa Fluor 588-conjugated anti-rabbit
1261 antibodies (Invitrogen), all diluted 1:1000 in PBS-T. All non-RNAscope sections were mounted
1262 on Superfrost Plus microscope slides (Fisher) and counterstained with Fluoromount-G
1263 containing DAPI (SouthernBiotech). Sections were stored long-term at 4˚C.

1264 ***RNAscope fluorescence* in situ *hybridization***

1265 RNAscope sections were cut at 15 µm on a CM 1950 cryostat (Leica) and mounted on
1266 Superfrost Plus slides and stored at -80˚C until processing via RNAscope within a month of
1267 mounting. RNAscope was performed in an RNA-free environment according to manufacturer
1268 instructions using the Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics).[107]
1269 RNAscope was performed using the probes *mm-Slc17a7* in the C2 channel, and *mm-Slc17a6* in
1270 the C3 channel, dyed with Opal 520 and Opal 690 in a counterbalanced manner at 1:15,000
1271 concentration to reduce background fluorescence and allow quantitation of unsaturated, clearly-
1272 distinguishable puncta.[108] Processed RNAscope sections were then mounted with Prolong
1273 Antifade Diamond (ThermoFisher) and stored long-term at 4˚C.

1274 ***Fluorescence image acquisition***

1275 Non-RNAscope Images were acquired at 10X magnification with an VS120 slide scanner
1276 (Olympus), with settings held constant within experiments. Confocal fluorescence images for
1277 RNAscope were acquired on an SP8 (Leica) confocal laser scanning microscope using a
1278 40x/1.30NA oil immersion objective. Serial Z-stack images were acquired using the LASX
1279 software at a thickness of 1 µm per Z stack, with 14-21 planes taken per image. Images were
1280 acquired with identical settings for laser power, detector gain, and amplifier offset for each set of
1281 counterbalanced probe-fluorophore combinations.

1282 **Sequencing Data Acquisition**

1283 ***Tissue extraction and cryopreservation for single-cell sequencing***

1284 Once unconscious, mice animals were transcardially perfused with ice-cold, carbogen-bubbled
1285 (95% O2, 5% CO2), nuclease-free, 0.22 µm sterile-filtered artificial cerebrospinal fluid (ACSF)
1286 with a composition of 93 mM N-methyl-D-glucamine, 2.5 mM KCl, 1.2 mM NaH2PO4, 30 mM
1287 NaHCO3, 20 mM HEPES, 25 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM
1288 sodium pyruvate, 13.2 mM trehalose, 12 mM N-acetyl-cysteine, 0.5 mM CaCl2, 10 mM MgSO4,
1289 and 93 mM HCl, at pH 7.3-7.4.[42,109] Following transcardial perfusion, brains were immediately
1290 extracted and submerged into ice-cold carbogen-bubbled ACSF, with less than 5 minutes
1291 between the beginning of perfusion and final submersion after extraction. Brains were serially
1292 sectioned in ice-cold, carbogen-bubbled ACSF on a VT1000S vibratome (Leica) with
1293 polytetrafluoroethane-coated razor blades (Ted Pella) at 0.15 mm/sec and 100 Hz, dividing the
1294 whole cerebrum into 400 µm coronal slices. Target regions were microdissected from these
1295 slices under a stereomicroscope using a sterile blunt-end needle (22 gauge for CeA, ASt, and
1296 tail of striatum, 16 gauge for dorsal striatum). All regions were targeted using Paxinos &
1297 Franklin, 5th Edition, as reference.[36] Extracted tissue samples were recovered in ice-cold,
1298 nuclease-free, 0.22 µm sterile-filtered cryoprotective nuclear storage buffer, composed of 0.32
1299 M sucrose, 5 mM CaCl2, 3 mM magnesium acetate, 10 mM Trizma hydrochloride buffer (pH
1300 8.0), 1 mM dithiothreitol, 0.02 U/µl SUPERase•In RNAse Inhibitor (Invitrogen), and 1X cOmplete
1301 Protease Inhibitor Cocktail with EDTA (Roche). Tissue was then snap frozen using a metal
1302 CoolRack M90 (Biocision) pre-chilled to -80˚C and stored at -80˚C until nuclear isolation.
1303 Following extraction of tissue regions of interest, remaining portions of sections were fixed in 4%
1304 paraformaldehyde and 4′,6-diamidino-2-phenylindole (DAPI) was applied to sections at 1 µg/ml.
1305 After fixation and staining, sections were mounted and imaged on an VS120 slide scanner
1306 (Olympus). From these images, dissection accuracy was assessed for each region, and
1307 individual samples were only selected for downstream nuclear isolation if the extracted tissue
1308 fell entirely within the defined target regions.

1309 Nuclear isolation procedures were adapted from multiple methods described previously.[102,110] All
1310 procedures were performed on ice, and all solutions were ice-cold, nuclease-free, and 0.22 µm
1311 sterile-filtered. Cryopreserved tissue pieces were slow thawed by incubation at 4˚C for 1 hour
1312 prior to isolation. Tissue pieces were then pooled and resuspended in nuclear isolation medium
1313 composed of 0.25 M sucrose, 25 mM KCl, 5 mM MgCl2, 10 mM Trizma hydrochloride buffer (pH
1314 7.4), 1 mM dithiothreitol, 0.04 U/µl RNasin Plus RNAse Inhibitor (Promega), 1X cOmplete
1315 Protease Inhibitor Cocktail with EDTA (Roche), and 0.1% Triton-X. The pooled tissue pieces in

1316 nuclear isolation medium were transferred to a 2 mL Dounce tissue grinder. Tissue was
1317 homogenized by 5 strokes from the loose pestle and 15 followed by the tight pestle, and the
1318 resulting homogenate was filtered through a 40 µm Flowmi cell strainer (Bel-Art) into a 1.5 ml
1319 Lo-Bind tube (Eppendorf). The homogenate was then centrifuged with a swinging bucket rotor
1320 at 4˚C and 1000 x g for 8 minutes. Nuclei were then washed with nuclear flow buffer composed
1321 of DPBS with 1% bovine serum albumin, 1 mM dithiothreitol, and 0.04 U/µl RNasin Plus RNAse
1322 Inhibitor (Promega) and centrifuged at 4˚C and 500 x g for 5 minutes, which was subsequently
1323 repeated. Nuclei were finally resuspended in nuclear flow buffer containing 3 µm DRAQ7 (Cell
1324 Signaling Technology) and again filtered through a 40 µm Flowmi cell strainer into a 5 ml round-
1325 bottom polystyrene tube. Each isolation took under 45 minutes to perform, from homogenization
1326 to final suspension.

1327 ***Fluorescence-activated nuclei sorting (FANS)***

1328 FANS was carried out on a FACSAria II SORP (BD Biosciences) using a 70 µm nozzle at 52
1329 PSI sheath pressure. For FANS, debris was first excluded by gating on forward and side scatter
1330 pulse area parameters (FSC-A and SSC-A), followed by exclusion of aggregates (FSC-W and
1331 SSC-W), and finally gating for nuclei based on DRAQ7 fluorescence (APC-Cy7). Nuclei were
1332 successively sorted into 1.5 ml LoBind tubes (Eppendorf) under the purity sort mode. The tube
1333 contained 10X RT master mix without RT Buffer C. 16,000 total nuclei were targeted for
1334 downstream processing, and to account for cytometer errors and subsequent loss of nuclei,
1335 21,000 were sorted into the tube. Nuclei were then immediately processed for snRNA-seq.
1336 FANS conditions were optimized for isolation of debris-free nuclei using the LIVE/DEAD
1337 Viability/Cytotoxicity Kit for Mammalian Cells (Molecular Probes), adding to the final suspension
1338 according to manufacturer instructions and examining on a hemocytometer using an EVOS FL
1339 Cell Imaging System (Thermo Fisher) for enrichment of ethidium homodimer-1-positive nuclei
1340 and the absence of Calcein AM-labeled cellular debris.

1341 ***Tissue extraction and sample preparation for spatial transcriptomics***

1342 Mice were euthanized with CO_2 followed by decapitation, either in the dark or in the light. Brain
1343 hemispheres were collected and placed in OCT and then flash frozen in isopentane in liquid
1344 nitrogen. One hemibrain from each mouse was cryosectioned at -18°C sagittally to a thickness
1345 of 10 mm (~2.8 mm from the midline) using a standard Leica CM1860 cryostat and processed
1346 according to the recommended protocols (Tissue optimization: CG000240 Visium 10X
1347 Genomics; Gene expression: CG000239). The tissue was immediately mounted on a Visium
1348 spatially barcoded slide (10X Genomics). The tissue was covered with OCT and kept at -80
1349 degrees C until it was cryosectioned again starting at the same position to a thickness of 10mm
1350 and mounted onto a Superfrost plus microscope slide (Fisherbrand) for staining. Each section
1351 covered approximately 80% of the 5,000 total spots within their fiducial frame. Slides were
1352 stored at -80˚C until use.

1353 **Library Preparation**

1354 ***Library preparation for single nucleus sequencing***

Nuclear suspensions were converted into barcoded snRNA-seq libraries using the Chromium Next GEM Single Cell 3' v3.1 Reagent Kits v3.1 Single Index (10X Genomics). Library preparation for both assays was performed in accordance with the manufacturer's instructions. 10,000 nuclei were targeted during each snRNA-seq library preparation run. 10X libraries were first sequenced at low depth on a NextSeq 550 Sequencing System (Illumina) to estimate quality and number of nuclei for each library, followed by deep sequencing on a NovaSeq 6000 Sequencing System. All runs were performed using 2 x 100-bp paired-end reads, outputting data in 28/8/91-bp read format for snRNA-seq runs.

Library preparation for Visium spatial transcriptomics

Visium spatial gene expression slides and reagents were used according to the manufacturer instructions (10X Genomics). Each capture area was 6.5mm x 6.5 mm and contained 5,000 barcoded spots that were 55 µm in diameter (100 µm center to center between spots) provide an average resolution of about 1 to 10 cells per spot. Optimal permeabilization time was measured at 24 min. Libraries were prepared according to the Visium protocol (10X Genomics) and sequenced on a NovaSeq4 (Illumina) at a sequencing depth of 182 million read pairs. Sequencing was performed with the recommended protocol in a 28/10/10/100-bp read format. H&E (Hematoxylin, Thermo; Dako bluing buffer, Dako; Eosin Y, Sigma) staining and image preparation was performed according to the Visium protocol. H&E-stained sections were imaged using a Nanozoomer slide scanner (Hamamatsu) Spatial gene expression assay was performed according to the protocol CG000239.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Analysis

All statistical details can be found in the figure legends. Sample sizes for behavioral studies were chosen based on past optogenetic studies for each behavior, which had used 6-15 animals per group. Blinding experimenters was not possible for behavioral, imaging, or sequencing experiments, given familiarity with subjects, but manual quantitation for connectivity experiments was performed blinded to group with random assignment. All statistical tests were performed in R (v4.2.3) unless otherwise specified. All statistical tests were performed with two tails. Group comparisons were made using two-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests, except where otherwise specified. All behavioral experiments were performed by multiple experimenters across multiple cohorts each composed of multiple litters, with littermates distributed across control and treatment groups, with all such cohorts yielding similar results (data not shown), and topography stimulation experiments were performed across multiple facilities and institutions. Numbers of mice used for all non-sequencing experiments are reported within the relevant figures, figure legends, and the text.

Calcium Imaging Data Analysis

Image processing

Data analysis for calcium imaging experiments was adapted from methods previously described elsewhere.[25] Ca^{2+} imaging data were first motion-corrected using the non-rigid motion correction algorithm NoRMCorre.[111] Afterwards, neural traces were extracted from the motion-corrected data using constrained nonnegative matrix factorization (CNMF).[112,113] Spatial components identified by CNMF were inspected by eye to ensure they were not artifacts. A Gaussian Mixture Model (GMM) was used to estimate the baseline fluorescence of each neuron. To account for potential low-frequency drift in the baseline, the GMM was applied along a moving window of 2,500 frames (500 seconds). The fluorescence of each neuron at each time point t was then normalized to the moving baseline to calculate $\Delta F/F = F_t - F_{baseline} / F_{baseline}$. All subsequent analysis was performed using custom code written in MATLAB (R2022b).

Hierarchical clustering of pooled averaged responses

ΔF/F in response to all 6 odors were averaged across trials then Z-scored. The resulting trial-average values from the following time bins were averaged across time: the first second during each odor, the last second during each odor, and the first second after each odor. The resulting 18-element vectors were sorted into 6 clusters after agglomerative hierarchical clustering using Euclidean distance and Ward linkage.

Responsiveness criteria

To determine how many neurons were responsive to a given odor, we compared ΔF/F at each frame during the 2 second odor period against a pooled distribution of ΔF/F values from the 2-seconds prior to odor onset using a Wilcoxon rank sum test. The resulting p-values were evaluated with Holm-Bonferroni correction to ensure that familywise error rate (FWER) was below 0.05. We also counted the number of neurons that were significantly responsive for at least 4 frames during the odor period to report the total percentage of responsive neurons during odor.

Single-neuron 6-odor classifiers

To test how reliably a single neuron's fluorescence could discriminate between the 6 odors, we assessed the performance of multinomial regression (MNR) classifiers trained on a single neuron's responses to 6 odors. For each neuron and odor pair, we averaged the ΔF/F during the last second of the odor exposure for each trial then Z-scored across all trials. The resulting 120-element vector was used to train the MNR classifiers. The 5-fold cross-validated accuracies are reported and plotted as violin plots.[114] As a control for each neuron, 100 shuffled classifiers were trained on the data with the odor labels randomly assigned.

Pairwise euclidean distance

To quantify the differences among population-level responses to the 6 odors, we quantified the pairwise Euclidean distance between the trajectories of odor responses. First, we subtracted the ΔF/F values during the 2 seconds prior to odor delivery from each frame then averaged these values across trials for each odor. The pairwise Euclidean distance at each frame was computed for each odor pair and normalized to the maximum pairwise distance measured in all

odor pairs at any time bin. These calculations were carried out separately for each animal and then averaged across biological replicates to report the mean and the standard error.

Population classifiers

To assess the discriminability of odor responses in high-dimensional space, we measured the accuracy of error-correcting output codes (ecoc) classifiers. At each time point relative to odor delivery, we pooled ΔF/F values from all trials during which either odor was presented. These values were then normalized and used to train a multinomial ecoc classifier using a Support Vector Machine (SVM). The accuracy of the classifier was evaluated via 5-fold cross-validation. To compare the classifier accuracies across different numbers of neurons used for training, we randomly selected varying numbers of pooled neurons and used the ΔF/F values during the last second of odor exposure for training.

Behavioral Data Analysis

Behavioral metrics (i.e. performance index, port distance, center distance, open field time, and total distance) for the four-quadrant preference test, open field test, and elevated plus maze were calculated on sets of coordinates created by identifying the centroid of the mouse in real time in video collected from an overhead camera (Basler) at 4 Hz using custom Labview code and outputting the centroid's coordinates for each frame. The mouse was automatically identified by taking a background greyscale image of the behavioral assay's environment at the beginning of each trial and detecting shapes of a minimum size deviating from the background image by a specific threshold. The centroid was then determined by automated generation of a bounding box for the mouse in each frame in real time and recording the coordinate of the centroid of this rectangle.

Four quadrant task data analysis

Mice were tested as previously described.[3] Mice were placed in the chamber for 25 min experiments and tested no more than once per day. The first 10 min served as a baseline test for spatial or temporal bias within the chamber during the trial, and no stimulus of any sort was provided, while the last 15 min were the test of the manipulation. 15 minutes was chosen to balance time courses of odor responses observed in previous experiments, where appetitive odors tend to yield initial responses that decay, while aversive odors tend to yield responses that grow in magnitude over time. To ensure effects did not arise from ceiling or floor effects in the baseline or from a nonstandard baseline internal state, the mouse had to remain within the stimulus quadrant during the baseline test between ~20-30% of the time or else the experiment was terminated, and the mouse was tested again later. The first 2 minutes of data after the stimulus was introduced were excluded from the analysis to reduce variance and account for novelty of the stimulus without affecting the overall valence of the behavioral response, and the last minute of data was excluded to ensure no minor differences in frames captured could affect analysis. For chemogenetic odor response silencing experiments, animals with vehicle odor responses below an absolute value of 0.1 were excluded from experiments to avoid false negatives from attempting to silence a response that was not observed at baseline, which applied to less than a quarter of overall animals tested across experimental conditions.

1470 Performance index represents the percent difference from chance occupancy in the
1471 manipulation quadrant, calculated as PI = (P - 25) / 0.25; where P is the percentage of time the
1472 animal spends in the manipulation quadrant. Mean port distance represents the mean distance
1473 of each point to the deepest point into the manipulation quadrant observed at baseline.

1474 ***Open field test data analysis***

1475 For elevated plus maze analysis, all chemogenetic inhibition trials used the entirely of the 10
1476 min test length as a single period, while optogenetic stimulation trials used the mean of the
1477 three "OFF" periods to compare to the mean of the two "ON" periods. Three metrics of interest
1478 were calculated. Center time was calculated as the proportion of time spent in the middle
1479 square of the open field comprising 50% of its total area. Corner time was calculated as the
1480 proportion of time spent in the corner squares bounded by the walls and the lines bounding the
1481 center region. Time immobile was calculated as the proportion of time when the animal moved
1482 less than 1 cm/s for at least a one-second period. Location of the open field and bounding
1483 regions was kept constant from trial to trial by registering the apparatus to a bounding box with
1484 the same top-down dimensions, and every measured centroid outside of the registered region
1485 resulting from shadows cast and other artifacts was interpolated between the closest points
1486 before and after within the region.

1487 ***Elevated plus maze data analysis***

1488 For elevated plus maze analysis, all chemogenetic inhibition trials used the entirely of the 10
1489 min test length as a single period, while optogenetic stimulation trials used the mean of the two
1490 "OFF" periods to compare to the "ON" period. Three metrics of interest were calculated. Time in
1491 the open arms was calculated as the proportion of time spent in the open arms compared to the
1492 whole period of interest and did not include time in the center between the two arms. Open arm
1493 entries measures number of episodes where the centroid is observed outside of the bounds of
1494 the closed arms or the center region, without any minimum time or distance out onto the open
1495 arms. Finally, distance was simply calculated as the distance traveled during each period of
1496 interest. Location of open and closed arms was kept constant from trial to trial by registering the
1497 apparatus to a cross-shaped bounding box with the same top-down dimensions, and every
1498 measured centroid outside of the registered region due to factors like the mouse leaning over
1499 the edge of the open arms, among others, was interpolated between the closest points before
1500 and after within the region.

1501 **Analysis of Single-Nucleus RNA Sequencing Data**

1502 ***Sequence alignment***

1503 All samples were processed using Cell Ranger (v5.0.0).[37] All processing was done by using Cell
1504 Ranger's implementation of STAR to align sample sequence reads to their pre-built mm10
1505 vm23/Ens98 reference transcriptome index 2020-A, with predicted and non-validated transcripts
1506 removed. All sequencing reads were aligned to both the exons and the introns present in the
1507 index. Samples were demultiplexed to produce a pair of FASTQ files for each sample. FASTQ
1508 files containing raw read sequence information were aligned to the Cell Ranger index using the

1509 cellranger count command with --chemistry SC3Pv3 and --include-introns flags enabled. Cell
1510 Ranger corrected sequencing errors in cell barcodes to pre-defined sequences in the 10X v3
1511 single-index whitelist within Hamming distance 1. PCR duplicates were removed by selecting
1512 unique combinations of corrected cell barcodes, unique molecular identifiers, gene names, and
1513 location within the transcript. Raw unfiltered count data was read into R (v4.2.1) using the
1514 Seurat package (v4.2.0).[115–118] The final result of the pipeline was a barcode x gene expression
1515 matrix for further analysis downstream.

1516 ***Quality control***

1517 We used the raw, unfiltered matrix output from Cell Ranger as the input to the beginning of the
1518 pipeline. However, to apply a more stringent filter, the emptyDrops dirichlet-multinomial model
1519 from the DropletUtils package (v1.10.2) was applied to each library individually.[119,120] Droplets
1520 with less than 100 total counts were used to construct the ambient RNA profile and an FDR
1521 threshold below 0.001 was used to select putatively occupied droplets. All barcodes with greater
1522 than 1000 UMIs were further assumed non-empty. Most quality filtration choices were heavily
1523 influenced by the recommendations presented in pipeComp.[121] All quality control was performed
1524 on each library individually prior to merging. Minimal quality filtering for each barcode was
1525 performed by setting a floor of 1000 features per barcode for downstream inclusion to ensure
1526 the dataset is entirely composed of high-quality nuclei. Next, to remove highly likely multiplet
1527 barcodes, barcodes were filtered out if their count depth was more than 5 median absolute
1528 deviations above the median count depth. Barcodes were then removed if their proportion of
1529 ribosomal or mitochondrial reads was more than 5 interquartile ranges above the 75th percentile
1530 (median absolute deviations cannot be used, because in many cases the median absolute
1531 deviation is 0). Heterotypic doublets were identified by creating simulated artificial doublets in
1532 scDblFinder (v1.4.1), which uses a DoubletFinder-like model to remove barcodes similar to
1533 simulated doublets, with an assumed doublet rate of 1% per 1000 nuclei in the library.[122,123]
1534 Scater (v1.18.3) was used to produce initial diagnostic tSNE and UMAP plots for visually
1535 checking the influence of each above metric on the structure of the data.[124]

1536 ***Data processing/transformation***

1537 All datasets (initially for all nuclei and again for selected subclusters) were formatted into Seurat
1538 objects (v4.0.0), merged, and then normalized and transformed individually using the
1539 SCTransform (v2) variance stabilizing transform, which performs best according to prior
1540 comparisons in pipeComp.[121,125,126] Following the merge, all genes expressed in 3 or fewer
1541 nuclei of interest were removed from analysis. SCTransform was run returning Pearson
1542 residuals regressing out mitochondrial gene expression, retaining the top 5000 highly variable
1543 features. Dimensionality of the dataset was first reduced using principal component analysis, as
1544 implemented in Seurat's RunPCA function, retaining the top 50 principal components.[127]
1545 Principal components were selected for downstream use by using the lower value of either the
1546 number of principal components where the lowest contributes 5% of standard deviation and all
1547 cumulatively contribute 90% of the standard deviation, or the number of principal components
1548 where the percent change in variation between the consecutive components is lower than 0.1%.
1549 These principal components were used as input to the non-linear tSNE and UMAP

1550 dimensionality reduction methods as implemented by Seurat's RunTSNE and/or RunUMAP
1551 functions with 1000 epochs at 0.5 minimum distance, with otherwise default settings.[128–130]

1552 Clusters were identified via Leiden clustering in latent space using the previously selected
1553 principal components as input.[131] Optimal clustering resolution was identified in a supervised
1554 manner using clustree, finding the highest resolution for each dataset where clustering remains
1555 stable.[132] Cluster annotation was performed in a semi-hierarchical semi-supervised manner,
1556 where known marker genes were first used to separate all nuclei into neuronal and non-
1557 neuronal cell types, and then these cells were re-analyzed and neurons were respectively
1558 separated into glutamatergic and GABAergic neurons, while non-neuronal cells were separated
1559 into astrocytes, microglia, macrophages, oligodendrocytes and their precursors/lineage, mural
1560 cells, endothelia, and vascular leptomeningeal cells. This lower level of cells was then
1561 reanalyzed, and novel cell types were then identified within these more-granular known cell
1562 types. For identification of known cell types, clusters expressing the same marker genes were
1563 manually merged to ensure all cells of a known type were analyzed together, which did not
1564 occur for novel cell type identification. Clusters resulting from specific difference in nuclei quality
1565 instead of true changes in gene expression (i.e. markedly lower mean UMI/features per nucleus,
1566 increased ribosomal/mitochondrial gene expression proportion) were removed prior to final
1567 clustering. Relationships between cell type proportion and plCoA zone were quantitated using
1568 propeller, treating each library as an independent replicate.[133]

1569 ***Differential expression***

1570 Marker genes were identified using Wilcoxon rank-sum test as implemented by the
1571 FindConservedMarkers function in Seurat, using the region as a grouping variable. Genes were
1572 accepted as differentially expressed with a minimum proportion cutoff at 0.1 and minimum fold
1573 change at 1.5-fold (log2-fold change of 0.585), with a p-value cutoff of 0.01 after Bonferroni
1574 correction. To identify genes differentially expressed by region, single-cell values were
1575 converted to pseudo-bulk by batch using the run_de function as implemented in the Libra
1576 package (v1.0.0) using default settings with a minimum proportion cutoff at 0.1, and tested for
1577 differential expression using edgeR's likelihood ratio test.[134] Zone-specific gene expression was
1578 identified by comparing batches from the two isolated zones.

1579 **Analysis of Spatial Transcriptomics Data**

1580 ***Sequence and image alignment***

1581 All samples were processed using Space Ranger (v1.3.0). All processing was done by using
1582 Space Ranger's implementation of STAR to align sample sequence reads to their pre-built
1583 mm10 vm23/Ens98 reference transcriptome index 2020-A, with predicted and non-validated
1584 transcripts removed, as in snRNA-seq data alignment. Samples were demultiplexed to produce
1585 a pair of FASTQ files for each sample. FASTQ files containing raw read sequence information
1586 were aligned to the index using the spaceranger count command. Space Ranger corrected
1587 sequencing errors in cell barcodes to pre-defined sequences in the single-index whitelist within
1588 Hamming distance 1. PCR duplicates were removed by selecting unique combinations of
1589 corrected cell barcodes, unique molecular identifiers, gene names, and location within the

transcript. Imaging data was processed using automatic fiducial alignment and tissue detection on a brightfield input.

Data processing/transformation

We used the image-filtered matrix output from Space Ranger as the input to the beginning of the pipeline. In a similar manner to snRNA-seq data, all datasets were formatted into Seurat objects (v5.0.0), merged, and then normalized and transformed using the SCTransform (v2) variance stabilizing transform. SCTransform was run returning Pearson residuals regressing out mitochondrial gene expression, retaining the top 5000 highly variable features. Dimensionality of the dataset was first reduced using principal component analysis, as implemented in Seurat's RunPCA function, retaining the top 50 principal components, all of which were used in downstream processing. These principal components were used as input to the non-linear tSNE and UMAP dimensionality reduction methods as implemented by Seurat's RunTSNE and/or RunUMAP functions with 1000 epochs at 0.2 minimum distance, with otherwise default settings. Clusters were identified via Leiden clustering in latent space using all 50 principal components as input. Optimal clustering resolution was identified in a supervised manner using clustree, finding the highest resolution for each dataset where clustering remains stable, choosing a resolution of 0.7. Cluster annotation was performed in a semi-supervised manner, observing where in captured plCoA regions each cluster's spots localized to. For clusters that could not be annotated from spatial location alone (e.g. OLG), marker genes were examined to determine the molecular identity of relevant spots. Spatial data was projected onto neuronal molecular cell types from snRNA-seq data and cell type likelihood was predicted using Seurat's FindTransferAnchors and TransferData functions using snRNA-seq data as a reference and plCoA spatial data as the query, using all 50 PCs. Prediction of a minority of subtypes failed, likely due to low abundance in tissue and/or due to mediolateral spatial differences, alluded to in a separate study, causing the section not to intersect with the part of the tissue containing the relevant neuronal subtypes.[135] Glutamatergic and GABAergic molecular subtype likelihoods were predicted separately to remove noise and increase modeled prediction confidence.

Histological Image Analysis

Registration and localization

Histology for all animals and samples was examined prior to inclusion. Localization within the coronal plane was determined by registering the coronal slice to the Allen Brain Atlas via the ABBA plugin, using elastix to sequentially perform affine and spline registration of the DAPI channel of the slice to the Nissl channel of the atlas.[136,137] The region of interest was then compared to the Paxinos and Franklin atlas to confirm localization, and find the region's anteroposterior distance from bregma.[36] This combined method was used because sections cannot be accurately registered to the Paxinos and Franklin atlas due to low Z-resolution, while the Allen Brain Atlas lacks information about anteroposterior distance from bregma. Exclusion based on histology would occur when most of the intervention fell outside of the region of interest. Due to these differences, individual representative images use the individually registered Allen Reference Atlas schematics with the comparable Paxinos and Franklin

1630 anteroposterior coordinates noted, while consolidated targeting schematics use the Paxinos and
1631 Franklin atlas for visualization.

1632 ***Quantification of histological fluorescence***

1633 In anterograde tracing experiments, output quantification was performed based on background-
1634 corrected total fluorescence. For all non-collateralization anterograde experiments, fluorescence
1635 intensities were quantified using FIJI (v2.9.0) throughout the whole brain in a series of evenly-
1636 space 50 µm coronal sections, manually segmenting by region with all settings held constant
1637 within experiments.[138] For collateralization experiments, we exclusively examined fluorescence
1638 in the MeA and NAc. We calculated background-corrected total fluorescence using the equation
1639 $F_{total} = ID - (Area \times F_{background})$, where F_{total} is the background-corrected total fluorescence, *ID* is
1640 the integrated density, and $F_{background}$ is the mean background fluorescence measured from four
1641 randomly selected areas per section not receiving input from plCoA. Overall proportion was
1642 calculated by taking the sum of background-corrected fluorescence values across all sections
1643 for a given region and dividing it by the sum of all background-corrected values. For retrograde
1644 experiments, we quantified number of cells using the Cell Counter plugin (v3.0.0) in FIJI. The
1645 sagittal brain slices containing the plCoA were then compared to Paxinos and Franklin, 5th
1646 Edition to count the number of cells found per distance away from bregma from -1.3 to -2.5 mm
1647 in increments of 100 µm.[36] At least two sections per region per animal were analyzed.
1648 Representative images were produced from slide scanner image output, with background
1649 subtraction and uniform brightness and contrast thresholds applied equally to all fluorescent
1650 channels in FIJI to avoid potential distortion of visible fluorescence levels.

1651 ***Quantification and analysis of RNAscope images***

1652 RNAscope images were analyzed as previously described.[86] Images were opened in FIJI and
1653 individual Z-planes encompassing the entire ROI were selected from each image for further
1654 image processing. Background was subtracted from all channels in all images using the
1655 subtract background feature. Masks of each region were drawn based on the mouse brain atlas,
1656 and images were then saved as 8bit TIFFs for further cell and puncta identification in
1657 CellProfiler (v4.2.4).[36,139] Image TIFFs were run through CellProfiler using an optimized version
1658 of the CellProfiler Colocalization pipeline. The pipeline was optimized to identify DAPI labelled
1659 cells (15-45 pixels in diameter) and then subsequently identify mRNA puncta (4-10 pixels in
1660 diameter). DAPI cell detection was further restricted by shrinking DAPI ROIs by 1 pixel. Puncta
1661 overlapping with DAPI-identified cells (using the relate objects module) were considered for
1662 analysis to assess the level of mRNA expression per cell. To determine if cells were expressing
1663 mRNA, a threshold of 5 or more puncta within twice the diameter of nucleus centered over the
1664 nucleus was used.[140] Total number and density of *Slc17a6*+ and *Slc17a7*+ cells in each region
1665 of interest were calculated from CellProfiler .csv outputs using custom R scripts.

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