

1 **The Neural Basis for a Persistent Internal State in Drosophila Females**
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19

20 **Abstract**

21 Sustained changes in mood or action require persistent changes in neural activity, but it has
22 been difficult to identify the neural circuit mechanisms that underlie persistent activity and
23 contribute to long-lasting changes in behavior. Here, we show that a subset of Doublesex+ pC1
24 neurons in the Drosophila female brain, called pC1d/e, can drive minutes-long changes in
25 female behavior in the presence of males. Using automated reconstruction of a volume electron
26 microscopic (EM) image of the female brain, we map all inputs and outputs to both pC1d and
27 pC1e. This reveals strong recurrent connectivity between, in particular, pC1d/e neurons and a
28 specific subset of Fruitless+ neurons called alPg. We additionally find that pC1d/e activation
29 drives long-lasting persistent neural activity in brain areas and cells overlapping with the pC1d/e
30 neural network, including both Doublesex+ and Fruitless+ neurons. Our work thus links minutes-
31 long persistent changes in behavior with persistent neural activity and recurrent circuit
32 architecture in the female brain.

33

34 **Introduction**

35 Social behaviors are known to be affected by persistent internal states (Anderson, 2016;
36 Berridge, 2004; Lorenz and Leyhausen, 1973). These states correspond with levels of arousal
37 or drive, and can impact whether and how individuals interact, with consequences for mating
38 decisions and reproduction (Chen and Hong, 2018; Kennedy et al., 2014; Stowers and Liberles,
39 2016). The neural mechanisms underlying arousal states remain largely unknown.

40

41 In male flies, a small population of male-specific neurons (P1) that express the sex-specific
42 transcription factors *Fruitless* and *Doublesex* (Auer and Benton, 2016), drive both male-
43 aggression and male-mating behaviors (Hooper et al., 2015; Koganezawa et al., 2016; von
44 Philipsborn et al., 2011). Brief optogenetic activation of P1 neurons drives both persistent song
45 production in solitary males and persistent aggression upon introduction of another male, both
46 over minutes Bath et al., 2014; Hooper et al., 2015; Inagaki et al., 2014). While P1 activation is
47 sufficient for eliciting the persistent behavioral phenotypes, other groups of neurons, such as
48 pCd also called pC3 (Rideout et al., 2010)), are involved in maintaining the persistent state
49 (Jung et al., 2020; Zhang et al., 2019) - these neurons are not synaptically coupled to P1, and
50 the circuit architecture that mediates persistence remains unresolved. P1 neurons also receive
51 dopaminergic input that affects mating drive over longer timescales of hours (Zhang et al.,
52 2016), suggesting neuromodulation is also important for persistent changes in behavior driven
53 by P1. Work on P1 in flies bears some similarity to work in mice. In male mice, optogenetic
54 activation of SF1 (steroidogenic factor 1) expressing neurons in the dorsomedial part of the
55 ventromedial hypothalamus ($\text{VMHdm}^{\text{SF1}}$) drive multiple defensive behaviors that outlast the
56 stimulation by up to one minute (Kunwar et al., 2015; Wang et al., 2015). In addition, activity of
57 $\text{VMHdm}^{\text{SF1}}$ neurons can persist for over a minute following the presentation of a fear stimulus.
58 Computational modeling work suggests a mix of recurrent circuitry and neuromodulation
59 underlie the persistence (Kennedy et al. 2020), but this has not yet been tested.

60

61 We know little about the persistence of social behaviors in females across taxa. P1 neurons are
62 a subset of the larger *Doublesex*+ pC1 neural subset Kimura et al., 2008). While *Drosophila*
63 females lack P1 neurons, they have *Doublesex*+ pC1 neurons (Rideout et al., 2010; Robinett et
64 al., 2010; Zhou et al., 2014), including a subset that are female-specific (Wu et al., 2019).
65 Activation of pC1 neurons affects receptivity toward males (Wang K., et al., 2020; Zhou et al.,
66 2014), drives chasing of males (Rezával et al., 2016; Wu et al., 2019), and aggressive
67 behaviors toward females (Palavicino-Maggio et al., 2019; Schretter et al., 2020). These data

68 suggest that female pC1 neurons can drive an arousal state, similar to male P1 neurons, but
69 whether female pC1 neurons can drive persistent changes in behavior and persistent neural
70 activity, has not yet been investigated. Importantly, a complete electron microscopic volume is
71 currently available for the entire adult female brain (Zheng et al., 2018), making it possible to
72 link brain activity to complete wiring diagrams in females. Direct measurements of synaptic
73 connectivity can determine whether recurrent neural networks, known to be important for
74 persistent neural activity lasting for seconds (Aksay et al., 2007), also underlie minutes-long
75 persistent activity and changes in behavior, as has been recently proposed for male mice and
76 flies (Jung et al. 2020; Kennedy et al. 2020).

77

78 Here, we show that pC1 activation drives persistent changes in female behavior for minutes
79 following stimulus offset, and We identify the subset of pC1 neurons (called pC1d/e (Wang F. et
80 al., 2020); also referred to as pC1-Alpha (Wu et al., 2019)) that affects the persistent aggressive
81 and male-like behaviors. A companion study (Schretter et al., 2020) demonstrates that pC1d,
82 but not pC1e, drives female aggressive behaviors. By leveraging the automated segmentation
83 of an electron microscopic volume of the female brain (Dorkenwald et al., 2020; Zheng et al.,
84 2018), we map all inputs and outputs of both pC1d and pC1e and find a strong recurrent neural
85 network with Fruitless+ alPg neurons. Using pan-neuronal calcium imaging, we find that pC1d/e
86 activation can elicit persistent activity for minutes among multiple cell types. The persistent
87 activity is present in Doublesex+ and Fruitless+ neurons, including pC1 neurons themselves.
88 We thus link minutes-long persistent neural activity and behavior with reciprocal connectivity in
89 the female brain.

90

91 **Results**

92 **Female pC1 activation persistently modulates both female receptivity and song
93 responses**

94 To investigate the neural basis of a persistent internal state in the female brain, we focused on
95 pC1 neurons, one of eight Doublesex-expressing cell types in the central brain (Figure
96 1A;(Kimura et al., 2015)). We used an intersection between two driver lines (Dsx-GAL4 and
97 R71G01-LexA , hereafter referred to as pC1-Int; see Fly genotype table for list of genotypes
98 used in this study), to label pC1 neurons, as done previously (Rezával et al., 2016; Zhou et al.,
99 2014) – prior work shows this line labels, per hemisphere, ~6 neurons, all of which project to the
100 lateral junction (Zhou et al. 2014). We tracked male and female body parts (head and thorax) in
101 addition to recording all sounds (Figure 1B, Figure 1 – figure supplement 1A, Video 1; see
102 Methods for details on song segmentation and tracking of flies on a non-homogenous
103 background). Silencing pC1-Int neurons in females affects receptivity (Zhou et al., 2014); we
104 corroborated these results (Figure 1C) and additionally showed that silencing pC1-Int neurons
105 diminished responses to male song (Figure 1D). Persistent changes in the behavioral state of
106 Drosophila males have been studied by examination of social behaviors following optogenetic
107 activation of P1 neurons (Hooper et al., 2015; Jung et al., 2020). Similarly, we activated pC1-Int
108 in a solitary virgin female for 5 minutes, followed by a variable delay period, after which a virgin
109 male was introduced to examine female behaviors in the context of courtship (Figure 1E) - there
110 was no optogenetic activation following the first 5 minutes. The activity of stimulated neurons
111 should decay during the variable delay period (d0 (0-minute delay), d3 (3 minute delay), or d6 (6
112 minute delay)) – below, we test this explicitly with neural imaging, and also examine shorter
113 activation periods. This paradigm therefore allowed us to examine the effects of differing levels
114 of persistent activity on behavior.

115

116 Experimental flies were fed all-trans-retinal (ATR), which is required for ReaChR (red-shifted
117 Channelrhodopsin) function in flies (Inagaki et al., 2014)). Control flies shared the same
118 genotype but were not fed ATR. We found that activation of pC1-Int neurons induces a
119 persistent effect on female receptivity and responses to male song, but that this effect
120 diminishes with a delay period between neural activation and introduction of a male. pC1-Int
121 activated females copulated significantly faster than controls in the d0 condition, with reduced
122 copulations following a delay between optogenetic activation and introduction of a male (Figure
123 1F). About 75% of the flies copulated within 5 minutes in the d0 and d3 conditions, compared
124 with fewer than 50% in the control and d6 groups (Figure 1F, inset). Another set of Dsx+

125 neurons called pCd1 (Figure 1A) was also shown to control female receptivity (Zhou et al.,
126 2014) and, additionally, to enable P1 induced persistent activity in males (Jung et al., 2020).
127 While we confirmed that pCd1 silencing in females reduced receptivity (Figure 1 – figure
128 supplement 1B), we found that 5 minute pCd1 activation had no persistent effect on female
129 receptivity (Figure 1 – figure supplement 1C).

130

131 Activating pC1-Int also produced a persistent effect on responses to male song, overall with the
132 strongest effect at the d0 delay (Figure 1G) - d0 females, in comparison with controls,
133 accelerated more in response to all song elements, behaving like unreceptive females (Coen et
134 al., 2014). This effect was not due to changes in wild type male song structure for the d0
135 condition, although male song bouts were shorter and less frequent in the d3 and d6 conditions
136 (Figure 1 – figure supplement 1D), possibly due to the strong effect of pC1-Int activation on
137 male-female interactions at longer delays, as shown below. Because at d0, females are both
138 hyper-receptive (mate quickly) but also accelerate in response to song, we suspect that different
139 subsets of pC1 neurons may control these two effects.

140

141 **Female pC1 activation drives persistent female shoving and chasing**

142 To quantify other behaviors elicited by pC1-Int activation, we used an unsupervised approach,
143 and first decomposed male and female movements and interactions into 17 parameters
144 (Calhoun et al., 2019) (Figure 2A). We then used a Support-Vector Machine (SVM) framework
145 (Cortes and Vapnik, 1995; Cristianini et al., 2000) to find the weights that best classify single
146 frames as belonging to sessions of control vs experimental groups (all delay conditions, d0-d6).
147 We found that the weights of 8 out of 17 parameters were significantly different from zero
148 (Figure 2B and Figure 2 – figure supplement 1A), with the strongest weight being fmAngle,
149 defined as the degrees the female needs to turn in order to point toward the male centroid. The
150 weight of fmAngle is negative because this parameter is smaller in the experimental flies
151 compared with controls, indicating that pC1-Int activated females spend more time facing the
152 male (Figure 2 – figure supplement 1B). When separated by experimental condition, the SVM
153 classifier performed best on the d3 condition versus control (Figure 2C), indicating that male-
154 female movements and interactions are most distinct following this delay.

155

156 Next, we clustered individual video frames based on the values of the 8 parameters identified as
157 most important by the SVM (Figure 2 – figure supplement 1A, asterisks), and found that the
158 largest 7 clusters accounted for over 90% of all frames (Figure 2D, inset). The weights of the 8

parameters were different for each cluster (Figure 2 – figure supplement 1C), representing different behaviors. Five of the clusters describe behaviors that are the same or reduced following pC1-Int activation (clusters 1 and 3, male chasing female, and 5-7, increased male-female distance). Two clusters, however, describe behaviors that occur with higher probability following pC1-Int activation (clusters 2 and 4; Figure 2D). Cluster 2 is characterized by small fmAngle and small mfAngle (indicating that the male and female are facing each other), decreased male-female distance, and large fmFV (indicating that the female is close to the male and moving in his direction). Cluster 4 is characterized by small fmAngle and large mfAngle (indicating that the female is behind the male), decreased male-female distance, and large fmFV (indicating that the female is moving in the direction of the male). Based on the weight values, and verified by inspection of the videos following clustering (Video 2), we termed cluster 2 ‘female shoving’ and cluster 4 ‘female chasing’. For both female shoving and female chasing, the amount of each behavior was highest in the d3 condition relative to control (Figure 2D).

We then used JAABA (Kabra et al., 2013) to train a classifier to recognize epochs (groups of video frames) of female chasing and female shoving in the data (Figure 2 - figure supplement 1; see Methods). This analysis confirmed that the amount of female shoving and chasing was greatest in the d3 condition relative to control (Figure 2E), in addition to revealing that the duration of female chasing and shoving bouts was longer (Figure 2 - figure supplement 1E). In many cases females transitioned directly between shoving and chasing, and the conditioned probability (transition probability, given that a transition occurred) for shoving →chasing was more than double at the d3 condition compared to the d0 and d6 conditions. By examining shoving and chasing probabilities over time (Figure 2F), following the introduction of a male ($t = 0$), we found that both behaviors persisted for as long as 30 minutes in the d3 condition (Figure 2F), but not in the d0 and d6 conditions, suggesting a complex interaction between neural activity in the female brain at the time of introduction of the male and feedback or social cues. While the percent of time females spent shoving or chasing in the first two minutes after the male was introduced was similar in the d0 and d3 conditions (19.7/21.3% for shoving, 2.8/3.1% for chasing), shoving and chasing probabilities rose over time in the d3, but not in the d0 condition. In the d6 condition, shoving probability was comparable to the probability in the d0-d3 conditions in the first two minutes (15%), but decayed to control level after 6 minutes. The transition into ‘chasing’ from ‘shoving’ or from ‘other’ (no shoving and no chasing) epochs also peaked at d3 (Figure 2 – figure supplement 2). In all cases, we only examined female behaviors prior to copulation. We also activated another Dsx+ cell type, pCd1, which is part of a circuit that

193 drives persistent behavior in the male brain (Jung et al. 2020), but observed neither shoving nor
194 chasing following 5 minutes of pCd1 activation in females (Figure 2 - figure supplement 2A).
195 This suggests that the persistent behavioral effects we observe are specific to pC1 activation.

196
197 Manual inspection of the videos identified several additional behaviors produced by females
198 following pC1-Int activation (Figure 2 - figure supplement 3). These include ‘female
199 approaching’, ‘circling’, ‘head-butting’, and ‘female wing extension’ (Figure 2 - figure supplement
200 3A-D; Videos 2,3). We found that some of these behaviors were coupled; for example, ‘circling’
201 was often preceded by ‘female shoving’ (Figure 2 - figure supplement 3B, inset and Video 3)
202 and ‘female wing extension’ was often coincident with ‘female chasing’ (Figure 2 - figure
203 supplement 3E and Video 2), similar to male behavior during courtship (although we did not
204 observe sounds from the females that resembled male courtship song (Figure 2 - figure
205 supplement 3D)). Our automated behavioral classifier (Figure 1B) did not find these behaviors
206 because we only tracked the head and thorax of each fly, which did not provide enough
207 information to automatically identify these behaviors, or to keep accurate track of identities
208 during behaviors in which the male and female often overlap (e.g., during ‘circling’).

209
210 In sum, we found that for minutes following pC1-Int activation, females produced a variety of
211 behaviors directed at the male. Some of these appear aggressive, such as shoving and head-
212 butting (Nilsen et al., 2004; Palavicino-Maggio et al., 2019; Schretter et al., 2020), while others
213 resemble male courtship behaviors, such as chasing and unilateral wing extension. These
214 behaviors typically peaked in the d3 condition, where they remained high after male
215 introduction. In contrast, the effect on female receptivity and female responses to male song,
216 were both strongest in the d0 condition, ruling out the possibility that the effect on female
217 receptivity is an indirect consequence of modified male courtship behavior in response to
218 changes in female behaviors. Below we identify the pC1 cell types that drive persistent shoving
219 and chasing, determine that it is part of a recurrent neural network using a new EM connectomic
220 resource, and demonstrate that it can drive persistent neural activity on timescales similar to
221 behavior.

222
223 **pC1 cell types**
224 We propose three possible circuit configurations to explain our behavioral results (Figure 3A). In
225 the first configuration, the same set of pC1 neurons activate different downstream circuits, each
226 one controlling a different behavior. The differences in the temporal dynamics arise downstream

227 of pC1. In the second configuration, three non-overlapping subsets of pC1 neurons control the
228 different behaviors. In the third configuration, one pC1 subset controls female receptivity (that
229 peaks at d0), and another set controls chasing and shoving (both peaking at d3). The second
230 and third models assume some functional heterogeneity in the pC1 population. To evaluate
231 these circuit models, we examined the behavioral consequences of activating distinct subsets of
232 pC1 neurons. To define pC1 cell types, we used automated reconstruction of neurons in an EM
233 volume of a female brain (FAFB; Zheng et al., 2018); neuron segmentation and reconstruction
234 were accomplished using a novel platform for visualization and proofreading called FlyWire
235 (Dorkenwald et al. 2020). We examined the morphologies of neurons that send projections to
236 the lateral junction through a thin neuronal bundle, as pC1 neurons are known to (Figure 1A, red
237 arrow; Figure 3B, red arrow; see also (Deutsch et al., 2019 and Zhou et al., 2014)).

238

239 We systematically checked all cell segments that pass through a cross section in the pC1
240 bundle (Figure 3 - figure supplement 1A) and excluded neurons that do not project to the lateral
241 junction (Figure 3 - figure supplement 1B-D), as all pC1 cells characterized so far project to the
242 lateral junction (Kimura et al., 2015; Rezával et al., 2016; Wu et al., 2019; Zhou et al., 2014).
243 We found 5 pC1 cells per hemisphere (Table 1), consistent with the cell types found from
244 manual tracing in the same EM volume, although with differences in some projections (Figure
245 3C; one cell/hemisphere for pC1a-e was also found in the hemibrain, a second EM dataset of
246 the adult female brain (Scheffer et al., 2020)).

247

248 **The effects of pC1 subtypes on behavior**

249 We used genetic intersections to label two non-overlapping pC1 subpopulations. pC1-A labels a
250 single pC1d and a single pC1e neuron in each hemisphere ((Wu et al., 2019); same as line
251 pC1dSS3 (Schretter et al., 2020)) and no cells in the VNC (Figure 3D and Figure 3 - figure
252 supplement 2; 2 ± 0 , n =7). the second intersection (pC1-S, a split-Gal4 intersection between
253 Dsx.DBD (Pavlou et al., 2016) and R71G01.AD ; Figure 3D and Fly genotype table) does not
254 label pC1d or pC1e cells, as evidenced by absence of the medial projections of pC1d/e neurons
255 (Figure 3C-D). The pC1-S line labels 7.7 ± 4 pC1 cells per hemisphere in the brain, and no cells
256 in the VNC (Figure 3 - figure supplement 2C). All cells in the pC1-S line project to the lateral
257 junction (Figure 3D).

258

259 Next, we tested if activation of these two non-overlapping pC1 sub-populations drives persistent
260 behavioral phenotypes. Activation of either line pC1-A or pC1-S did not affect female receptivity

261 (Figure 4A, D), but activation of pC1-A drove persistent shoving and chasing (Figure 4B-C),
262 while activation of pC1-S did not (Figure 4E). These results are consistent with model 3 (Figure
263 3A), in which female receptivity and female shoving/chasing are driven by different populations,
264 and also consistent with prior work, showing that 10 minutes of thermogenetic pC1-A activation
265 drives persistent chasing in females (Wu et al., 2019). pC1d/e neurons are female-specific (Wu
266 et al., 2019), and prior work reveals that pC1 neurons in females, but not males, respond to
267 courtship song (Deutsch et al., 2019). Using *in vivo* whole-cell patch clamp recordings of
268 neurons labeled in the pC1-A line, we found that pC1d/e neurons in virgin females respond with
269 depolarizing responses to features in conspecific courtship song (Figure 4 – figure supplement
270 1). This finding indicates that pC1d/e neurons can be activated during courtship by male song.

271

272 When examining the fraction of time spent shoving or chasing across the three conditions
273 (control, d0 and d3) to compare results of activating neurons in pC1-A and pC1-Int lines (Figure
274 4F), we found that levels of female chasing are increased with pC1-A activation (relative to pC1-
275 Int) at d3. The levels of shoving are slightly decreased at d0, but this effect was not significant.
276 This suggests that pC1 cell types in the pC1-Int driver, other than pC1d/e (and other than the
277 cells in the pC1-S driver), have a modulatory effect on the shoving and chasing behaviors, at
278 distinct timepoints.

279

280 pC1-Int, but not pC1-A, activation drives persistent female receptivity (Figure 1F vs Figure 4A),
281 indicating that pC1-Int cells other than pC1d/e drive female receptivity (though high copulation
282 rates in control flies could mask a potential effect of pC1-A on female receptivity). We
283 hypothesized that the same pC1-Int cells that drive female receptivity, also affect the probability
284 of shoving and chasing. If true, we would expect a different rate of shoving and chasing in
285 receptive and non-receptive females. Indeed, we found that following pC1-Int activation,
286 females that eventually copulated (receptive) showed a slightly higher level of shoving
287 compared with females that did not eventually copulate (unreceptive), though this effect was not
288 statistically significant (Figure 4G). In contrast, receptive females showed a strong and
289 significant reduction in chasing (Figure 4H). Taken together, our results suggest that cells within
290 the pC1-Int line that control receptivity modulate the amount of female chasing (and possibly
291 also shoving) (Figure 4I). We also tested shorter activation durations and found robust
292 persistent shoving and chasing following 2 minutes activation, but reduced persistent shoving
293 and chasing following 30 seconds activation (Figure 4J). Below we address how minutes-long
294 pC1d/e activation affects neural activity.

295

296 **pC1d is reciprocally connected to a specific subset of Fruitless+ neurons**

297 The pC1-A line (same as pC1dSS3 (Schretter et al., 2020)) includes two cells per hemisphere,
298 one pC1d and one pC1e cell. Schretter et al., (2020) have demonstrated that optogenetic
299 activation of genetic lines that contain pC1d, but not pC1e, drive female aggressive behaviors,
300 such as those we refer to here as ‘shoving’. We therefore first focused on pC1d, and mapped
301 the major inputs and outputs, searching for circuit motifs that could account for pC1d’s ability to
302 drive a persistent behavioral state in females. We used automated reconstruction of all neurons
303 within an EM volume of an entire adult female brain called FAFB ((Zheng et al., 2018);
304 Dorkenwald et al. 2020). This volume contains both brain hemispheres, enabling complete
305 reconstruction of pC1d cells that send projections across the midline (Figure 3C). Focusing on a
306 single pC1d cell (Figure 5A) we first manually detected synaptic connections (Figure 5 - figure
307 supplement 1A; see Methods), as done previously for other circuits in FAFB (Felsenberg et al.,
308 2018; Sayin et al., 2019; Zheng et al., 2018) - while our manual detection process was
309 unbiased, in that we looked for synapses between pC1d and any other overlapping segment in
310 FlyWire (see Methods), we only sampled a subset of all pC1d synapses (see below for analysis
311 based on automated synapse detection). After proofreading the pC1d cell and its input and
312 output cells, and excluding neurons with weak connections (using 3 synapses as a threshold,
313 see Methods), we counted a total of 417 presynaptic and 421 postsynaptic sites (Figure 5B,
314 Video 6). We sorted all pC1d synaptic partners by cell type, based on morphology, and
315 examined the distribution of synapses by type for input (presynaptic partners) and output
316 (postsynaptic partners) neurons separately (Figure 5C). The three output types with the largest
317 number of synapses with pC1d share a common morphology: all pass through a single neurite
318 bundle (Figure 5D-E, Figure 5 - figure supplement 1B-C and Video 7) and all send projections to
319 the ring (Figure 5A), including dense projections in the lateral junction (Figure 5E).

320

321 The top matches (using NBLAST (Costa et al., 2016)) for all three types were sexually
322 dimorphic Fru+ neurons called aIPg ((Figure 5E and Figure 5 - figure supplement 1B, see
323 (Cachero et al., 2010). FlyWire cells that share the aIP-g morphology were sorted into three
324 types, aIPg-a, aIPg-b, and aIPg-c, based on the three separate bundles through which their
325 projections pass (Figure 5E). According to our manual synapse detection, aIPg-a cells have 131
326 sites postsynaptic to pC1d and only 5 presynaptic sites, while aIPg-b,c cells have stronger
327 reciprocal connections with pC1d, with an output:input ratio of ~1:1 (38:39) for aIPg-b, and 2.8:1
328 (39:14) for aIPg-c.

329

330 FlyWire provides a mapping of the publicly available, automatically detected synapses from
331 ((Buhmann et al., 2019); see Methods for details). We re-evaluated synaptic partners using
332 automatic detection, and focused on cells with strong connections with pC1d using two criteria:
333 (1) minimum of 6 synapses, (2) the cell belongs to a cell type (based on morphology) with at
334 least one cell with 15 synapses or more with pC1d. Consistent with manual detection, we found
335 that aIPg-a cells are postsynaptic to pC1d, while aIPg-b,c are reciprocally connected to pC1d
336 (Figure 5F). Some cells from the aIPg-b,c groups are also interconnected (Figure 5G).
337 Interestingly, the most interconnected cells within the aIPg-b group were also the ones that are
338 reciprocally connected to pC1d (Figure 5G, red lines).

339

340 We also examined synaptic connectivity between pC1d and aIPg cells in a second EM database
341 that consists of a portion of the adult female brain (the ‘hemibrain’ (Scheffer et al., 2020)), and
342 found a set of 13 neurons identified as aIPg (also evaluated in (Schretter et al., 2020)),
343 compared with 39 neurons we identified as aIPg in FlyWire. Twelve of these aIPg cells (denoted
344 as types aIPg1-3 in the hemibrain) share the aIPg-b morphology (Figure 5 - figure supplement
345 1D), and are synaptically connected to pC1d (excluding 1 connection with less than 3
346 synapses). One (aIPg4) shares the aIPg-c morphology. Consistent with our results in FlyWire,
347 pC1d in the hemibrain has more presynaptic sites than postsynaptic sites with aIPg-b
348 (hemibrain aIPg1-3) cells, and aIPg-b neurons form many recurrent connections with each other
349 (Figure 5H). Note that while our classification of aIPg cell types was based on morphology
350 alone, the classification in the hemibrain is based on both morphology and connectivity. In the
351 hemibrain v1.1, we found additional cells that match (morphologically) neurons we term aIPg-a
352 and aIPg-c – these neurons are called SMP555/556 and SMP558, respectively.

353

354 Finally, in FlyWire, we found that pC1d forms connections with other Dsx+ cells, including direct
355 connections with pMN1(DNp13) and pMN2 (vpoDN) (Figure 5I), other pC1 cells (Figure 5J), and
356 pC2l neurons (Figure 5K, Video 8 and Table 1). These results indicate that pC1d may serve as
357 a hub within the central brain for Dsx+ and Fru+ neurons (Figure 5L).

358

359 Using automated synapse detection in FlyWire, we examined all the major inputs and outputs of
360 both pC1d (Figure 6A) and pC1e (Figure 6 – figure supplement 1), as our driver line pC1-A
361 labeled both neurons in each hemisphere (see full list of inputs and outputs and public links to
362 FlyWire neurons in Table 1). aIPg-b neurons have the most reciprocal connections with pC1d,

363 and the 3 strongest output types of pC1d are alPg-a (588 synapses), alPg-c (300 synapses),
364 and alPg-b (232 synapses). The pC1d output:input ratio with alPg (pC1d-to-alPg:alPg-to-pC1d)
365 was 588:0 for alPg-a, 2.6:1 for alPg-b and 14.3:1 for alPg-c (Figure 5F) – consistent with the
366 results obtained by manual detection, showing that alPg-b has the most ‘balanced’ reciprocal
367 connectivity with pC1d. For pC1e (Figure 6 – figure supplement 1), of the top inputs and
368 outputs, the only reciprocal connections were with alPg-b. Shared inputs between pC1d and
369 pC1e also include pC1a, a cell previously shown to control female receptivity (Wang K. et al.,
370 2020). Shared outputs include alPg-a,c, and cell types that share the hemibrain morphology of
371 SIP024 and LAL003. Some of the top connections of pC1d were cross-hemispheric neurons or
372 neurons with synapses contralateral to pC1d (Figure 6B) – these neurons and connections fall
373 outside of the volume of the hemibrain. However, several of the top inputs and outputs could be
374 identified in the hemibrain, and we have indicated the corresponding cell type name where we
375 could find matches (Figure 6 and Figure 6 – figure supplement 1). Seven of the alPg cells that
376 are pC1d synaptic partners, also synapse with pC1e (Figure 6 – figure supplement 1).

377

378 As recurrent connectivity between neurons is known to support persistent neural activity
379 (Goldman-Rakic, 1995; Major and Tank, 2004; Zylberberg and Strowbridge, 2017), we next
380 examined whether activating pC1d/e could drive long-lasting changes in brain activity, with a
381 spatial distribution that matches the pC1d or pC1e connectomes.

382

383 **Activation of pC1d/e neurons drives persistent neural activity**

384 Persistent neural activity is defined as activity that continues after a triggering stimulus ends
385 (Zylberberg and Strowbridge, 2017). To relate our findings above to persistent neural activity,
386 we first activated either neurons in the pC1-A or pC1-S lines using 5 minutes of optogenetic
387 stimulation (similar to behavioral experiments, see Methods), and imaged responding cells via
388 GCaMP6s expressed pan-neuronally (Figure 7A). To compare activity across flies and to map
389 activity onto a reference atlas, we used a recently developed pipeline for two-photon volumetric
390 calcium imaging, motion correction, registration, and region of interest (ROI) segmentation
391 (Pacheco et al., 2019), and scanned the entirety (in the anterior-posterior axis) of the dorsal half
392 of a single brain hemisphere (either left or right) in each fly (Figure 7A) – we mirrored all activity
393 onto one hemisphere for display. We measured neuronal activity during the 5 minutes of
394 optogenetic activation in addition to 9.5 minutes following activation offset (Video 4), and found
395 that out of 47,882 ROIs with activity segmented across 28 brains and all genotypes (Figure 7B;

396 see Methods), 4,254 ROIs showed significant responses to optogenetic stimulation (Figure 7C;
397 $Ft1 > 3\sigma_0$, σ_0 = standard deviation of activity during baseline).

398

399 We then clustered these ROIs based on response patterns (Figure 7D), which revealed four
400 types of responses. Transient responses - ROIs with elevated activity during the optogenetic
401 stimulus (t1), but not following the stimulus (t2) - could be grouped into two clusters (response
402 types 3 and 4). The other two types showed sustained activity lasting at least 5 minutes after the
403 optogenetic stimulus offset (response types 1 and 2; $Ft2 > 3\sigma_0$, see Methods). The temporal
404 dynamics of persistent neural activity, continuing to at least 10 minutes following stimulation, is
405 consistent with our observation of female shoving and chasing of a male introduced 6 minutes
406 after stimulation offset (Figure 2E-F). In addition, response type 2 could be split into two clusters
407 (Fig 6D, right) based on response decay dynamics.

408

409 While response type 1 had low spatial consistency across animals, response types 2-4 showed
410 higher spatial consistency, and the spatial distribution of ROIs differed between controls, pC1-S,
411 and pC1-A activated flies (Figure 7E). Activation of pC1d/e neurons (in line pC1-A) drove
412 persistent activity (response type 2) in more than 30% of the imaged flies, and in 24.7 times
413 more voxels than in controls, and 6.8 times more voxels compared with pC1-S activation.
414 Making use of neuropil segmentation of an in vivo brain atlas to which all ROIs were registered
415 (Pacheco et al., 2019), we evaluated the distribution of pC1d/e-elicited activity by brain neuropil
416 (Ito et al., 2014). Persistent activity (response type 2) was clustered in the posterior-dorsal
417 portion of the brain spanning the Superior Medial, Lateral and Intermediate Protocerebrum
418 (SMP, SLP, SIP), the Anterior Optic Tuber (AOTU), and the Inferior and Superior Clamp (ICL
419 and SCL; Figure 7F and Figure 7 – figure supplement 1A); these brain regions contain a large
420 number of projections from sexually dimorphic neurons expressing either Doublesex or Fruitless
421 (Rideout et al., 2010; Yu et al., 2010). We found that 61% (4717/7759) of all the presynaptic
422 terminals and 85% (3283/3848) of all the postsynaptic terminals for the group of 11 aIPg1-3 (all
423 share the aIPg-b morphology) cells in hemibrain v1.1 are in these 6 areas, with the SMP, SIP
424 and AOTU being the most dominant input and output regions for the aIPg1-3 cells.

425

426 Our behavioral results indicated that female brain state must differ between the d0, d3, and d6
427 conditions (Figure 2F and Figure 4B-C) – we therefore quantified neural activity at these specific
428 time points (0, 3 min, and 6 min) following optogenetic activation. In the neuropils with highest
429 activity, we found that responses were highest immediately following stimulation ($t = 0$) and

430 decayed significantly by $t = 3$ min, and further still by $t = 6$ min (Figure 7G; Figure 7 – figure
431 supplement 1B).

432

433 In order to measure the overlap of pC1-elicited activity with Dsx+ neurons, we generated
434 anatomical labels for the lateral protocerebral complex (LPC), a diffuse brain area to which all
435 Dsx+ neurons send their projections, and also for all major groups of Dsx+ somas (pC1, pC2I,
436 pCd1, and pCd2) within the *in vivo* brain atlas (Figure 7F, see Methods). We found that ROIs
437 with persistent activity (response type 2) overlap with the LPC, in addition to the regions
438 occupied by pC1 somas, and to a lesser extent with regions occupied by pC2, pCd1 and pCd2
439 somas, suggesting that Dsx+ pC1 neurons carry persistent activity. We also looked for overlap
440 between ROIs with persistent activity and the projections of individual aIPg neurons, all
441 registered into the same reference brain (Figure 7H; see Methods). While persistent activity
442 (response type 2; Figure 7D-E) spans only 4.3% of the central brain (Figure 7E; see Methods),
443 we found response type 2 activity in 20.14% (union of the overlap) of the voxels that include
444 aIPg-a/b/c example cell (one of each type) from FlyWire (Figure 7H). In addition, activity driven
445 by 5 minutes of activation was not aberrant. DF/F values following 5 min optogenetic activation
446 (data from Figure 7C) fell within the distribution of DF/F values observed in separate
447 experiments (Pacheco et al. 2019) in which activity was driven by auditory stimuli rather than
448 optogenetic activation (Figure 7 – figure supplement 1C).

449

450 We also examined persistent activity following a shorter activation period of 2 minutes, and
451 found ROIs with persistent activity (response Type 2; Figure 7I-J). The persistent activity
452 following 2-minute activation was weaker in this condition compared to 5-minute activation,
453 suggesting that persistent activity scales with the activation period.

454

455 We next expressed GCaMP6s in only Dsx+ neurons, to confirm the specific Dsx+ cells with
456 persistent neural activity – this is possible because Dsx+ somas are clustered by cell type
457 (Figure 1A). We activated pC1d/e neurons for 5 minutes (Figure 8A and Video 5) and recorded
458 activity in 273 cells (ROIs drawn manually) across 16 flies. We examined the responses during
459 (t_1) and after (t_2) optogenetic stimulation (same as for the pan-neuronal dataset), and compared
460 these responses to controls in which pC1d/e neurons were not activated ($n = 11$ flies, 192 ROIs;
461 See Fly genotype table for full genotypes; Figure 8B). A number of Dsx+ pC1 cells showed
462 strong persistent activity (Figure 8B; same definition as for the pan-neuronal screening, $Ft_2 >$
463 $3\sigma_0$) following optogenetic activation. We observed some heterogeneity in responses across the

464 pC1 cells (Figure 8B), with some cells showing faster decay than others following stimulus
465 offset, consistent with the two clusters underlying response type 2 (Figure 7D, green and
466 brown). We did not observe persistent activity in any non-pC1 Dsx-expressing cell types (Figure
467 8 - figure supplement 1), including pC2 neurons or pCd1 neurons, previously shown to be
468 necessary for P1 induced persistent activity in males (Jung et al., 2020; Zhang et al., 2019).

469

470 Last, using the same methodology, we examined neural activity in single Fru+ cells following
471 pC1d/e activation, by expressing GCaMP6s via the Fru-LexA driver (Figure 8C, top). We found
472 persistent activity in two group of cells, denoted as ‘Group 1’ and ‘Group 2’ (Figure 8C, bottom),
473 that often lasted over a minute following activation (Figure 8D-E) – ROIs drawn manually
474 individual cells within each group. By comparing the location of Fru+ cell bodies with persistent
475 activity with the position of single Fru+ cells and to the location of pC1/pC2/aIPg cells in FlyWire
476 (Figure 8 – figure supplement 2). We conclude that Group 2 includes pC1 neurons, while Group
477 1 likely includes pC2/pIP5 neurons and possibly also aIP-g cells. The persistent activity is most
478 likely not in Dsx+/Fru+ pC2 neurons given our observations that Dsx+ pC2 cells do not show
479 persistent activity following pC1d/e activation, but could be pIP-e (also called pIP5 in the
480 hemibrain). This is consistent with our analysis in FlyWire, showing that pIP5 is both pre- and
481 post-synaptic to pC1d.

482

483 In sum, our pan-neuronal imaging reveals that female brain state is different at 0 and 3 minutes
484 following activation, providing an explanation for the differences in behaviors produced following
485 introduction of a male at these different delays. In addition, by clustering response types, we
486 were able to map pC1d/e driven persistent neural activity to brain regions containing both Dsx+
487 neurons and Fru+ aIPg neurons, and with follow-up experiments showed that several Dsx+ pC1
488 neurons as well as Fru+ putative pC1 and aIPg cells contain persistent neural activity. This is
489 consistent with the recurrent circuit architecture we found using EM reconstruction (Figures 5-6).

490

491 **Discussion**

492 We find that pC1 neurons drive a persistent internal state in the *Drosophila* female brain that
493 modulates multiple behaviors over timescales of minutes (receptivity, responses to male
494 courtship song, aggressive behaviors, and male-like courtship behaviors (Figures 1-2 and 4)).
495 The behavioral effects we observe may be similar to the effects of ‘emotion states’ observed in
496 other animals, such as mice, fish, and primates (Anderson and Adolphs, 2014; Kunwar et al.,
497 2015; Posner et al., 2005; Russell, 2003; Woods et al., 2014). In general, effects on behavior of

498 such emotion states are thought to scale with levels of persistent neural activity (Hoopfer et al.,
499 2015; Lee et al., 2014). We found that neural activity decays during a delay period following
500 stimulation (Figure 7-8), and that behavioral effects of activation were also different following
501 different delays. Specifically, we found that the highest levels of pC1 activation enhance
502 receptivity but have an opposing effect on responses to male song (speeding females up
503 instead of slowing them down). Slightly lower levels of pC1 activation (following a delay) bias
504 females toward aggression and male-like behaviors. These effects on behavior may not
505 naturally co-occur, but our optogenetic activation paradigm uncovers the scalable relationship
506 between activation of different pC1 subtypes, their individual levels of activity, and distinct
507 behavioral programs.

508

509 Our study also provides new insight into the neural mechanisms that contribute to changes in
510 state on timescales of minutes (Figures 7-8). We used pan-neuronal imaging with registration to
511 map responses that continue following pC1 optogenetic activation (previously this technique
512 had only been used to map sensory activity (Pacheco et al., 2019) and spontaneous activity
513 (Mann et al., 2017)). We found that activation of pC1d/e neurons drives robust persistent neural
514 activity throughout the posterior dorsal regions of the central brain (known to contain the
515 processes of sexually dimorphic neurons (Cachero et al., 2010; Kimura et al., 2015), and
516 overlapping with the Fru+ aIPg neurons we identified as reciprocally connected with pC1d/e),
517 lasting for minutes following activation. This is consistent with our behavioral observations -
518 females still show elevated shoving and chasing even following a 6-minute delay between
519 optogenetic activation and the introduction of a male fly. Importantly, whether or not pC1
520 neurons themselves carry persistent neural activity has been debated (Inagaki et al., 2014; Jung
521 et al., 2020; Zhang et al., 2018). Here we find that in females, Dsx+ pC1 neurons, as well as
522 multiple Fru+ neurons, including putative pC1 and aIPg cells, do indeed carry persistent neural
523 activity in response to our activation protocol (Figure 8).

524

525 **pC1 neurons drive both aggression and receptivity in *Drosophila* females**

526 We used unsupervised methods to identify the most prominent behaviors (beyond receptivity
527 and responses to courtship song) produced following activation of pC1 neurons in virgin females
528 - these include behaviors that resemble male courtship (female chasing the male) and
529 aggression (female shoving the male) (Figure 2). Both behaviors are not typically observed in
530 mature virgin females interacting with a male; this suggests that sensory cues from the virgin
531 male do not inhibit these aberrant behaviors, but rather may enhance the persistent effects of

532 pC1 activation (Figure 2F), most likely via visual inputs to aIPg neurons (Schretter et al., 2020).
533 pC1 neurons also drive aggressive behaviors towards females during stimulation (Palavicino-
534 Maggio et al., 2019; Schretter et al., 2020), but whether the quality of aggression generated
535 towards males versus females is similar remains to be determined. As one of our manually
536 scored behaviors, ‘female approaching’ (Figure 2 - figure supplement 3A), begins from a
537 distance greater than 4 body lengths from the male fly (a distance at which it may be difficult to
538 discern male from female (Borst, 2009)) and often ends with shoving or circling (see Video 3),
539 we hypothesize that pC1 activation most likely drives persistent behaviors towards another fly,
540 and not specifically a male or female fly, consistent with (Schretter et al. 2020).

541

542 What is the role of female aggression? Female aggression, whether towards males or females,
543 has been previously reported across model systems (Huhman et al., 2003; Stockley and Bro-
544 Jørgensen, 2011; Woodley et al., 2000). In *Drosophila*, female-female fights over food source
545 are strongly stimulated by the receipt of sperm at mating (Bath et al., 2017), and include both
546 patterns that are common with male aggression (such as shoving and fencing) and female-only
547 patterns (Nilsen et al., 2004). Female-male aggression was reported in the context of rejecting
548 behavior in mated, immature, or older females (Cook and Connolly, 1973). The behavioral
549 changes in our study do not mimic those in a mated female, as we also observe that pC1
550 activation drives enhanced receptivity. While we have not confirmed which pC1 cell types
551 control receptivity, our work reveals a separation: pC1d/e neurons are sufficient to drive
552 persistent shoving/chasing, but do not have a persistent effect on female receptivity (Figure 4A-
553 C), while separate pC1 neurons that control receptivity modulate the pathways that control
554 chasing and aggression (Figure 4G-I). Recent work reveals that pC1a neurons are modulated
555 by the sex peptide receptor pathway, such that following mating (when sex peptide is
556 transferred), pC1a neurons are inhibited (Wang K. et al., 2020). Because we find that pC1a
557 provides direct input to pC1d and pC1e neurons (Figure 6A, Table 1; see also (Schretter et al.,
558 2020)), we speculate that pC1a provides this receptivity information. Interestingly, work in male
559 flies suggests a separation in pC1 subsets that control courtship versus aggression
560 (Koganezawa et al., 2016), with reciprocal inhibitory influences between persistent courtship
561 and aggression, following pC1 activation (Hoopfer et al., 2015). Though the phenotypes are
562 sex-specific (male singing vs female receptivity; male tussling vs female shoving), and the pC1
563 subsets driving these behaviors are sex-specific (P1 in males, pC1d/e in females), this suggests
564 some common architecture. Ultimately, comparing the connectomes of male and female brains,
565 combined with functional studies, should elucidate both similarities and differences.

566

567 Because courtship interactions unfold over many minutes (Coen et al. 2014), we postulate that
568 the changes in brain state we observed following pC1d/e activation may occur naturally as
569 females receive continual drive to pC1 neurons. Our connectomic analyses reveals inputs to
570 pC1d/e neurons from auditory cell types in the AVLP (Wang F. et al., 2020; Deutsch et al.,
571 2020) – this is consistent with our patch clamp recordings of pC1d/e neurons (Figure 4 – figure
572 supplement 1), showing auditory activity, and also with prior work on auditory responses in
573 female, but not in male pC1 neurons (Deutsch et al., 2019). Thus, during natural courtship
574 interactions, male song should drive pC1d/e neurons – in combination with other inputs, this
575 may shift behaviors from receptive ones towards chasing and shoving.

576

577 **Recurrent circuitry and persistent neural activity**

578 FlyWire enabled a systematic search for all of the synaptic partners of a single pC1d and pC1e
579 cell. Using manual and automatic synapse detection (Buhmann et al., 2019) we found that pC1d
580 is reciprocally connected with aIPg-b and aIPg-c cells, and that aIPg-b and aIPg-c cells are also
581 interconnected – pC1e also shows reciprocal connectivity with aIPg-b neurons. We also
582 identified pC1d and aIPg-c cells in a separate EM volume of an adult female brain, the
583 hemibrain (Scheffer et al., 2020), and found similar results (see (Schretter et al., 2020) for a
584 thorough analysis of the pC1d/e connectomes in the hemibrain). Because FlyWire is based on
585 the FAFB dataset of the entire adult female brain (Zheng et al., 2018), our search for synaptic
586 partners of pC1d and pC1e could completely cover both hemispheres, showing that, for
587 example, pC1d is reciprocally connected to itself in the contralateral hemisphere, and is
588 presynaptic to some contralateral aIPg cells. Comparisons between connectivity diagrams in the
589 two female EM volumes of FAFB (segmented in FlyWire) and the hemibrain will continue to be
590 of value for future studies.

591

592 Synaptically recurrent neural networks have been proposed to contribute to persistent neural
593 activity lasting for seconds, and to underlie processes like accumulation of evidence and
594 working memory (Aksay et al., 2007; Barak and Tsodyks, 2007; Major and Tank, 2004; Mante et
595 al., 2013; Seung, 1996; Wang, 2008; Zylberberg and Stroudbridge, 2017). For example, in
596 Drosophila, recurrent excitatory loops in the ellipsoid body of the central complex contribute to
597 stabilization of a heading navigation signal over timescales of seconds (Turner-Evans et al.,
598 2019). Internal states underlying social behaviors, however, persist on much longer timescales
599 of minutes to hours (Chen and Hong, 2018). Neuromodulation, via hormones or peptides, is

600 thought to support longer timescales of persistence (Adolphs and Anderson, 2018; Bargmann,
601 2012; Marder, 2012; Zelikowsky et al., 2018). Our work now links a strongly recurrent neural
602 network (pC1d/e to aIPg) to both minutes-long persistent neural activity and minutes-long
603 changes in behavior. Work from (Schretter et al., 2020) demonstrates that pC1d, pC1e, and
604 aIPg neurons are all cholinergic, suggesting these neurons make up an excitatory neural
605 network. While our study found persistent changes in behavior following pC1d/e activation, the
606 (Schretter et al., 2020) study did not – this discrepancy could arise from a number of
607 methodological reasons, including differences in the optogenetic activator, timescale of
608 activation, or in behavioral protocols. Nonetheless, Schretter et al., (2020) demonstrate that
609 aIPg-b activation drives persistent female aggression following activation, and that increasing
610 stimulation of aIPg-b, increases the amount of persistent aggression. This is consistent with a
611 model in which strong activation of pC1d/e neurons recruits aIPg-b neurons (via their excitatory
612 interconnections) to drive, at least, persistent shoving.

613

614 Work in male flies has proposed a recurrent circuit motif including pCd neurons that contributes
615 to persistent aggressive behaviors (Jung et al., 2020) or even longer-term changes in mating
616 drive (driven by male-specific P1 neurons, but without mapping the synaptic connections). Our
617 studies of pCd, revealed that, in females, it does not contribute to the persistent arousal state
618 (Figure 2 – figure supplement 2), nor is it persistently active following pC1d/e activation (Figure
619 8 – figure supplement 1). In males, activating P1 neurons for 5 seconds was enough to induce
620 some persistent activity in pCd cells (Jung et al., 2020). We did not measure neural responses
621 to pC1d/e stimulation shorter than 2 minutes, and so whether shorter activation periods drive
622 some persistent neural activity in females remains open. While pC1d/e neurons are female-
623 specific, aIPg neurons are present in both males and females – it will be interesting to determine
624 what role the aIPg neurons play in male brains and whether they represent a shared component
625 of persistence in the two sexes.

626

627 Finally, it is important to point out that recent EM connectomic work in *Drosophila* using FAFB
628 (via manual tracing) has revealed a number of recurrent or reciprocal circuits throughout the
629 brain (Jefferis Neuron 2018, Kadow Neuron 2019, Turner-Evans 2019). Half of the outputs of
630 pC1d are the aIPg neurons, and many of these neurons (several aIPg-b and aIPg-c cells) are
631 strong inputs. Our identification of a recurrent circuit motif containing pC1d links it to the
632 persistent neural activity and changes in behavior we observe following activation, especially in

633 Fru+ somas. This is an important first step towards determining how recurrent neural networks
634 contribute to such long timescales of persistence.

635

636

637 **Acknowledgements:**

638 We thank Barry Dickson, David Anderson, Annegret Falkner and Christa Baker for comments
639 on the manuscript and the entire Murthy lab for helpful discussions. We thank Barry Dickson for
640 sharing the pC1-A split GAL4 line ahead of publication. We thank Stephan Thibierge for
641 assistance with two-photon imaging, Nat Tabris for assistance with software development, Josh
642 Shaevitz for development of modifications to LEAP, Shruthi Ravindranath for assistance with
643 identifying neurons in FlyWire, and Junyu Li for assistance with proofreading behavioral data.
644 We thank Joseph Hsu (Janelia) and other members of the Cambridge Drosophila Connectomics
645 Group for contributing to FlyWire tracing of neurons in this study, and Maria Dreher (Janelia) for
646 assistance with matching cell types across datasets. We thank Katie Schretter and Gerry Rubin
647 for exchanging information prior to publication. This study was supported by an NIH BRAIN
648 Initiative RF1 MH117815-01 to MM and HSS and an NIH BRAIN R01 NS104899 and HHMI
649 Faculty Scholar award to MM.

650

651 **Declaration of Interests:**

652 The authors declare no competing interests.

653

654 **Materials and methods**

655

656 **Key resources table**

657

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (<i>D. melanogaster</i>)	NM91	Coen et al. (2014)		A gift from Peter Andolfatto
Genetic reagent (<i>D. melanogaster</i>)	Dsx-Gal4 (III)	Rideout et al. (2010)		A gift from Stephan Goodwin

Genetic reagent (<i>D. melanogaster</i>)	Dsx-LexA::P65 (III)	(Zhou et al., 2015)		A gift from Bruce Baker
Genetic reagent (<i>D. melanogaster</i>)	UAS-2xEGFP (II)		BDSC: 6874; RRID:BDSC_6874	
Genetic reagent (<i>D. melanogaster</i>)	R71G01-LexA (II)	(Pan et al., 2012)	BDSC: 54733; RRID:BDSC_54733	
Genetic reagent (<i>D. melanogaster</i>)	R41A01-LexA (II)	Zhou et al. (2014)	BDSC: 54787; RRID:BDSC_54787	
Genetic reagent (<i>D. melanogaster</i>)	8xLexAop2-FLP (II)		BDSC: 55819; RRID:BDSC_55819	
Genetic reagent (<i>D. melanogaster</i>)	UAS>STOP>TNT (III)	Stockinger et al. (2005)		A gift from Barry Dickson
Genetic reagent (<i>D. melanogaster</i>)	UAS-ReachR (III)	(Inagaki et al., 2014)	BDSC: 53748; RRID:BDSC_53748	
Genetic reagent (<i>D. melanogaster</i>)	VT25602.p65ADZp (II)	(Wu et al., 2019)		A gift from Barry Dickson
Genetic reagent (<i>D. melanogaster</i>)	VT2064.ZpGAL4DBD (III)	(Wu et al., 2019)		A gift from Barry Dickson
Genetic reagent (<i>D. melanogaster</i>)	UAS(FRT.mCherry)Reac hR (III)	(Inagaki et al., 2014)	BDSC: 53743; RRID:BDSC_53743	A gift from David Anderson
Genetic reagent (<i>D. melanogaster</i>)	R71G01-p65.AD (II)	(Dionne et al., 2018)	BDSC: 70798; RRID:BDSC_70798	A gift from Gerry Rubin
Genetic reagent (<i>D. melanogaster</i>)	Dsx.DBD (III)	Pavlou et al., (2016)		A gift from Stephan Goodwin
Genetic reagent (<i>D. melanogaster</i>)	10xUAS-IVS-myr::GFP (II)		BDSC: 32198; RRID:BDSC_32198	

Genetic reagent (<i>D. melanogaster</i>)	W+,NorpA(X)		BDSC: 9048; RRID:BDSC_9048	
Genetic reagent (<i>D. melanogaster</i>)	VT25602-Gal4 (III)			A gift from Barry Dickson
Genetic reagent (<i>D. melanogaster</i>)	R57C10-LexA (II)		BDSC: 52817; RRID:BDSC_52817	A gift from Gerry Rubin
Genetic reagent (<i>D. melanogaster</i>)	8xLexAop-mCD8tdTomato (III)			A gift from Yuh Nung Jan
Genetic reagent (<i>D. melanogaster</i>)	13xLexAop-IVS-GCaMP6s (III)		BDSC: 44590; RRID:BDSC_44590	
Genetic reagent (<i>D. melanogaster</i>)	10xUAS-IVS-Syn21-Chrimson-tdT-3.1 (X)	(Hoopfer et al., 2015)		A gift from allan M. wong
Genetic reagent (<i>D. melanogaster</i>)	13xLexAop2-IVS-Syn21-opGCaMP6s (X)			A gift from allan M. wong
Genetic reagent (<i>D. melanogaster</i>)	20xUAS-IVS-CsChrimson.mVenus (X)		BDSC: 55134 RRID:BDSC_55134	A gift from Vivek Jayaraman
Genetic reagent (<i>D. melanogaster</i>)	Fru[P1.LexA] (III)		BDSC: 66698 RRID:BDSC_66698	A gift from Bruce Baker
Antibody	mouse anti-brp	mAb DSHS - developmental studies Hybridoma bank	#nc82; RRID:AB_2314866	(1:20 dilution)
Antibody	chicken anti-GFP (Polyclonal)	Invitrogen	#A10262, RRID:AB_2534023	(1:2000 dilution)
Antibody	Rabbit anti-dsRed (Polyclonal)	Takara Bio	#632496, RRID:AB_10013483	(1:500 dilution)

Antibody	Goat anti-mouse Alexa-Flour 568 (Polyclonal)	Invitrogen	#A-11004, RRID:AB_2534072	(1:400 dilution)
Antibody	Goat anti-chicken Alexa-Flour 488 (Polyclonal)	Invitrogen	#A11039, RRID: AB_142924	(1:300 dilution)
Antibody	Goat anti-rabbit Alexa-Flour 633 (Polyclonal)	Invitrogen	#A-21070, RRID:AB_2535731	(1:400 dilution)
Chemical compound, drug	All trans-Retinal	Sigma-Aldrich	#R2500	
Software, algorithm	MATLAB	Mathworks	RRID:SCR_001622	
Software, algorithm	SLEAP	https://sleap.ai/		
Software, algorithm	Illustrator	Adobe	RRID:SCR_010279	

658

659

660 Fly genotype table

Figure panel	Genotype	Additional information
1A	UAS-2xEGFP; Dsx-Gal4	Immunostaining
1C-D	W;R71G01-LexA/8xLexAop2-FLP;Dsx-Gal4/UAS>STOP>TNT	Express TNT in pC1-Int
1S1B	W;R41A01-LexA/8xLexAop2-FLP;Dsx-Gal4/UAS>STOP>TNT	Express TNT in pCd1 neurons
1C-D, 1S1B	W;+/8xLexAop2-FLP;Dsx-Gal4/UAS>STOP>TNT	Control for TNT expression in pC1-Int and in pCd1
1F-G, 2, 4F-H, 1S1D, 2S1, 2S2B, 2S3, 2S2C	W;R71G01-LexA/8xLexAop2-FLP;Dsx-Gal4/10xUAS>STOP>ReachR	Express ReachR in pC1-Int neurons; ATR+ experimental, ATR- control
1S1C, 2S2A	W;R41A01-LexA/8xLexAop2-FLP;Dsx-Gal4/10xUAS>STOP>ReachR	Express ReachR in pCd1 neurons; ATR+ experimental, ATR- control

4D-E	W;R71G01.AD/+;DSX.DBD/UAS-ReachR	Express ReachR in 'pC1-s' neurons (behavioral experiment); ATR+ for experimental, ATR- for control
4A-C, F, J	W;VT25602.AD/+;VT2064.DBD/10xUAS-ReachR	Express ReachR in 'pC1-A' neurons (behavioral experiment); ATR+ for experimental, ATR- for controls. pC1-A is similar to pC1dSS3 in Schrutter et al., (2020).
4S1	W;UAS-2xEGFP/+;VT25602-Gal4/+	Express GFP in 'pC1-A' neurons for patch clamp recordings
3D (top), 3S2C	W;R71G01.AD/UAS-10x-IVS-myr::GFP;Dsx.DBD/+	Immunostaining (pC1-S)
3D (bottom), 3S2B	W;VT25602.AD/UAS-10x-IVS-myr::GFP;VT2064.DBD/+	Immunostaining (pC1-A)
3S2A	R71G01-LexA/8xLexAop2-FLP; Dsx-Gal4/UAS>STOP>myrGFP	Immunostaining (pC1-Int)
7B-G, 7S1A	W+NorpA[36],20xUAS-csChrimson.mVenus;R71G01.AD/R57C10-LexA;Dsx.DBD/8xLexAop-mCD8tdTomato,13xLexAop-GCaMP6s	pC1-S activation, measure pan-neuronal Ca response; ATR+
7B-J, 7S1A-B	W+NorpA[36],20xUAS-csChrimson.mVenus;VT25602.AD/R57C10-LexA;VT2064.DBD/8xLexAop-mCD8tdTomato,13xLexAop-GCaMP6s	pC1-A activation, measure pan-neuronal Ca response; ATR+
7B-E,I-J, 7S1A	W+NorpA[36],20xUAS-csChrimson.mVenus;R57C10-LexA/CyO;8xLexAop-mCD8tdTomato,13xLexAop-GCaMP6s/TM6B,tb	Control; ATR+
8A-B, 8S1	10xUAS-Chrimson.tdTomato,13LexAop2-GCaMP6s/W+NorpA[36],20xUAS-csChrimson.mVenus;VT25602.AD/CyO;Dsx-LexA/TV2064.DBD	pC1-A activation, measure Ca response in Dsx+ neurons; ATR+
8A-B, 8S1	10UAS-Chrimson.tdTomato,13LexAop2-GCaMP6s/+; Sp/CyO;Dsx-LexA/TM6B,tb	Control; ATR+
8C-E, 8S1	10xUAS-Chrimson.tdTomato,13LexAop-GCaMP6s/+;VT25602.AD/CyO; Fru-LexA /VT2064.DBD	pC1-A activation, measure Ca response in fru+ neurons; ATR+
8C-E	10UAS-Chrimson.tdTomato,13LexAop-GCaMP6s;Sp/CyO;Fru-LexA/Tm6Tb	Control; ATR+

662

663 **Fly stocks**

664 All flies were raised at 25°C on standard medium in a 12 hr light/12 hr dark cycle at 60% relative
665 humidity. Female flies used for optogenetic experiments were fed with food that contained all-
666 trans-retinal (Sigma R2500-100MG; ATR concentration is 1 mM) for a minimum of three days
667 post eclosion. Control flies were raised on regular fly food after eclosion. Both experimental and
668 control female flies used for optogenetic experiments, were reared post-eclosion in dark blue
669 acrylic boxes (acrylic available from McMaster-Carr, #8505K92).

670

671 The full details on genotypes for the flies used in this study and their source are in Key
672 resources table and in Fly genotype table.

673

674 **Behavioral experiments**

675 All behavioral experiments were carried out using a behavioral chamber (diameter ~25mm) tiled
676 with 16 microphones (NR23158, Knowles Electronics; Figure 1B and Videos 1-3), and
677 connected to a custom amplifier (Arthur et al., 2013)). Audio signals were recorded at 10KHz,
678 and the fly song was segmented as previously described (Arthur et al., 2013; Coen et al., 2014).
679 A point grey camera (FL3-U3-13Y3M; 1280X960) was used to record fly behavior from a top
680 view (see Figures 1B, S1A) at 60 frames per second using custom written software in Python
681 and saved as compressed videos (H.264). Virgin females (see Fly genotype table for genotype
682 used in each experiment) and wild type NM91 virgin males (both males and females were 3-7
683 days old) were used for all behavioral experiments. For inactivation experiments (Figures 1C-D,
684 Figure 1 – figure supplement 1B), a male and female were introduced into the behavioral
685 chamber simultaneously. For activation experiments, a female was positioned in the behavioral
686 chamber, and red light (a ring of 6 LEDs, 627nm, LuxeonStar) was then delivered at
687 1.1mw/mm² ($\pm 5\%$ across the chamber) at 100Hz (50% duty cycle) for 5 minutes (Figure 1E) or
688 for 2 minutes (Figure 4J). Following stimulus offset, a male was introduced with either no delay
689 (d0), or after a 3- or 6-minute delay (d3, d6). We collected data from the time the male was
690 introduced (t = 0) until 30 minutes or when the flies copulated, whichever came first. We chose
691 to use ReachR (Inagaki et al., 2014) for activation experiments rather than using the more
692 sensitive red shifted channelrhodopsin csChrimson (Klapoetke et al., 2014) to minimize
693 optogenetic activation due to background light. The percent of flies that copulated as a function
694 of time (Figure 1C,F, Figure 1 – figure supplement 1B-C) was calculated from the time the male
695 was introduced (t = 0) and until t = 30 minutes.

696

697 **Tracking centroids and headings**

698 Each video frame was analyzed by first finding the fly centroid, then detecting the location of the
699 thorax and head which formed the heading vector for each fly. Having microphones at the
700 chamber bottom results in a highly inhomogeneous background (Figure 1- figure supplement
701 1A) posing a major challenge for accurate tracking.

702 The centroid was found in one of two ways that yielded similar results. In the first method, the
703 inhomogeneous background of the video was found by taking the median across all frames.
704 Because the animals move throughout the video, finding the median pixel usually does not
705 contain any pixels containing an animal's body. However, as animals occasionally sit for long
706 periods, they can become part of the background. To avoid this, we divided the video into 10
707 shorter videos of equal length and found the median 'frame' (median set of pixel values) for
708 each sub-video. We then created a median frame by computing the median across these
709 medians. Each video frame then had this background subtracted to identify pixels that were
710 potentially part of each fly. These pixels were smoothed by a series of operations using the
711 OpenCV Python package and then thresholded. Using OpenCV, we identified all contours
712 surrounding collections of pixels and any smaller or larger than some predefined threshold (less
713 than half the size of a typical 'fly' or more than twice its size) were discarded. The remaining
714 pixels were then clustered via k-means. The number of clusters were iteratively increased until
715 the compactness of each cluster reached some threshold. The least-compact clusters were
716 discarded, and the remaining pixels were clustered again with k-means with k=2 to identify the
717 two clusters corresponding to the animals. These clusters were then fit with an ellipse to identify
718 the centroid of each animal. In the second method, we trained a deep convolutional network to
719 detect all instances of individual body parts (head, thorax) within each frame using a modified
720 version of LEAP ((Pereira et al., 2019), or SLEAP (Pereira et al., 2020) <https://sleap.ai/>; 544
721 labeled frames were used for training; Figure 1- figure supplement 1Ai-ii and Video 1). Using the
722 same software and neural network architecture, a separate network was then trained to group
723 these detections together with the correct animals by inferring part affinity fields (Figure 1- figure
724 supplement 1Aiii; Cao et al., 2017). This enabled estimation of the vector that represents fly
725 heading for both flies (Figure 1- figure supplement 1Aiv).

726

727 **Linear classification of single frames**

728 17 parameters were extracted for each frame based on the tracking of male and female centroid
729 and heading (Figure 2A), describing either the female movements (fFV – female forward

730 velocity; fLS – female lateral speed; fFA/fLA – female forward/lateral acceleration; fRS – female
731 rotational speed), male movements (similar to female movements – mFV/mLS/mFA/mLA/mRS),
732 or male-female interaction (mfDist – male-female distance; fmAngle – female heading relative to
733 the female-male axis, mfAngle – male heading relative to male-female axis; fmFV or fmLS –
734 female speed in the male direction or in the perpendicular axis; mfFV or mfLS – male speed in
735 the female direction or perpendicular). Using 17 parameters for each frame, we trained binary
736 support vector machine (SVM) linear classifiers to find the parameters (dimensions) that best
737 separate the groups. We first trained classifiers that separate between frames that belong to
738 experimental flies (class 1, pC1-Int activated, either one condition - d0/d3/d6 or all groups
739 together, d0-d6), and controls (class 2). We trained 90 classifiers, randomly choosing a set of
740 3000 frames from each class ('training set'; non-overlapping - the same frame was never used
741 in two classifiers; increasing the number of frames beyond 3000 did not increase performance).
742 We used the MATLAB R2019b procedure fitcsvm (MathWorks, Natick, MA), with a linear kernel.
743 We then used a separate set of 30,000 frames per class for each classifier ('validation set; the
744 same frame was never used twice, either between classifiers or between sets) to test the
745 performance of each classifier (fraction of frames correctly classified). We then choose the 30
746 best-performing classifiers (Figure 2B for control vs d0-d6). We used a third set of frames for
747 each classifier (30,000 frames/class, again – with no overlap with other sets) to measure the
748 performance of each classifier. The MATLAB function predict was used to find the SVM-
749 predicted class for each frame in the validation or train set. Performance was calculated as the
750 percent of frames correctly classified (Figure 2C). For each weight (out of the 17; control vs d0-
751 d6), we looked at the distribution coming from the 30 independent classifiers, and tested
752 whether the mean was significantly different than zero (Figure 2 – figure supplement 1A).
753 We used a two-sample t-test to measure the probability that the mean weight associated with
754 each parameter is different from zero (Figure 2 – figure supplement 1A). We found 8 out of the
755 17 parameters to be highly significant (*P<0.0001 in S1E).

756

757 **Clustering behaviors based on single frames**

758 The 8 most significant parameters found by the SVM classifier (see previous section) were used
759 for classification. We took the same number of frames from each group (control/d0/d3/d6) -
760 357997 frames (99.4 minutes) per group, corresponding to the number of frames in the smallest
761 group (d6). We repeated the clustering 30 times (Figure 2D, black dots), each time selecting
762 99.4 minutes of data from each one of the other groups (d0, d3, d6, control) randomly (with
763 replacements – the same frame could be used in multiple repeats), therefore having >1.4 million

764 frames for clustering on each repeat. The sets are not independent (overlapping frames
765 between repeats) and no statistical test was performed over the repeats. After z-scoring each
766 parameter (over all the frames in a given repeat), k-means clustering was performed (using
767 MATLAB function kmeans), allowing 20 clusters and a maximum of 500 iterations (other
768 parameters set to default). We found that the first 7 largest clusters (cluster size being the
769 number of frames in the cluster) capture 90.4% of the frames, averaged over repeats (Figure
770 2D, inset). To match clusters between repeats (for each cluster number in repeat 1, find the
771 corresponding cluster number in repeats 2-30), we used the smallest distance between clusters,
772 by calculating the mean square error over the weights (the variability in weight size across
773 repeats is shown in Figure 2 – figure supplement 1C).

774

775 **Machine learning based classification of behavioral epochs**

776 The Janelia Automatic Animal Behavior Annotator (JAABA; <http://jaaba.sourceforge.net/>; Kabra
777 et al., 2013) was used to detect epochs of ‘female shoving’ and ‘female chasing’. Two
778 independent classifiers (‘shoving classifier’, ‘chasing classifier’) were trained, one for each
779 behavior. We used the automatic segmentations to find examples for shoving and chasing
780 epochs, used as a first step in training each classifier. We then added example epochs (positive
781 and negative examples are used for each classifier), in an iterative manner (using examples
782 where the classifiers made wrong predictions). Altogether we used 24,222 frames (6.7 minutes)
783 to train the ‘shoving classifier’, and 11,941 frames (3.3 minutes) for the ‘chasing classifier’.

784 The classifiers was based on the 17 parameters defined above (denoted as ‘per-frame’
785 features), as well as on ‘window features’ (‘mean’, ‘min’, ‘max’, ‘change’, ‘std’,
786 ‘diff_neighbor_mean’, ‘diff_neighbor_min’, ‘diff_neighbor_max’, ‘zscore_neighbors’ with a
787 window radius of 10 and default ‘windows parameters’), therefore taking into account longer
788 timescales for classification, rather than the single frames we used for SVM classification and k-
789 means clustering (see Figure 2 – figure supplement 1C for comparison). We cross validated
790 each classifier before applying the classification on all of the data, using the cross-validation
791 procedure available in JAABA package (with default parameters). 94.2% of the frames
792 annotated by the user as shoving were correctly classified as shoving, while 92.8% of the
793 frames annotated as no shoving were classified as no-shoving. For the ‘chasing classifier’, we
794 got 96% and 90.8% success in classifying chasing and no-chasing. The trained shoving
795 classifier was used to annotate each frame as belonging or not belonging to ‘female shoving’
796 epoch, and the trained chasing classifier was used independently to classify each frame as

797 belonging or not-belonging to a ‘female chasing’ epoch. The same classifiers were used for all
798 experimental conditions (5-minute or 2-minute activation duration, d0/d3/d6 delay conditions).

799

800 **Manual tracking annotation of behaviors**

801 We annotated a subset of the data manually (pC1-Int, d0-d6), to confirm our automatic behavior
802 detection, as well as in search for more rare events, or events that are not captured due to
803 tracking issues. Three behaviors were annotated by two observers: female shoving, female
804 chasing and circling (Figure 2 - figure supplement 3A-C). The two observers annotated different
805 sets of movies, while a small subset ($n = 5$ movies) were annotated by the two observers and we
806 confirmed that both detected three behaviors (shoving, chasing, circling) similarly. Female
807 circling was not detected by our automated procedures for two reasons. First, during circling
808 male and female bodies often overlap, causing large errors in heading detection. Second, these
809 events are relatively sparse. One observer also detected three other rare behaviors: head
810 butting, female mounting (Figure 2 – figure supplement 3C, Video 3) and wing extension (Figure
811 2 – figure supplement 3D-E, Video 2).

812

813 **Statistical analysis**

814 Statistical analysis was performed using MATLAB (Mathworks, Natick, MA) procedures, and
815 corrected for multiple comparisons using the Bonferroni correction when appropriate. The
816 details on the statistical test used are listed under the Results section and the Figure legends.
817 Black lines between two groups indicate a statistically significant difference between the groups
818 after multiple comparison correction, while a red line indicates that the difference is statistically
819 significant only when multiple comparisons test is not used. To test for significant differences in
820 copulation rate, we used Cox’s proportional hazards regression model, using the MATLAB
821 procedure coxphfit. ‘Censoring’ was used to account for the fact that some flies copulated within
822 the 30-minute time window (after which the experiment was terminated), while others did not.
823 The correlation between female velocity and male song (Figure 1D,G) was done as previously
824 described (Clemens et al., 2015). Briefly, female absolute speed and male song were averaged
825 over 1-minute windows. In each window we calculated the mean value of female (absolute)
826 speed, bout amount (the total amount of song in the window), bout number (the number of song
827 bouts in the window) and bout duration (the mean bout duration in the window). Then, for each
828 condition, we calculated the correlation between female speed and male song by pooling all
829 windows for a given group together. The MATLAB procedure corr was used to calculate the
830 Pearson correlation, and one way analysis of covariance (ANOCOVA) was used to compare the

831 slopes (x,y being the male song and the female speed) between groups using aoctool
832 (MATLAB). The 30 SVM (Support Vector Machine) classifiers (Figure 2B-C, Figure 2 – figure
833 supplement 1C) were trained using non-overlapping sets of frames and are therefore
834 considered independent. One-sample t-test was used to calculate a test decision for the null
835 hypothesis that the 30 weight values (for a given parameter) come from a normal distribution
836 with a mean of zero (and unknown variance). For each parameter, -log(P) is shown, and a
837 vertical dashed indicates P<10⁻⁴ (Figure 2 – figure supplement 2C).

838

839 **Immunostaining**

840 Flies were dissected in S2 insect medium (Sigma #S0146). Dissected brains were moved
841 through 6 wells (12 μ l/well) containing a fixation solution (4% paraformaldehyde, Electron
842 microscopy sciences #15713 in PBT (0.3% Triton in PBSX1; Triton X-100 Sigma Aldrich #X100;
843 PBS - Cellgro #21-040), before sitting for 30 minutes on a rotator at room temperature.
844 Following fixation, brains were moved through 6 wells containing PBT, 15 minutes in each well.
845 Then, brains were transferred through 4 wells containing a blocking solution (5% Goat Serum in
846 PBT; Life Technologies #16210-064), and sitting in the last well for 30 minutes. Brains were
847 then moved to a solution containing primary antibodies (see below) and then incubated for two
848 nights at 4C (sealed and light protected). After 8 washes (20 minutes per wash) in PBT, brains
849 were incubated overnight with secondary antibodies. After 8 washed (20 minutes each) in PBT,
850 brains were placed on a slide (Fisher Scientific #12-550-15), between two zero numbered
851 coverslips used as spacers (Fisher Scientific 12-540B) and under a coverslip (Fisher Scientific
852 #12-542B), and Vectashield (Vector Laboratories) was applied. Nail polish was used to seal
853 around the center coverslip edges, and brains were stored in dark at 4C overnight to harden,
854 before imaging. See Ker Resources Table for the list of antibodies used in this study. Imaging
855 was done using a Leica confocal microscope (TCS SP8 X). Figure 1A was modified from
856 (Deutsch et al., 2019).

857

858 **Identification and proofreading of neurons in FlyWire**

859 Neurons in a complete EM volume of an adult female brain (Zheng et al., 2018) were
860 automatically reconstructed in FlyWire (flywire.ai, (Dorkenwald et al., 2020)). Within FlyWire, we
861 first searched for reconstructed segments that match the morphology of known pC1 cells. We
862 used anatomical landmarks to find the bundle that projects dorsally from pC1 cells bodies
863 (Figure 1A, red arrow). We then looked at 2 cross sections of this bundle in each hemisphere
864 (Figures 3B and Figure 3 – figure supplement 1) and scanned systematically all the segments

865 that pass through this bundle. Based on known morphology of female pC1 cells (Deutsch et al.,
866 2019; Kimura et al., 2015; Zhou et al., 2014), we defined cells as pC1 when they crossed
867 through the pC1 bundle, and also projected to the lateral junction (Figure 1A). Similarly, pC2I
868 cells were found by looking through the pC2I bundle (see Deutsch et al., 2019; we refer to these
869 cells as pC2 below). The aIPg cells were first found by searching for neurons synaptically
870 connected to pC1d (see below), and other aIPg cells were found by systematically exploring a
871 cross section within the aIPg bundle (Figures 6E and Figure 5 – figure supplement 1). pMN1
872 and pMN2 were found when mapping the pC1d synaptic partners, and then named pMN1 and
873 pMN2 based on their morphology (Deutsch et al., 2019; Kimura et al., 2015). pC1 and pC2 cells
874 were sorted manually into subtypes based on morphology (Figure 3C and Figure 5 – figure
875 supplement 1C).

876

877 Proofreading of a neuron was performed using the tools available in FlyWire (flywire.ai,
878 (Dorkenwald et al., 2020)). In short, this process has two parts: (1) removing ('splitting') parts
879 that do not belong to the cell ('mergers'), such as parts of glia or parts of other neurons (for
880 example, when detecting two cell bodies in one segment), and (2) adding missing parts
881 ('merging'). We had an average of 5.4 splits and 10.7 merges per neuron, and proofreading a
882 single cell took 43 minutes on average (we measured the proofreading time for a subset of the
883 cells we proofread). Proofreading was complete when no additional obvious mergers were
884 found, and we couldn't identify missing parts at the edge of any processes. In some cases, the
885 known morphology of the cell (e.g., pMN1 or pMN2) or the existence of other cells with similar
886 morphology (in the same or the other hemisphere) were used to verify that no major processes
887 were missing. Sorting cells into types was done manually, based on their morphology. pC1 was
888 divided into five subtypes, and aIPg were divided into three subtypes.

889

890 Assigning names to known neurons we found in the EM volume was done solely based on
891 morphology. It is possible, that in some cases (e.g., for pC2 or pC1 cells), some of the neurons
892 we found are not actually Dsx+ cells. More work is needed to compare LM based and EM based
893 morphologies, and to classify cell types based both on morphology and connectivity (Scheffer et
894 al., 2020). Finding cell types in hemibrain version 1.1 with shared morphology was done for the
895 major inputs and outputs of pC1d/e manually.

896

897 **Mapping synaptic inputs and outputs in FlyWire**

898 We mapped all the direct inputs and outputs of a single pC1d neuron (Figure 3C) by manually
899 detecting pre- and postsynaptic partners for this cell. After proofreading the cell (see details
900 above), we looked systematically, branch by branch, for synaptic partners based on previously
901 defined criteria. For a contact to be defined as a chemical synapse, it had to meet three
902 conditions: (1) the presence of a synaptic cleft between the pre- and postsynaptic cells, (2)
903 presynaptic active zone with vesicles near the contact point, and (3) one of two (or both) must
904 exist: a presynaptic T-bar adjacent to the cleft (Fouquet et al., 2009) at the presynaptic terminal
905 or a postsynaptic density (PSD,(Ziff, 1997)). In flies, PSDs are variable, and are often unclear or
906 absent(Prokop and Meinertzhagen, 2006). Typically, we observed T-bars rather than PSDs, as
907 a T-bar is easier to identify. Our criteria was slightly more conservative than the one used in
908 (Zheng et al., 2018), possibly leading to less false positives (wrongly assigned synapses), and
909 more false negatives (missing synapses). On top of using a conservative criterion, synapses
910 were missed for several other reasons, including missed detections by the manual observers
911 (see comparison to automatic detection below) and signal to noise issues in the dataset. Once a
912 synapse was detected, we then looked for the post-synaptic partner. Around 10% of the inputs
913 to pC1d and about 60% of the outputs were short segments ('twigs'), that we could not connect
914 to backbones in order to identify or proofread the connected neuron. The twigs were not
915 restricted to a specific part of the pC1d cell, and we therefore believe that they do not impose a
916 bias on the distribution of pC1d connections, though it is possible that specific output types
917 (e.g., cells with thinner processes) are less likely to be detected.

918 Following the detection of pC1d synaptic partners, we mapped the inputs and outputs to pC1d
919 in three steps. First, we manually proofread the input and output segments. Second, we
920 eliminated cells that connect to pC1d with less than 3 synapses, to reduce the number of
921 potential false positives, and to focus on stronger connections. We ended up having 78 input
922 and 52 output cells. Third, we sorted cells manually into cell types based on morphology. Some
923 cells were classified based on known morphology from light microscopy (pMN1, pMN2, pC1,
924 pC2, aIPg). In order to look for connections between pC1 and pC2 cells (the largest sets of
925 Dsx+ neurons) in an unbiased way (not focusing on specific types or individual pC1 or pC2), we
926 first identified and proofread pC1 and pC2 cells. Synaptic connections between individual cells
927 of pC1 or pC2 type were detected by manually inspecting the volume plane by plane. Once a
928 pair of segments that came within proximity of one another was detected, we zoomed and
929 looked for synaptic connections based on the criteria defined above.
930 We also used automatic detection of synapses in FAFB. FlyWire provides a mapping of the
931 publicly available, automatically detected synapses from (Buhmann et al., 2019)

932 . We estimated the reliability of synapse detection by manually testing all the synapses between
933 pC1d (presynaptic) and two different output cells (a single aIPg-b and a single pC2-like). We
934 found 61.7% (71/115) of the tested synapses to be true positives according to our criteria,
935 24.3% (28/115) redundant (two coordinates for the same synapse) and 13.9% false positives.
936 By looking at pairwise distances between presynaptic coordinates, and using a minimum
937 threshold of 150nm, we were able to remove 27/28 of the redundant synapses, while eliminating
938 only 3 true synapses, thus ending up having 80% (68/85) true positives. We therefore used this
939 threshold whenever using the Buhmann detector. The corrected Buhmann detector found all the
940 cells we previously detected manually (32/32), while adding 36 true positive synapses that were
941 missed during the manual search, suggesting that the number of true synapses are at least
942 double the number of the synapses we estimated with a manual search. We detected all the
943 inputs and outputs of a single pC1d (FlyWire coordinates 145817, 41367, 5139) and a single
944 pC1e (FlyWire coordinates 142741, 44980, 4908) cells. Cells were included in the count only if
945 they followed the following two criteria: (1) Connected to the inspected pC1d or pC1e with 6
946 synapses or more, and (2) is part of a cell type (based on morphology, using manual
947 classification) that includes at least one cell with strong connection (defined as 15 synapses or
948 more) with pC1d or pC1e. Only synapses that belong to these cells were counted (see Table 1;
949 Figure 5F; Figure 6; Figure 6 – figure supplement 1).

950

951 Finding the best match in the single clone dataset FlyCircuit for a given EM segment was done
952 in two steps. First, an .swc file was generated for a given segment (using the automatically
953 segmented cells rather than the proofread ones for technical reasons). Second, we performed
954 an NBLAST search (Costa et al., 2016) either online
955 (http://nblast.virtualflybrain.org:8080/NBLAST_on-the-fly/) or using ‘natverse’, an R package for
956 neuroanatomical data analysis (Bates et al., 2020). For visualization purposes, we first created
957 mesh files (.obj) for proofread neurons, and then used either Meshlab (<http://www.meshlab.net/>)
958 to create images, or Blender (<https://www.blender.org/>) to create movies (see support/FAQ in
959 <https://flywire.ai/> for instructions on creating .swc and .obj files).

960

961 **In vivo calcium imaging**

962 We imaged brain activity following pC1 optogenetic activation (through the microscope objective)
963 under a two-photon custom made microscope (Pacheco et al., 2019) in females, using the
964 calcium indicator GCaMP6s (Chen et al., 2013). Both GCaMP6s and the structural marker
965 tdTomato (Shaner et al., 2004) were expressed pan-neuronally in blind flies (NorpA[36] mutant)

966 using the nsyb enhancer (Bussell et al., 2014). For pC1 activation, we used the same temporal
967 pattern as the one used in the behavioral experiments: 5 or 2 minutes of light on, at 100Hz and
968 50% duty cycle. Imaging started 5 minutes before stimulus onset, where baseline activity was
969 measured, and lasted 9.5 minutes after stimulus offset for whole-brain imaging and 30 minutes
970 after stimulus offset for doublesex imaging. While the red shifted channelrhodopsin ReachR was
971 used for behavior experiments to minimize optogenetic activation by background light, we used
972 Chrimson for two-photon imaging for two reasons. First, to minimize the amount of bleed-
973 through from the optogenetic activation light to the green photomultiplier tube (PMT). For this
974 reason we also choose a longer wavelength of 700nm (M700L4, Thorlabs, Newton, NJ with a
975 band pass filter FF01-708/75-25, Semrock, Rochester, NY) that is well separated from the
976 range of light that cross the green PMT entrance filter (Semrock FF01-593/40-25). Second, we
977 wanted to minimize the amount of light needed for neuronal activation by using a more sensitive
978 effector, to reduce the amount of heat accumulating in the brain during imaging (on top of the
979 heating caused by the two-photon laser, whose power was limited to 15mW). We are aware of
980 possible differences in pC1 activation level between the behavioral and imaging experiments.
981 Based on existing literature, we tried to choose an activation level for the imaging experiments
982 that will roughly match the activation induced during behavior. In order to match the activation
983 level between the behavioral experiments (ReachR, 627nm light, intact fly) and the imaging
984 experiments (csChrimson, 700nm light, cuticle removed above the fly brain) we used data from
985 existing literature. By comparing the amount of light needed for driving proboscis extension
986 reflex (PER) in 100% of adult flies in (Inagaki et al., 2014) (ReachR, 1.1mW/mm², 627nm) to the
987 level of light used to saturate PER score in (Klapoetke et al., 2014) (CsChrimson, 0.07mW/mm²,
988 720nm), taking into account the different duty cycles used in the two studies and given the
989 penetration rate through the cuticle (Based on (Inagaki et al., 2014) Figure 1A, around 6% of the
990 light penetrates at 627nm), we choose a light intensity of 0.013 mw/mm².

991
992 A volume of ~307x307x200 μm^3 from the dorsal part of the central brain was scanned at 0.1 Hz
993 (1.4x1.2x2 μm^3 voxel size), covering a complete dorsal quadrant (full anterior-posterior axis of
994 the central brain) which represents about 58.02 +/- 3.97 % of the whole hemisphere (mean +/-
995 SD, n =28 animals). Volumetric data was processed as described in (Pacheco et al., 2019). In
996 brief, tdTomato signal was used to motion-correct volumetric time-series of GCaMP6s signal in
997 XYZ axis (using the NoRMCorre algorithm (Pnevmatikakis and Giovannucci, 2017). Volumes
998 were spatially resampled to have isotropic XY voxel size of 1.2x1.2x2 μm^3 (bilinear interpolation
999 on X and Y axes), and temporally resampled to correct for different slice timing across planes of

1000 the same volume, and to align timestamps of volumes relative to the start of the optogenetic
1001 stimulation (linear interpolation). Next, the GCaMP6s signal was 3D-ROI segmented using the
1002 Constrained Nonnegative Matrix Factorization (CNMF) algorithm to obtain temporal traces and
1003 spatial footprints per segmented ROI as implemented in CalmAn (Giovannucci et al., 2019;
1004 Pacheco et al., 2019). In this algorithm each ROI is defined as a contiguous set of pixels within
1005 the field of view that are correlated in time, these ROIs are initialized around locations with
1006 maximum variance across time. Spatially overlapping ROIs with correlated activity are merged
1007 (correlation coefficient > 0.9); therefore, ROIs could have different sizes. Code to perform these
1008 processing steps are available at <https://github.com/murthylab/FlyCalmAn>. In addition, to further
1009 remove residual motion artifacts from the GCaMP6s signal, in particular slow drift over tens of
1010 minutes, we performed independent component analysis (ICA) on the tdTomato (F_{tdTomato}) and
1011 GCaMP6s (F_{GCAMP}) signal for each ROI independently, similar to (Scholz et al., 2018). To
1012 remove opto-related artifact bleeding through the red channel, F_{tdTomato} was linearly interpolated
1013 from 20 seconds before stimulus onset to 20 after stimulus offset (to ignore opto-related artifact
1014 bleeding through the red channel) and random noise (from normal distribution centered at 0)
1015 added to interpolated timepoints. F_{tdTomato} was then smoothed (moving average with a window of
1016 50s), and ICA was used (rica function implemented in MATLAB) to extract background and
1017 signal components from F_{tdTomato} and F_{GCAMP} . Independent component highly correlated to F_{tdTomato}
1018 (absolute correlation coefficient > 0.9) was considered the background component
1019 ($\text{ICA}_{\text{Background}}(F_{\text{tdTomato}}, F_{\text{GCAMP}})$), while the other component considered the signal component
1020 ($\text{ICA}_{\text{Signal}}(F_{\text{tdTomato}}, F_{\text{GCAMP}})$). Sign of $\text{ICA}_{\text{Signal}}(F_{\text{tdTomato}}, F_{\text{GCAMP}})$ was corrected using the sign of the
1021 correlation between $\text{ICA}_{\text{Signal}}(F_{\text{tdTomato}}, F_{\text{GCAMP}})$ and F_{GCAMP} . For ROIs extracted from pan-neuronal
1022 data we report calcium signals as $\text{ICA}_{\text{Signal}}(F_{\text{tdTomato}}, F_{\text{GCAMP}})$ as shown in Figure 5B.
1023

1024 We defined responsive ROIs as ROIs with a mean activity during optogenetic stimulation ($Ft1$)
1025 higher than $3\sigma_0$ (σ_0 - standard deviation of activity during baseline). We then split ROIs into
1026 transiently and persistently active units using the mean activity after optogenetic stimulation
1027 ($Ft2$, from stimulus offset to 5 minutes after stimulus offset), transient ROIs had $Ft2 \leq 3\sigma_0$, while
1028 persistent ROIs had $Ft2 > 3\sigma_0$. To evaluate the diversity of these coarse activity types, we
1029 hierarchically clustered transient and persistent responses (we evaluated the number of clusters
1030 these response types split into using the consensus across Calinski-Harabasz, Silhouette, Gap,
1031 and Davies-Bouldin criteria), obtaining 2 clusters of transient responses and 2 clusters of
1032 persistent responses (Figures 5C-D).
1033

1034 For recordings of Dsx+ cell types, we imaged pC1, pC2, pCd1, and pCd2 cells (1-2 groups at a
1035 time), located in the dorsal side of the central brain, at a speed of 0.5-0.25 Hz ($0.5 \times 0.5 \times 1 \text{ } \mu\text{m}^3$ -
1036 $2.5 \times 2.5 \times 1 \text{ } \mu\text{m}^3$ voxel size). Similarly, for recordings of Fru+ cell types we imaged Fru+ cells
1037 (both within the same field of view), located in the dorsal side of the central brain, at a speed of
1038 0.5 Hz ($1.2 \times 1.2 \times 2 \text{ } \mu\text{m}^3$ voxel size). Volumetric time-series of GCaMP6s signal was motion-
1039 corrected in the XYZ axes (using the NoRMCorre algorithm (Pnevmatikakis and Giovannucci,
1040 2017) , and temporally resampled to correct for different slice timing across planes of the same
1041 volume, and to align timestamps of volumes relative to the start of the optogenetic stimulation
1042 (linear interpolation). Dsx+ and Fru+ somas were manually segmented by finding the center and
1043 edge of each cell body stack by stack (Deutsch et al., 2019)).

1044 **In vivo whole-cell patch clamp recordings**

1045 Stimulus design, recording techniques, and analysis methods are described in detail in
1046 (Clemens et al., 2015). Briefly, virgin female flies (1-2 days old) were mounted and dissected as
1047 described previously, and the cell bodies were accessed from the posterior surface of the head
1048 and visualized by expressing GFP in pC1-A neurons (see Fly genotype table) – 1 cell was
1049 recorded per fly. Raw data were further analyzed using MATLAB (Mathworks). To construct the
1050 IPI, sine frequency, and intensity tuning curves, we calculated the integral voltage in response
1051 to each stimulus, after subtracting the baseline voltage. Each response was then normalized by
1052 the duration of the stimulus. To compare across individuals, tuning curves were normalized to
1053 peak at 1.0.

1054

1055

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1254 **FIGURE LEGENDS**

1255

1256 **Figure 1: pC1-Int activation has a persistent effect on female receptivity and responses to male courtship
1257 song**

1258 (A) Dsx+ neurons in the female central brain. Max z-projection of a confocal stack in which Dsx+ cells are labeled
1259 with GFP (adapted from (Deutsch et al., 2019)). Dsx is expressed in 8 morphologically distinct cell types in the female
1260 brain (7 types are indicated by circling of their somas; the more anterior cell type aDN (Lee et al., 2002) is not
1261 shown). pCd has two morphologically distinct types, pCd1 and pCd2 (Kimura et al., 2015). Many of these cells project
1262 to a brain region known as the lateral junction (red square). pC1 cells project to the lateral junction through a thin
1263 bundle (red arrow). These bundles were used to identify these cells in the EM dataset (see Figure 3).

1264 (B) Behavioral chamber (diameter ~25mm) tiled with 16 microphones to record song. Male and female positions were
1265 tracked (a 1.5 second example trace is shown for the male (cyan) and female (magenta)), and male song was
1266 automatically segmented into sine and pulse (below).

1267 (C) Percent of male/female pairs that copulated as a function of time. Cox proportional hazards regression $P =$
1268 6.3×10^{-6} : pC1-Int TNT (red, $n = 68$ pairs) compared with controls (grey, $n = 40$ pairs).

1269 (D) Rank correlation between male song (bout amount, number and duration) and female speed for pC1-TNT
1270 females (dark red) or control females (grey) paired with wild type males. Significance between experimental groups
1271 was measured using ANOCOVA and multiple comparison correction (* $P < 0.01$). An asterisk on a bar indicates a
1272 significant correlation between a single male song measure and female speed (* $P < 0.01$).

1273 (E) Experimental design for pC1-Int activation. pC1-Int cells were activated (using ReaChR) for 5 minutes in a solitary
1274 female placed in the behavioral chamber. Following light offset, a wild type male was introduced at $t=0$, following a
1275 variable delay period (d_0 = no delay; d_3 = 3min delay; d_6 = 6min delay). All behavioral phenotypes were measured at
1276 $t > 0$.

1277 (F) Same as (C), but for females expressing ReachR in pC1-Int cells according to the protocol shown in (E). Inset:
1278 The percent of pairs copulated between $t = 0$ and $t = 5$ minutes for each condition. pC1-Int activated females in the d_0
1279 condition ($n = 57$) copulated significantly faster than controls ($n = 51$; vertical black line; $P = 0.0045$, Cox's
1280 proportional hazards regression model, accounting for censoring, as not all flies copulated in 30 minutes; black
1281 vertical line). Time to copulation was also shorter in the d_3 group ($n = 39$ pairs) compared with controls (d_0 , no ATR),
1282 but the difference was not significant after Bonferroni correction ($P = 0.034$; red vertical line), and no significant
1283 difference was found between the 6-min delay ($n = 30$) and control groups ($P = 0.21$).

1284 (G) Same as (D), but for females expressing ReachR in pC1-Int cells according to the protocol shown in (E).
1285 Asterisks show significance, using the same criteria as in (D). Numbers of pairs are the same as in (F).

1286

1287 **Figure 2: Automated identification of persistent female behaviors following pC1-Int activation**

1288 (A) For each video frame, 17 parameters were extracted based on the tracking of male/female position and heading
1289 (see Methods for parameter definition). An example trace (30 seconds) is shown for each parameter.

1290 (B) 30 independent (using non-overlapping sets of video frames) Support Vector Machine (SVM) classifiers were
1291 trained to classify frames (each frame represented by 17 values) as belonging to control or experimental group (all
1292 delays d_0-d_6 considered together; see Methods). Each classifier is represented by 17 points - one for each
1293 parameter. Each point is the weight associated with a given parameter for one classifier, and the bar height
1294 represents the mean over classifiers (* $P < 10^{-4}$, one-sample t-test with Bonferroni correction for multiple comparisons;
1295 see Methods).

1296 (C) The percent of frames correctly classified using the SVM classifier. Each dot is the prediction of a single SVM
1297 classifier, trained to classify frames as belonging to control or experimental group (d0, d3, d6 or d0-d6 together) – 30
1298 classifiers and their mean plotted for each group.

1299 (D) K-means was used to cluster frames based on the 8 most significant parameters (marked with asterisks in (B)).
1300 The largest 7 clusters include 90.4% of the frames (see inset). Clustering was performed 30 times (black dots; bars =
1301 mean), using different but overlapping sets of frames. The same number of frames was taken from each group (see
1302 Methods). Cluster 2 (blue box - ‘female shoving’) is more probable following pC1-Int activation (in d0, d3 and d6
1303 conditions) compared to control, while cluster 4 (green box- ‘female chasing’) is more probable in the d3 condition
1304 only compared to control. At right, schematic describing the male-female interaction in each cluster, based on the
1305 mean values of the weights.

1306 (E) JAABA-based classification of shoving (top) and chasing (bottom) behaviors. Each dot represents a single pair of
1307 flies. The fraction of time the male-female pair spent shoving (0.037/0.21/0.27/0.15 for control/d0/d3/d6) or chasing
1308 (0.013/0.030/0.079/0.022) are shown. Black lines represent significant differences with $p < 0.05$ after Bonferroni
1309 correction for multiple comparison. Red lines - significant before, but not after correction for multiple comparisons.

1310 (F) Fraction of time females spent chasing or shoving (moving average with a two-minute window), based on JAABA
1311 classification in each condition (control, d0, d3, d6). T = 0 is the time the male was introduced (see Figure 1E), and
1312 the vertical dotted line indicates the time, for each condition, when 80% of the pairs copulated. Behaviors are not
1313 scored after copulation.

1314

1315 **Figure 3: Defining pC1 cell types**

1316 (A) Models of how pC1 cell types control female receptivity, shoving, and chasing. In (i), a homogenous pC1
1317 population drives three distinct behaviors, and in (ii) and (iii) pC1 is a heterogenous group, with different behaviors
1318 controlled by different pC1 subsets.

1319 (B) EM reconstruction of pC1 cells and other example neurons that pass through a cross section in the pC1 bundle.
1320 See Figure 3, figure supplement 1 for all the cells that go through the pC1-bundle, including neurons that project to
1321 the lateral junction (considered pC1 cells), and neurons that do not project to the junction (not considered pC1 cells).

1322 (C) 5 pC1 cell types identified in FlyWire, mostly consistent with (F. Wang et al., 2020). The medial projection in pC1c
1323 (see black arrows) is ipsilateral to the soma in one cell and contralateral in the other, likely reflecting variability
1324 between individual cells. Both pC1d and pC1e share a horizontal projection from the ring, but only pC1d cells have an
1325 extra vertical projection (red arrows indicate difference between pC1d and pC1e projections). The right pC1e cell is
1326 also missing part of the ring.

1327 (D) (top) Split GAL4 line pC1-S (R71G01.AD; DSX.DBD; n = 8, 7.7 ± 5 cells per hemisphere) and (bottom) Split GAL4
1328 line pC1-A (VT25602.AD; VT2064.DBD; n = 7, 2 ± 0 cells per hemisphere). Neurons express GFP and are labeled
1329 with anti-GFP, see Key Resources Table for full genotype. pC1-A has a medial projection (red arrow), similar to pC1d
1330 neurons found in EM (C); the medial projection was found in 7/7 imaged pC1-A female brains, in both hemispheres.
1331 The pC1d and pC1e projections were not found in pC1-S imaged female brains (8/8 brains) - neurons labeled in pC1-
1332 S therefore may label some combination of pC1a-c neurons found in EM (C). The medial projection that is unique to
1333 the pC1c subtype was missing in all the 8 pC1-S flies we imaged; therefore it is likely that pC1-S includes only pC1a
1334 and/or pC1b cells.

1335

1336 **Figure 4: pC1d/e neurons drive female shoving and chasing, but do not affect receptivity**

1337 (A) pC1-A activation did not affect copulation rate in either the d0 or d3 conditions ($n = 40, 38, 40$ for control/d0/d3; P
1338 = 0.79 or 0.29 for control vs d0 or d3; Cox proportional hazards regression, see Methods).
1339 (B, C) Shoving (B) and chasing (C) probabilities (control/do/d3: 0.02/0.17/0.20 and 0.018/0.11/0.07 for shoving and
1340 chasing) were significantly higher in both the d0 and d3 conditions compared to control (two-sample t-test; *P<0.05).
1341 (D-E) same as (A-C), but for pC1-S activation. pC1-S activation did not affect neither copulation rate (D) nor shoving
1342 or chasing (E) probabilities (control/do: 0.02/0.02 and 0.01/0.01 for shoving and chasing).
1343 (F) Probability distribution of the fraction of time the female spent shoving (left) or chasing (right) following pC1-A
1344 activation (line) or pC1-Int activation (dashed line). Arrows indicate the difference in peak shoving (at d3; two-sample
1345 t-test, P = 0.11) or chasing (at d0; two-sample t-test, *P = 4.5*10⁻⁴) probability, between pC1-Int and pC1-A activation.
1346 Bonferroni correction was used for multiple comparisons.
1347 (G) Fraction of frames with shoving for pairs in which females copulated (R - Receptive) or did not copulate (NR –
1348 Non Receptive) for all experimental conditions taken together (d0-d6). Each dot is a single pair, and the bar value is
1349 the mean over all pairs (P = 0.92 and 0.13 for control and d0-d6).
1350 (H) Same as (G), for female chasing (P = 0.13 and control and P < 10⁻⁵ for d0-d6).
1351 (I) Female pC1d/e cells drive persistent shoving and chasing, but do not affect female receptivity. Female receptivity,
1352 controlled by a separate pC1 subset labeled in the pC1-Int line, suppresses female chasing, while possibly enhancing
1353 female shoving.
1354 (J) Shoving and chasing probabilities (control/do: 0.077/0.015 and 0.039/0.006 for shoving and chasing; P = 0.026
1355 and 0.035; n = 20/20 for control/d0) were significantly higher in the d0 condition compared to control following two
1356 minutes of pC1-A activation, but not following 30 seconds (n = 20/21 for control/d0) of pC1-A activation (P = 0.52 and
1357 P = 0.24 for shoving and chasing).
1358

1359 **Figure 5: The connectome of pC1d reveals recurrent connections with alPg neurons**

1360 (A) The left pC1d cell (posterior view) from FlyWire after automatic segmentation and manual proofreading. The cell
1361 body is marked with a red arrow, and the pC1d long medial projection (that does not exist in other pC1 types) is
1362 marked with black arrows.
1363 (B) Same cell as in (A), with manually detected synapses. Presynaptic terminals (inputs to pC1d-I) are marked in red,
1364 post-synaptic terminals (outputs) in green. After excluding segments that are connected with pC1d with less than 3
1365 synapses, we end up counting 417/421 manually-detected input/output synapses (see also Video 6).
1366 (C) **Left:** pC1d inputs (66 cells) and outputs (50 cells) were classified manually to cell types based on morphology.
1367 The number of input (top) or output (bottom) synapses are shown for each type, sorted (separately for inputs and
1368 outputs) based on the total number of synapses with pC1d for each type. **Right:** The cumulative fraction of synapses
1369 counted as a function of the number of types included (calculated separately for inputs/outputs). The three most
1370 common output types encompass 49.4% of the output synapses, while the 3 most common input types encompass
1371 30.5% of all input synapses.
1372 (D) pC1d major inputs (top) and outputs (bottom) based on manual synapse detection. Right: the cells that belong to
1373 the most common cell types (50% of all output synapses) are shown for pC1d output cells. One cell is shown per cell
1374 type. Note that pC1d has postsynaptic connections with both left (ipsilateral to cells body) and right (contralateral)
1375 alPg cells.
1376 (E) **Left:** Posterior view (same as in (A)) of pC1d (grey) and alPg cells. **Right:** rotated view, showing the separation
1377 between three subtypes of alPg cells (6/24/8 for alPg-a/b/c), that we sorted into alPg-a, alPg-b and alPg-c according
1378 to their projections. See Table 1 for the full list of FlyWire detected alPg cells in one hemisphere.

1379 (F) Synapses between pC1d (grey) and example alPg cells, color coded by alPg type as in (E). alPg example cells:
1380 alPg-a: 159548, 54417, 5069, alPg-b: 156131, 54718, 5430, alPg-c: 159565, 54293, 5261 (numbers listed here are
1381 FlyWire coordinates for each cell type). Synapse count for each type is summarized. Synapses were detected
1382 automatically (Buhmann et al., 2019). Only cells with 6 synapses or more with pC1d are included in the analysis (if
1383 the number of synapses is smaller than 6, both the cell and its synapses are excluded from the count). See Materials
1384 and Methods and Table 1 for more details.
1385 (G) Connection matrix between pairs of alPg cells. The number of synapses (Buhmann et al., 2019) between an
1386 given pair of alPg cells are indicated with a colorscale. Black lines separate alPg subtypes. Red lines denote the alPg-
1387 cells that are reciprocally connected with pC1d (with 6 synapses or more each way), and red arrows indicate an alPg-
1388 b cell that is reciprocally connected to the same alPg-b cells that also connect to pC1d.
1389 (H) The number of synapses within and between groups of alPg cells based on the fly hemibrain connectome
1390 ([Scheffer et al., 2020](#)). The number in parentheses indicates the number of cells per group (alPg-1-4). Round arrows
1391 indicate within-group connections (e.g., 61 synaptic connections between pairs of alPg-1 cells). Dotted arrows are
1392 shown for weak connections (under 5 synapses).
1393 (I) pC1d connections with putative Dsx+ pMN1 and Dsx+ pMN2 cells.
1394 (J) An example putative Dsx+pC1 cell (type pC1c) that is recurrently connected to pC1d.
1395 (K) pC1d connections with pC2-like cells (with similar morphology as hemibrain pIP5 neurons or Fru+ pIP-e clones
1396 from ([Cachero et al., 2010](#))).
1397 (L) A summary of pC1d connections with putative Dsx+ and Fru+ neurons. pC1d is a hub connecting Dsx+ or Fru+
1398 pC1, pIP5, pMN1, and pMN2 neurons with Fru+ alPg neurons.

1399
1400 **Figure 6: pC1d synaptic partners based on automatic synapse detection in FlyWire**
1401 (A) Top (≥ 80 synapses) pC1d inputs (left) and outputs (right). The number of pC1d presynaptic and postsynaptic
1402 terminals are shown in red/green. The number of input or output cells that are connected to pC1d with 6 synapses or
1403 more (see Methods) are shown in the purple circles. Cells with shared morphology were identified in hemibrain v1.1
1404 (names shown in blue) for each cell type. As the number of synapses is counted per type and only for cells with 6
1405 synapses or more, separately for the inputs and outputs, the numbers between the left and right plots do not
1406 necessarily match (see Table 1 for more details).
1407 (B) Example cells that are connected to pC1d. pC1d_In8 synapses with pC1d in the side ipsilateral to pC1d soma,
1408 but has its soma and dendrites in the contralateral hemisphere. pC1d_Out6 is connected to pC1d contralateral to
1409 pC1d soma.
1410 (C) Example pC1d input or output cells in FlyWire, compared to a similar cell in the hemibrain v1.1.

1411
1412 **Figure 7: pC1d/e neurons drive persistent neural activity in the central brain**
1413 (A) Experimental setup. pC1 cells (pC1-A or pC1-S) expressing csChrimson were activated through the objective
1414 using an LED (700nm). GCaMP6s and tdTomato were expressed pan-neuronally using the nsyb driver, and a
1415 custom-designed two-photon microscope was used to image brain activity before, during and after pC1 activation
1416 (see Key Resources Table for genotypes and Methods for more details on the experimental setup).
1417 (B) Brain activity recorded in response to optogenetic stimuli ($n = 28$ flies, all genotypes). GCaMP6s signal was
1418 motion corrected and 3D-ROI segmented based on correlated activity in neighbor voxels (see Methods).
1419 The z-scored signal of all ROIs ($n = 47882$ ROIs from both pC1-S and pC1-A activation and control experiments) are
1420 plotted in units of standard deviations (see scale bar), and shown 5 minutes before activation, during activation (t1),

1421 and 9.5 minutes post-activation (t2 marks the first 5 minutes post-activation). Red dashed line depicts the optogenetic
1422 stimulus onset and offset.

1423 **(C)** pC1 activation evokes transient and persistent activity. Subset of ROIs from panel **(B)** were selected based on
1424 mean z-scored activity during (t1) and after photoactivation (t2), and ROIs were sorted by hierarchical clustering of
1425 temporal dynamics. We found 4254 responsive ROIs, defined as ROIs with $F_{t1} > 3\sigma_0$ (σ_0 - standard deviation during
1426 baseline, F_{t1} is the mean fluorescence during t1), including transient ($F_{t2} \leq 3\sigma_0$, blue and cyan; F_{t2} is the mean
1427 fluorescence during t2) or persistent response types ($F_{t2} > 3\sigma_0$, red and purple).

1428 **(D)** Mean \pm SD for response types 1-4. In response types 1 and 2 the activity level (calcium response) persists after
1429 activation offset, while for types 3 and 4, the activity is high during, but not after photoactivation. The major 2 sub
1430 clusters of response type 2 are shown at right.

1431 **(E)** Maps of transient and persistent activity types. ROIs from response types 1-4 per animal were registered to an in
1432 vivo intersex atlas (Pacheco et al., 2019) to generate probability density maps across animals per brain voxel (each
1433 voxel is $0.75 \times 0.75 \times 1 \mu\text{m}^3$). Activity maps are maximum-projected along the anterior-posterior axis, and overlaid onto
1434 the brain template, color coded by the fraction of flies showing activity at each voxel (ranging from 30-100%). We
1435 considered a voxel to consistently have a particular response type if active in over 30% of flies. Response type 2
1436 shows persistent activity following pC1-A activation, and occupies 4.3% of half the central brain (does not include
1437 optic lobes), compared with 0.6% following pC1-S and 0.2% in control flies.

1438 **(F)** Brain regions containing persistent responses (type 2). We used both anatomical segmentation of the in vivo brain
1439 atlas (Pacheco et al. 2019) and segmentation of the Dsx+ circuit (also registered to the same atlas) into processes in
1440 the LPC and major groups of cell bodies (pC1, pC2, pCd1, pCd2) to assign ROIs to neuropils (red) or overlap with
1441 Dsx+ neurons (green). For each of these regions, we calculated the average number of voxels or volume (across-
1442 individuals) occupied by all ROIs belonging to response type 2, following pC1-A activation. Neuropils were sorted by
1443 the number of voxels, and the top 6 neuropils are shown. pC2m and pC2l are shown together as pC2, as they are not
1444 always spatially separable in females. For responses in other conditions (pC1-S, control) and other neuropils see
1445 Figure 7 – figure supplement 1A.

1446 **(G)** Mean response (DF/F) over all flies and ROIs per brain neuropil from **(F)**, at $t = 0$ (stimulus offset), $t = 3$ minutes
1447 and $t = 6$ minutes. Time points relative to stimulus are shown in arrows in the schematic. Each ROI's activity was z-
1448 scored relative to the baseline; therefore, DF/F units are plotted in standard deviation (SD) relative to baseline
1449 activity.

1450 **(H)** The percent of voxels that belong to the persistent cluster (response type 2) out of half on the central brain
1451 (4.3%), out of the voxels that include pC1d (29.04%), or out of half of the voxels that include aIPg cells (20.14%; see
1452 Methods) – pC1d and aIPg neurons from FlyWire were registered into the in vivo atlas for comparisons.

1453 **(I)** Shorter duration (2-minute) pC1-A activation also evokes both transient and persistent activity. ROIs for both
1454 control and pC1-A activation (using the same criteria as in **(C)**) could also be clustered into four response types
1455 (purple, red, blue, and cyan) similar to **(D)**.

1456 **(J)** Map of persistent activity type 2 upon 2-minute pC1-A stimulation. ROIs from response type 2 were processed as
1457 described in **(E)**.

1458

1459 **Figure 8: pC1d/e neurons drive persistent neural activity in Dsx+ and Fru+ cells**

1460 **(A Left:** Activation of neurons in the pC1-A line and imaging of neural activity in all Dsx+ cell bodies. Activity in pC1
1461 neurons is shown 10 seconds before stimulus onset and 5 minutes after stimulus onset for an example experiment.

1462 **Right:** Normalized activity of Dsx+ pC1 somas $((F(t) - F_0)/\sigma_0$; F_0 and $F(t)$ are mean Fluorescence during baseline and

1463 fluorescence over time, respectively). Chrimson and TdTomato are expressed in pC1-A cells. GCaMP6s is expressed
1464 in Dsx+ cells. ~~imaged~~ imaged pC1 neurons for pC1-A activation and control with $F_{t1} > 3\sigma_0$ (σ_0 - standard deviation during
1465 baseline, F_{t1} is the mean fluorescence during t1) are shown (see (B) for full set of imaged pC1 neurons).
1466 **(B) Left:** Mean Calcium response in Dsx+ pC1 neurons during t1 (x-axis) versus during t2 (y-axis) for pC1-A
1467 activation and control. Normalized activity is defined as $(F - F_0)/\sigma_0$, where F_0 is the mean activity during baseline, σ_0 is
1468 the standard deviation during baseline, and F is the mean activity during t1 for x and t2 for y. Each dot represents a
1469 single segmented soma. Dots above the red line represent persistent responses following activation. All imaged pC1
1470 neurons are shown (n = 8 flies, 58 ROIs for pC1-A, n = 5 flies, 25 ROIs for controls). **Right:** Example traces of $(F -$
1471 $F_0)/F_0$ from two individual pC1 cells, showing different response decays after stimulus offset (matching the results
1472 from (Figure 7D)). Corresponding points are enlarged and marked in green and purple in Figure 8B (left).
1473 **(C) Top:** Activation of neurons in the pC1-A line and imaging of neural activity in Fru+ cell bodies. **Bottom:** Spatial
1474 pattern of Fru+ cell bodies imaged (here, a left hemibrain is shown (Z-projection of Fru+ neurons expressing
1475 GCaMP6s)). Fru+ cell body groups 1 and 2 are defined based on their spatial location - group 1 likely contains Fru+
1476 pC2 and alPg neuronal cell bodies, while group 2 likely contains Fru+ pC1 cell bodies (Figure 8 – figure supplement
1477 2).
1478 **(D)** Normalized activity of Fru+ cell bodies from group 1 (Top) or group 2 (Bottom).
1479 **(E)** Mean activity in Fru+ cell bodies from group 1 (left, n = 9 flies, 46 ROIs for pC1-A, n = 5 flies, 13 ROIs for control)
1480 and group 2 (right, n = 9 flies, 37 ROIs for pC1-A, n = 5 flies, 24 ROIs for control) during t1 (x-axis) versus during t2
1481 (y-axis) following pC1-A activation and in controls (see Key Resources Table for full genotypes). Data were analyzed
1482 and plotted as in **(B)**.

1483 **Supplementary Figures**

1484

1485 **Figure 1 – figure supplement 1**

1486 (A) (i) A single video frame of a male (with painted dot) and a female in the behavioral chamber. (ii) Confidence maps
1487 (Pereira et al., 2019) for male and female head (blue) and thorax (red). (iii) Part affinity vector fields (Cao et al.,
1488 2017). (iv) Heading of male (cyan) and female (magenta).

1489 (B) Percent of pairs that copulated as a function of time ($P = 0.0001$, Cox proportional hazards regression, see
1490 Methods; $n = 40$, 60 pairs for Control, d0). pCd1>TNT (see Key Resources Table for full genotype).

1491 (C) **Left:** Same as (B) for pCd1 activated female (pCd1>ReaChR, see Key Resources Table for full genotype). $P =$
1492 0.93, Cox proportional hazards regression, see Methods; $n = 42$, 47 pairs for Control, d0.

1493 **Inset:** percent of flies copulated in 5 minutes.

1494 (D) Song amount (left), number (middle) and duration are defined as in (Clemens et al., 2015), see Methods. Mean
1495 and standard error over 1-minute windows are shown for each condition.

1496

1497 **Figure 2 – figure supplement 1**

1498 (A) Bar height indicates $-\log(P\text{-value})$ for the probability that the mean distribution of SVM (Support Vector Machine)
1499 weights (over 30 independent classifiers) associated with each weight (Figure 2B) is significantly different than zero.
1500 Natural log is used. Dashed line indicates $P\text{-value} = 10^{-4}$. Asterisks indicate weights associated with distributions with
1501 $P\text{-value} < 10^{-4}$, using Benferroni correction for multiple comparisons.

1502 (B) Distribution of fmAngle and mfAngle (Figure 2A) are shown for 4 experimental conditions (4.5 deg bin size).
1503 fmAngle/mfAngle are the absolute number of degrees the female/male needs to turn in order to point to the centroid
1504 of the other fly (see cartoons).

1505 (C) The weights associated with each behavioral cluster (Figure 2D), for the 8 significant weights (Figure 2 – figure
1506 supplement 1A) that were used for clustering. Each dot represents a single clustering repeat (see Methods).

1507 (D) Frames that belong to the shoving (blue) or chasing (green) behavioral clusters (Figure 2D) are indicated as black
1508 horizontal lines. JAABA classification for the same 15 seconds is indicated as horizontal bars.

1509 (E) Violin plots (MATLAB violin) are shown for bout duration of female shoving (left) and chasing (right) bouts based
1510 on JAABA classification. Means are shown as black lines (0.99/1.47/1.88/1.7 seconds for control/d0/d3/d6). Black
1511 vertical line indicates a significant difference between groups ($p < 0.05$, two sample t-test). Red line indicates that the
1512 difference is significant only if multiple comparison correction is not applied. **Inset:** The fraction of all frames in the
1513 experiment that belong to long bouts (≥ 5 seconds). In the main plots (but not in the insets and not for statistical
1514 measures) the smallest and largest 5% bout durations were excluded for each condition.

1515

1516 **Figure 2 – figure supplement 2**

1517 (A) Fraction of time the female spent shoving (left) or chasing (right) following pCd1 activation, based on JAABA
1518 classification.

1519 (B) Fraction of time the female spent shoving or chasing following pC1-Int activation, based on manual scoring.

1520 (C) Conditioned probabilities (the probability for a given transition, given that a transition occurred) for chasing,
1521 shoving and other (no shoving and no chasing) for the d0, d3 and d6 conditions, following pC1-Int, 5-minutes
1522 activation. Arrow width is proportional to probability. Shoving and chasing classification is based on JAABA.
1523

1524 **Figure 2 – figure supplement 3**

1525 (A) **Left:** Distribution of mfDist (male-female distance) during female chasing (green) and shoving (blue) for d0-d6
1526 conditions. The horizontal arrow illustrates the criterion used for defining 'female approaching' events: the female is
1527 approaching the male from large distance (> 98 percentile mfDist during shoving or chasing) to short distance (< 95
1528 percentile for distance mfDist during shoving/chasing), while continuously heading towards the male (fmAngle < 30
1529 deg). **Right:** The percent of frames for each condition that belong to 'Female approaching' epochs. Black line
1530 indicates significant difference ($P < 0.05$, two sample t-test with Bonferroni correction for multiple comparisons).

1531 (B) **Left:** Four example frames from a single 'circling' epoch, separated by 90 deg in the female heading direction
1532 (see also Video 3). In this example, a female completed 270 deg in 2.1 seconds. **Right:** Fraction of time the male and
1533 female are spending 'circling' based on manual annotation. The difference was statistically significant between the
1534 control and conditions d0-d6 taken together ($p < 0.05$, two sample t-test), but not when taking each condition alone.
1535 **Inset:** The fraction of time the female spent shoving (d0-d6) aligned to circling onsets indicates high probability for
1536 shoving shortly before circling onset.

1537 (C) Number of bouts per minute are shown for manually detected behaviors: 'female headbutting', 'female mounting'
1538 and female extending one or two wings (see Videos 2,3). The two points with >5 represent 5.5 and 8.1 bouts per
1539 minute.

1540 (D) Example frames with female unilateral (top) or bilateral (bottom) wing extension (WingExt). **Middle:** example
1541 sound trace during female chasing with unilateral and bilateral wing extensions. **Right:** example sound trace during
1542 female shoving with bilateral wing extension. Note that the sound evoked by female wing extension during shoving
1543 was an order of magnitude larger than the sound evoked when the female extended one or two wings during chasing.

1544 (E) **Left:** Wing extension was manually detected in 9.3% of all frames (d0-d6 taken together) during chasing epochs,
1545 and in 1% of the frames during shoving epochs. **Right:** 50% of the frames detected as 'wing extension' were part of
1546 female chasing or shoving, and 67.9% of the frames with wing extension occurred during or around chasing or
1547 shoving bouts ('around': 2 seconds before epoch onset until 2 seconds after epoch offset).

1548

1549 **Figure 3 – figure supplement 1**

1550 (A) Cross section of left pC1-bundle. All segments going through this cross-section or through another cross section
1551 (separated by 140 slices, equal to 5.6 μm ; FlyWire coordinates: 148388, 39874, 4080) were tested. Similarly, two
1552 cross sections were tested in the right hemisphere. Automatically reconstructed segments crossing any of these 4
1553 cross sections were sorted based on morphology. Segments that included a projection to the lateral junction (colored
1554 in blue/green/cyan) or segments that were too short to judge were proofread, and considered pC1-like neurons.
1555 Neurons that passed through any of the cross sections, but did not project to the lateral junction (colored in brown)
1556 were not further analyzed.

1557 (B) Proofread neurons that go through the cross section in (A) and also project to the lateral junction. Dashed red
1558 circle marks the lateral junction (Cachero et al., 2010).

1559 (C) Neurons that go through the cross section in (A), and do not project to the lateral junction. Dashed red circle
1560 marks the lateral junction location.

1561 (D) The most common type of neuron from those shown in (C-D). This cell follows the pC1 bundle, but does not
1562 project to the lateral junction.

1563

1564 **Figure 3 – figure supplement 2**

1565 (A) pC1-Int neurons expressing GFP (see Key Resources Table for full genotype). **Left:** A maximum z-projection is
1566 shown for the pC1-Int processes. Red arrows mark the medial projections (left and right hemispheres) that exist in
1567 pC1d, but not in other pC1 cells types. **Right:** pC1-Int expression in the female VNC.

1568 (B) pC1-A in the female VNC expressing GFP (see Key Resources Table for full genotype).

1569 (C) pC1-S in the female VNC expressing GFP (see Key Resources Table for full genotype).

1570

1571 **Figure 4 – figure supplement 1**

1572 (A) Example Vm traces of pC1d/e cells (using pC1-A driver, see Key Resources Table) for pulse trains with different
1573 IPIs. Each line is a cell recorded in a different individual. The stimulus pattern is shown below each trace.

1574 (B) Example response (top) to natural song (bottom).

1575 (C) Tuning of pC1d/e for IPI (left, n = 6 cells), sine frequency (middle, n = 3 cells), and white noise intensity (right, n =
1576 3 cells). The response was calculated as the baseline subtracted Vm integrated over the stimulus duration. Each
1577 individual curve corresponds to the trial-averaged responses of pC1d/e from one individual and was normalized to
1578 peak at 1.0.

1579 (D) Response to the pulse, sine, and noise stimulus that drive the strongest response, normalized by the response to
1580 the overall stimulus that drove the strongest response (n = 3 cells).

1581

1582 **Figure 5 – figure supplement 1**

1583 (A) **Top:** Example synapses between pC1d and postsynaptic alPg-a (left; dyadic synapse) and alPg-b (right).
1584 **Bottom:** A cross section of the bundle that connects the alPg cell bodies (FlyWire coordinates: 160515, 53776,
1585 3390). One of two cross sections checked in the left hemisphere for alPg cell types (second cross section FlyWire
1586 coordinates: 161278, 55953, 4590).

1587 (B) **Top:** Example neurons, one of each type - alPg-a,b,c from FlyWire (following proofreading). **Bottom:** Fru+ clones
1588 (from <http://www.flycircuit.tw/>) that share similar morphology with alPg cell types found in FlyWire. These cells were
1589 classified as alP-g in VFB (virtualflybrain.org), following Cachero et al., 2010.

1590 (C) alPg cells in FlyWire. **Left:** neurons labeled as alPg in the hemibrain ([Scheffer et al., 2020](#)) were divided into 4
1591 subgroups (alPg-1,2,3,4). **right:** alPg-c cells from the FAFB dataset. Black arrows indicate common morphology
1592 between alPg-c neurons in the two datasets. Black points indicate the point where alPg neurons split into alPg-a,b,c
1593 (see Figure 6E). Red arrow - a projection that was found in both EM scanned brains, but shows longer projections in
1594 the hemibrain. This difference could reflect technical differences or biological variability.

1595 (D) **Left:** Hemibrain (version 1.1) cells that follow the morphology of alPg-a (SMP555/556; yellow arrow), alPg-b
1596 (alPg1-3 and 1 alPg4; cyan arrow), and alPg-c (SMP558 and a single alPg4 cell). **Middle:** All the alPg1-3 cells in the
1597 hemibrain (n = 11). **Right:** alPg cells in FlyWire, using the same view as for hemibrain alPg1-3. The same FlyWire
1598 alPg-b cells are presented in (C) and (D).

1599

1600

1601 **Figure 6 – figure supplement 1: pC1e synaptic partners based on automatic synapse detection in FlyWire**

1602 (A) Top (≥ 30 synapses) pC1e inputs (left) and outputs (right). The number of pC1e presynaptic and postsynaptic
1603 terminals are shown in red/green. The number of input or output cells that are connected to pC1d with 6 synapses or
1604 more (see Methods) are shown in the purple circles. Cells with similar morphology were identified in hemibrain v1.1
1605 (names shown in blue) for some of the cell types.

1606 (B) Top two input and top two output types (judged by number of synapses) that are connected to pC1e.

1607 (C) 10 aIPg-b cells that get inputs from pC1d were sorted based on their synaptic connectivity with pC1d and pC1e
1608 (one pC1d and one pC1e cell, both in the left hemisphere in posterior view). 7 out of the 10 aIPg cells that are pC1d
1609 synaptic partners, also synapse with pC1e.

1610

1611 **Figure 7 – figure supplement 1**

1612 (A) Average number of voxels occupied by all ROIs belonging to each response type (as in Figure 7F) across all 36
1613 central brain neuropils for each condition (pC1-A or pC1-S activation, or controls), sorted from left to right by the
1614 amount of response type 2.

1615 (B) Distribution of mean ROIs' response (DF/F) per flies at $t = 0$ (stimulus offset), $t = 3$ min, and $t = 6$ min from
1616 response type 2 (see Figure 7D) for pC1-A activation. Each ROI's activity was z-scored relative to the baseline;
1617 therefore, DF/F units are plotted in standard deviation (SD) relative to baseline activity. P-values: one-way ANOVA
1618 with Bonferroni corrections.

1619 (C) Sensory-evoked versus optogenetically-evoked responses. Distribution of neural responses to pulse, sine or
1620 white noise auditory stimuli at t_0 (auditory stimulus offset) form all auditory ROIs ($n = 33$ flies, 19,036 ROIs; see
1621 (Pacheco et al., 2019)) - blue, compared with optogenetically-evoked responses at t_0 (light offset) for ROIs with either
1622 transient or persistent activity - back (data from Figure 7C). Responses were z-scored relative to the baseline;
1623 therefore DF/F units are plotted in standard deviation (SD) relative to baseline activity (baseline = -8 to -0.5 s before
1624 auditory stimulus onset and -245 to -5 for before optogenetic light onset).

1625

1626 **Figure 8 – figure supplement 1**

1627 (A) Mean calcium responses during t_1 (x-axis) versus during t_2 (y-axis) as in Figure 6J for the major Dsx+ cell types
1628 except pC1 - pC2 ($n = 12$ flies, 154 ROIs for pC1-A, $n = 8$ flies, 166 ROIs for controls), pCd1 ($n = 5$ flies, 44 ROIs for
1629 pC1-A), and pCd2 ($n = 3$ flies, 16 ROIs for pC1-A, $n = 1$ flies, 1 ROIs for controls). Activity units are in $(F - F_0)/F_0$,
1630 where F_0 is the mean activity during baseline, and F is the mean activity during t_1 or t_2 for x and y axes, respectively.
1631 Each dot represents a single cell, and dot colors refer to different conditions (pC1-A activation and controls).

1632 (B) Example traces of pC2, pCd1 and pCd2 cells with stimulus-locked transient responses. In some cases, a
1633 transient response was locked to stimulus onset.

1634

1635 **Figure 8 – figure supplement 2**

1636 (A) Z-projection of Fru+ neurons (Fru-LexA driving GCaMP6s) from the left hemibrain from fixed brain (black and
1637 white) and Fru+ single neurons that were downloaded from virtualflybrain.org (source: FlyCircuit; Single cell clones
1638 from adult female brains (Cachero et al., 2010)) co-registered to JFRC2 atlas. pC1-like (blue, $n = 6$; classification:
1639 adult female pMP-e), pC2-like (green, $n = 6$; classification: adult female fruitless pIP-e), and aIP-g (red, $n = 6$;
1640 classification: adult female fruitless aIP-g).

1641 Fru+ group 1 cells (using the fru-LexA driver) overlap with registered single clones of pC1-like (pMP-e) and pC2-like
1642 (pIP-e) cells.

1643 (B) FlyWire cells that has a shared morphology with pC1 (blue), pC2 (green) and aIPg (red) cells.

1644

1645

1646

1647 **Supplementary Videos**

1648

1649 **Video 1**

1650 Confidence maps (head in blue, thorax in orange) and part affinity vector fields (white arrows) calculated by LEAP
1651 (Pereira et al., 2019) for the male and female. The male has a white painted dot on his back. Male chasing and
1652 singing are shown, as well as female shoving. Movie is slowed down 4 times.

1653

1654 **Video 2**

1655 A sequence of female shoving and chasing. The female is shoving the male while occasionally extending one or two
1656 wings, and is then chasing the male while occasionally extending a single wing or contacting the male with her front
1657 legs. Finally, the male attempts to copulate, the female spreads her wings and copulation occurs. Movie is in real
1658 time. Experimental condition: pC1-Int, d0.

1659 **Video 3**

1660 Multiple example behaviors: female approaching (Figure 2- figure supplement 3A), shoving and circling (Figure 2 –
1661 figure supplement 3B), female headbutting and ‘female mounting’ (Figure 2 – figure supplement 3C). Following
1662 ‘female approaching’ in this example, there is a short epoch of circling. In the ‘shoving and circling’ example, the
1663 female is shoving the male before a circling behavior starts (See Figure 2 - figure supplement 3B, inset). In the
1664 ‘female headbutting’ example, the female is extending two wings while headbutting the male, followed by a male
1665 jump. In the ‘female mounting’ example, the female is positioning herself behind the male and climbing on his back.
1666 Circling and Headbutting examples are from pC1-Int (d0) condition, and female approaching/mounting from pC1-Int
1667 (d3) condition.

1668

1669 **Video 4**

1670 Maximum z-projection (60µm in Z) of the calcium response in a female expressing GCaMP6s pan-neuronally.
1671 Calcium response ((F(t) -Fo/Fo), color coded) is shown 5 minutes before, 5 minutes during and 9.5 minutes after
1672 pC1-A activation (using csChrimson). The movie is sped up 20 times.

1673

1674 **Video 5**

1675 Maximum Z-projection of the calcium response in a female expressing GCaMP6s in Dsx+ cells. pC1 cells in the left
1676 hemisphere are shown. Calcium level ((F(t) -Fo/Fo), color coded) is shown 5 minutes before, 5 minutes during and
1677 9.5 minutes after pC1-A activation (using csChrimson). The movie is sped up 20 times.

1678

1679 **Video 6**

1680 A single pC1d neuron, automatically traced and manually proofread. Inputs (post-synaptic terminals, manual
1681 detection) are shown in red, outputs (pre-synaptic terminals, manual detection) in green (see also Figure 5A-B).

1682

1683 **Video 7**

1684 pCd (blue) is shown with example alPg-a,b,c cells. Synapses (detected manually) are marked in red for inputs (to
1685 pC1d) and in green for outputs. Cell type colors (yellow, cyan, magenta) are shown for alPg-a,b,c as in Figure 5E-F
1686 and Figure 5 – figure supplement 1.

1687

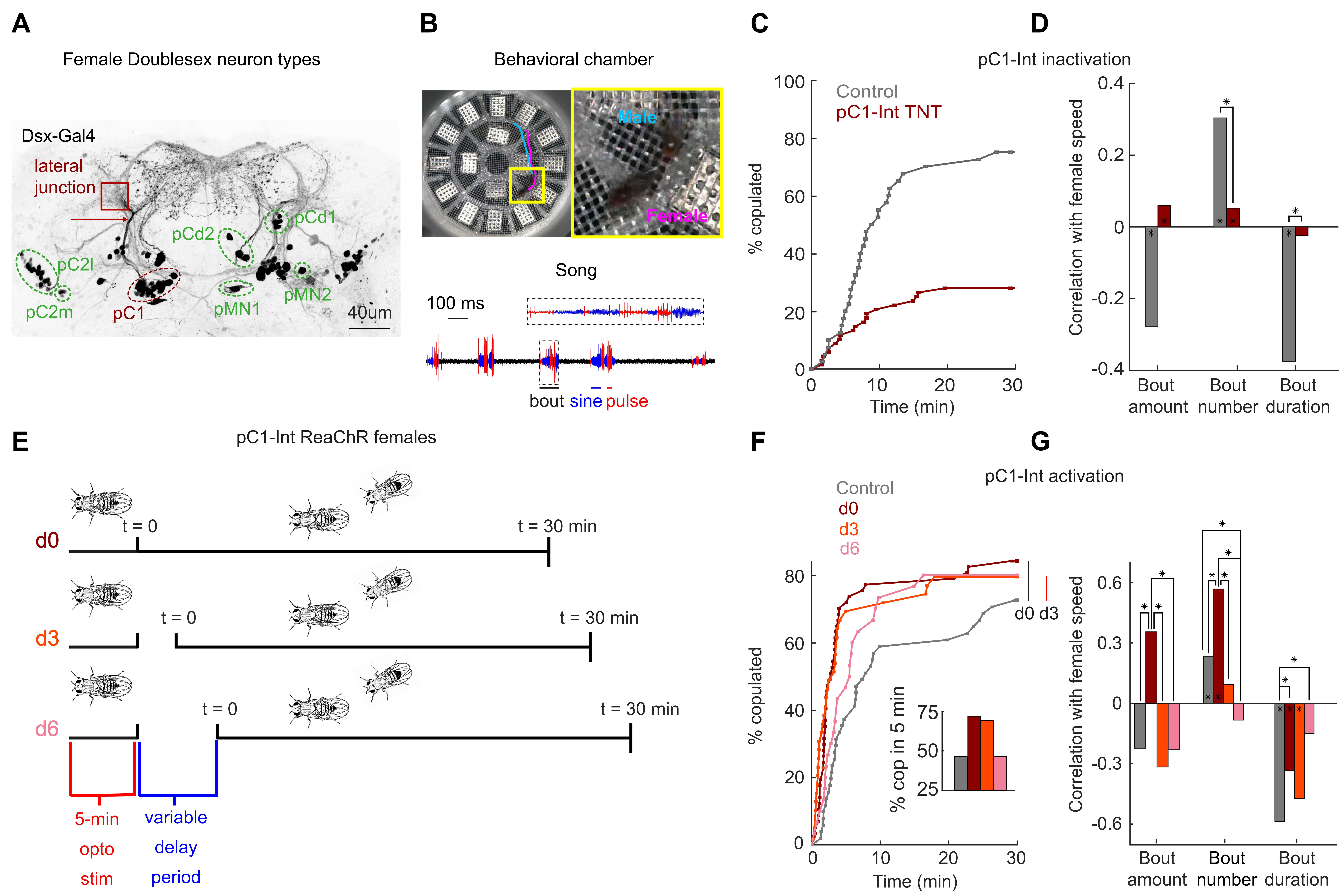
1688 **Video 8**

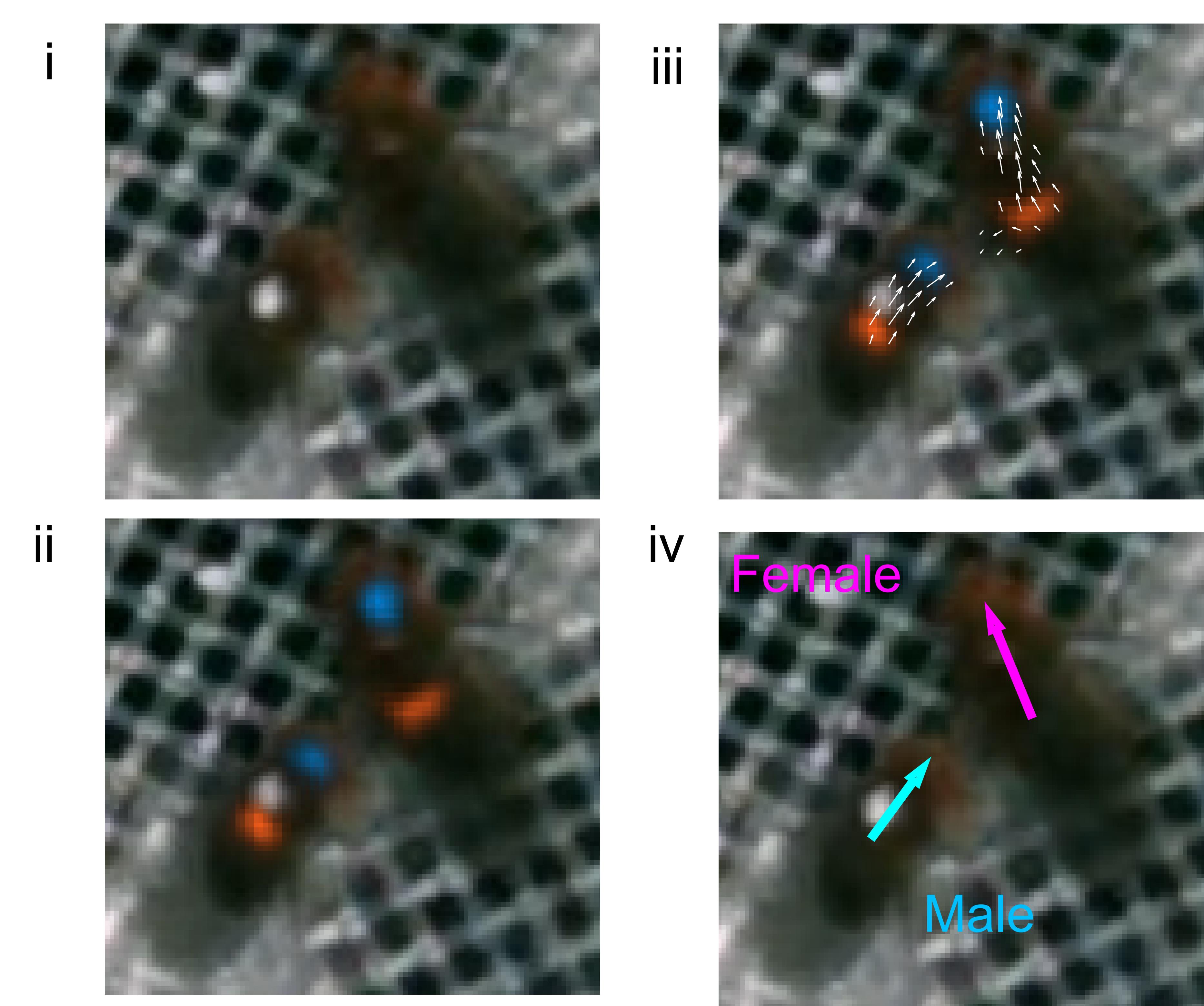
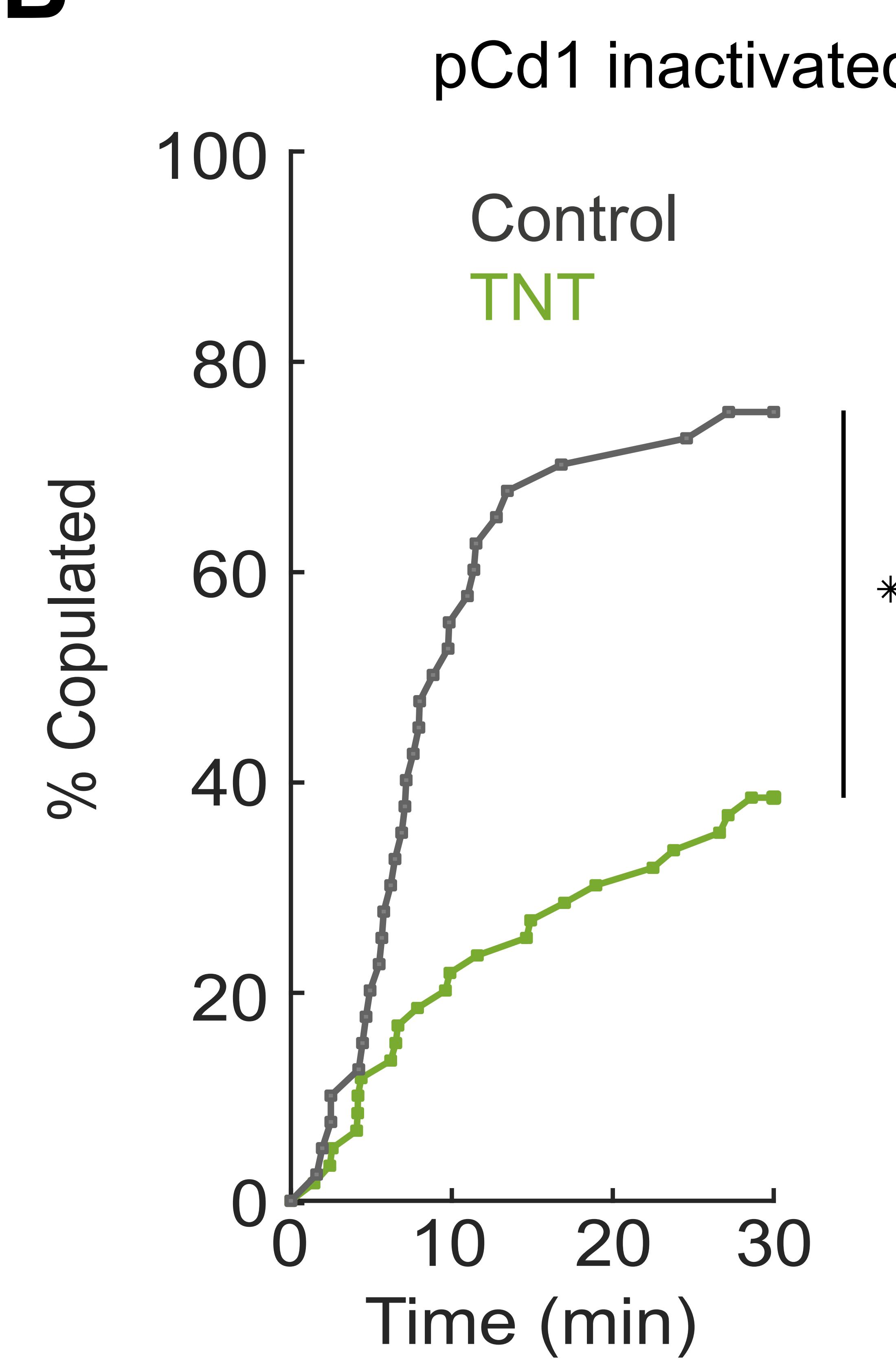
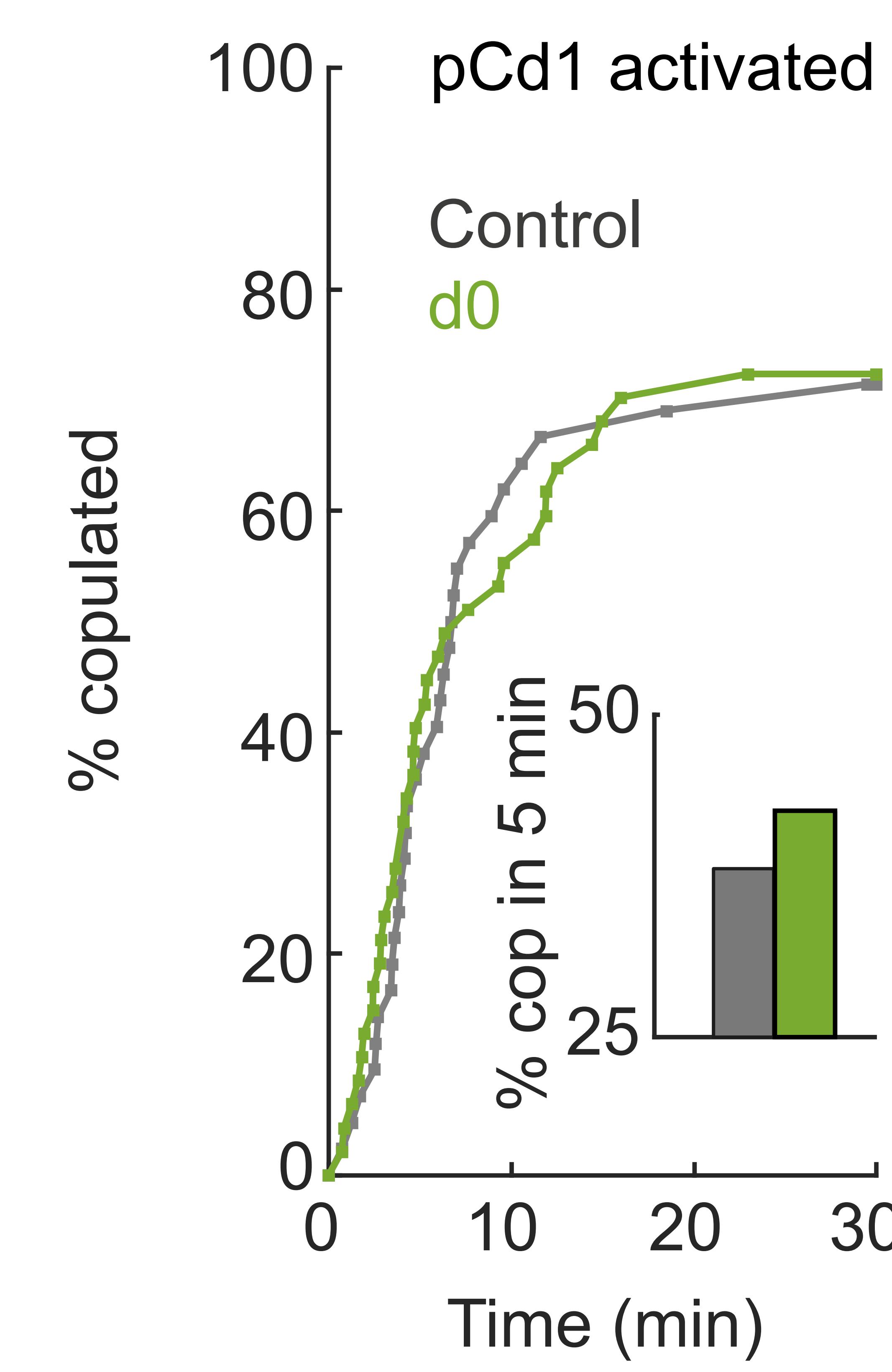
1689 pC1d (blue) is shown with neurons that have a similar morphology as known female Doublesex-expressing cells. pC1
1690 subtypes are shown in Figure 3C and two pC2 subtypes are shown in Figure 5K. pC1d input/output synapses
1691 (manual detection) with each example cell are shown in red/green.

1692

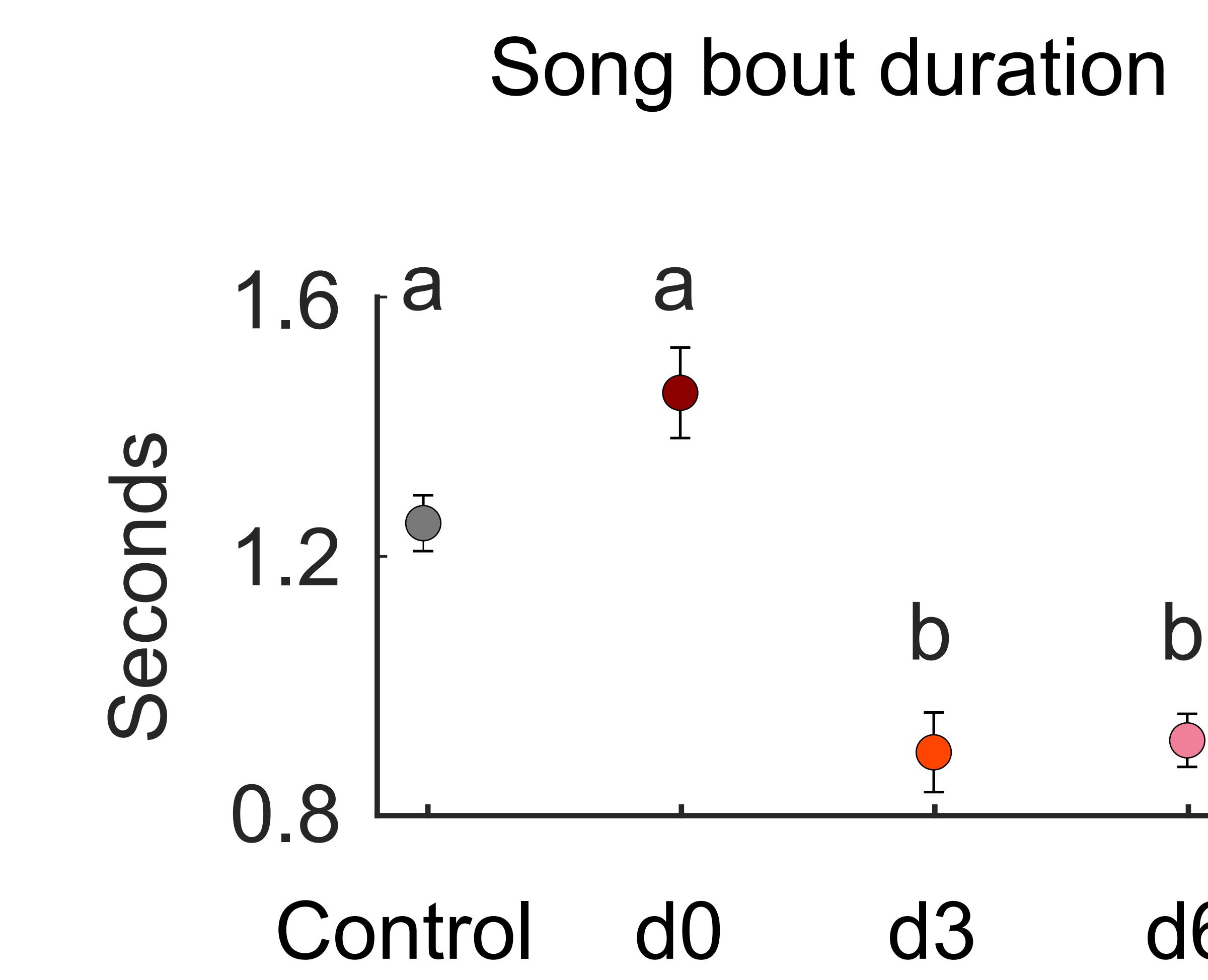
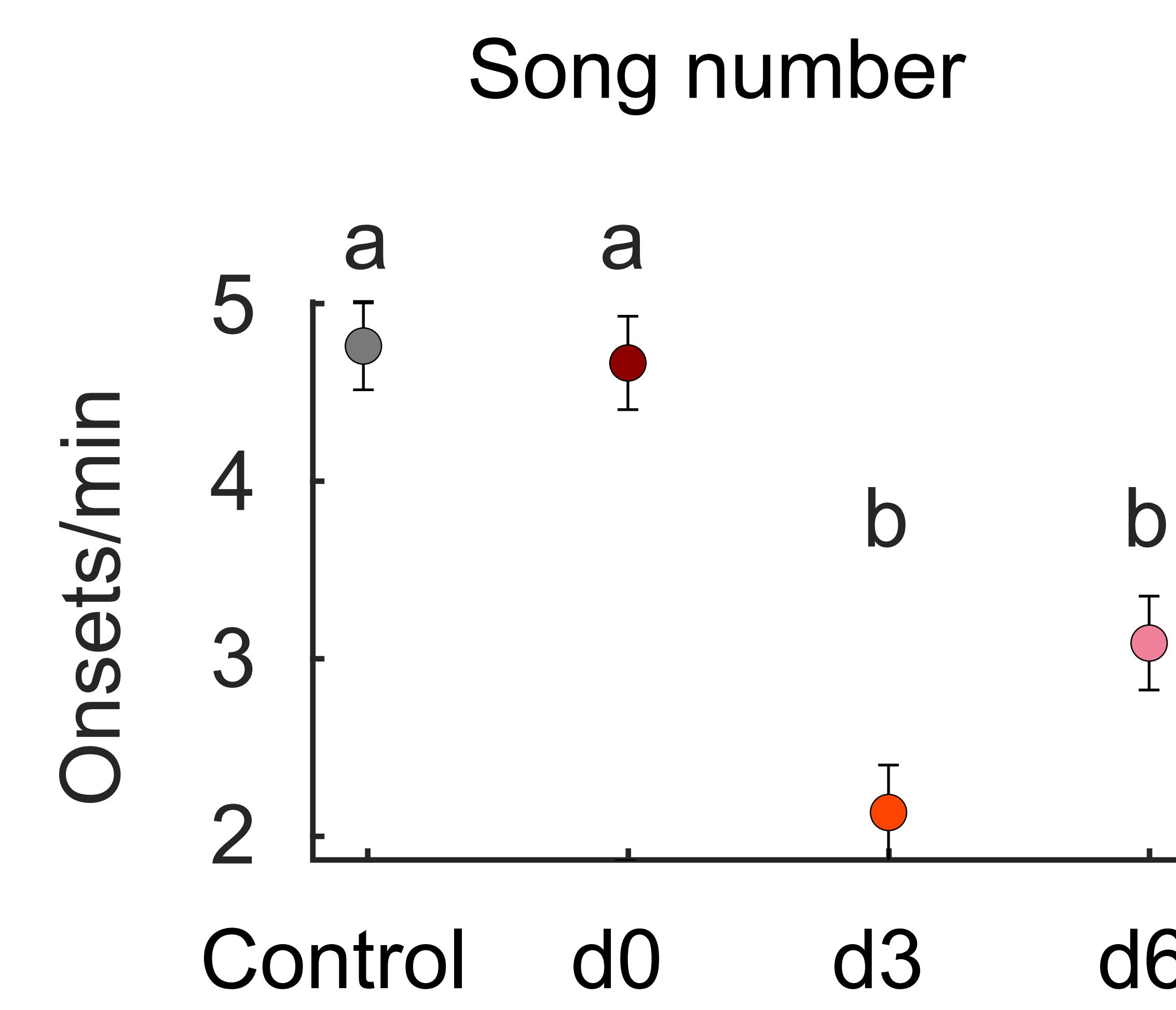
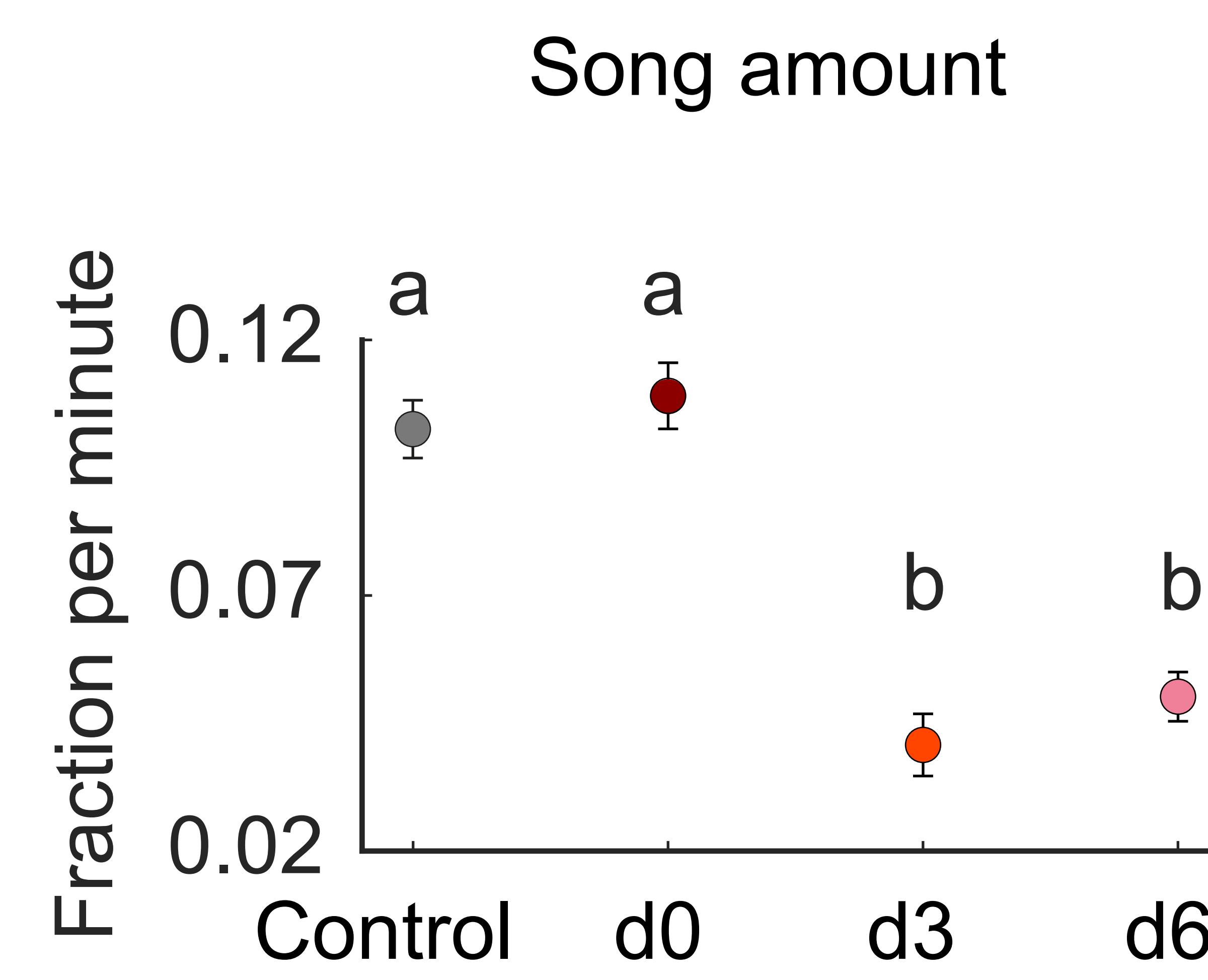
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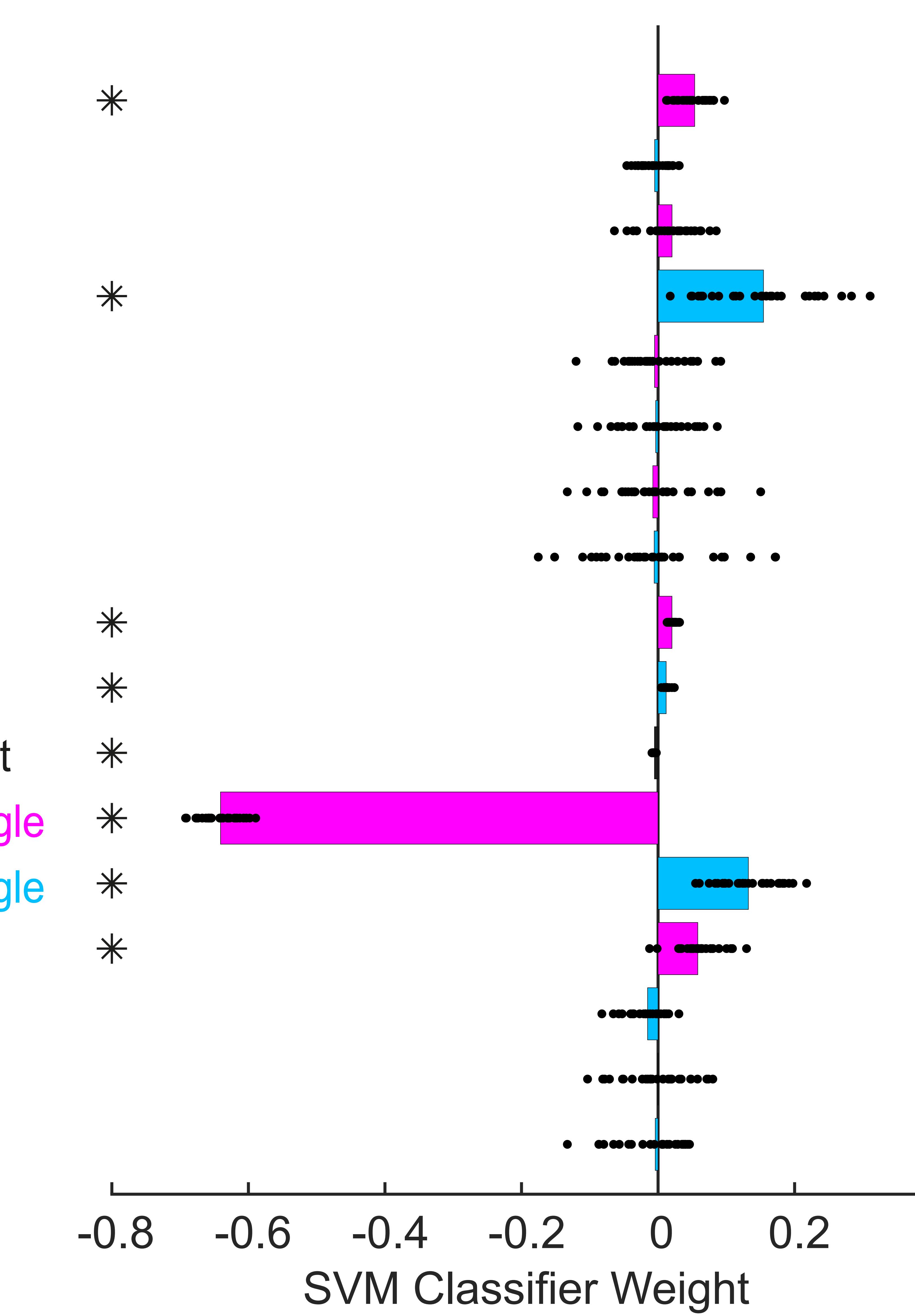
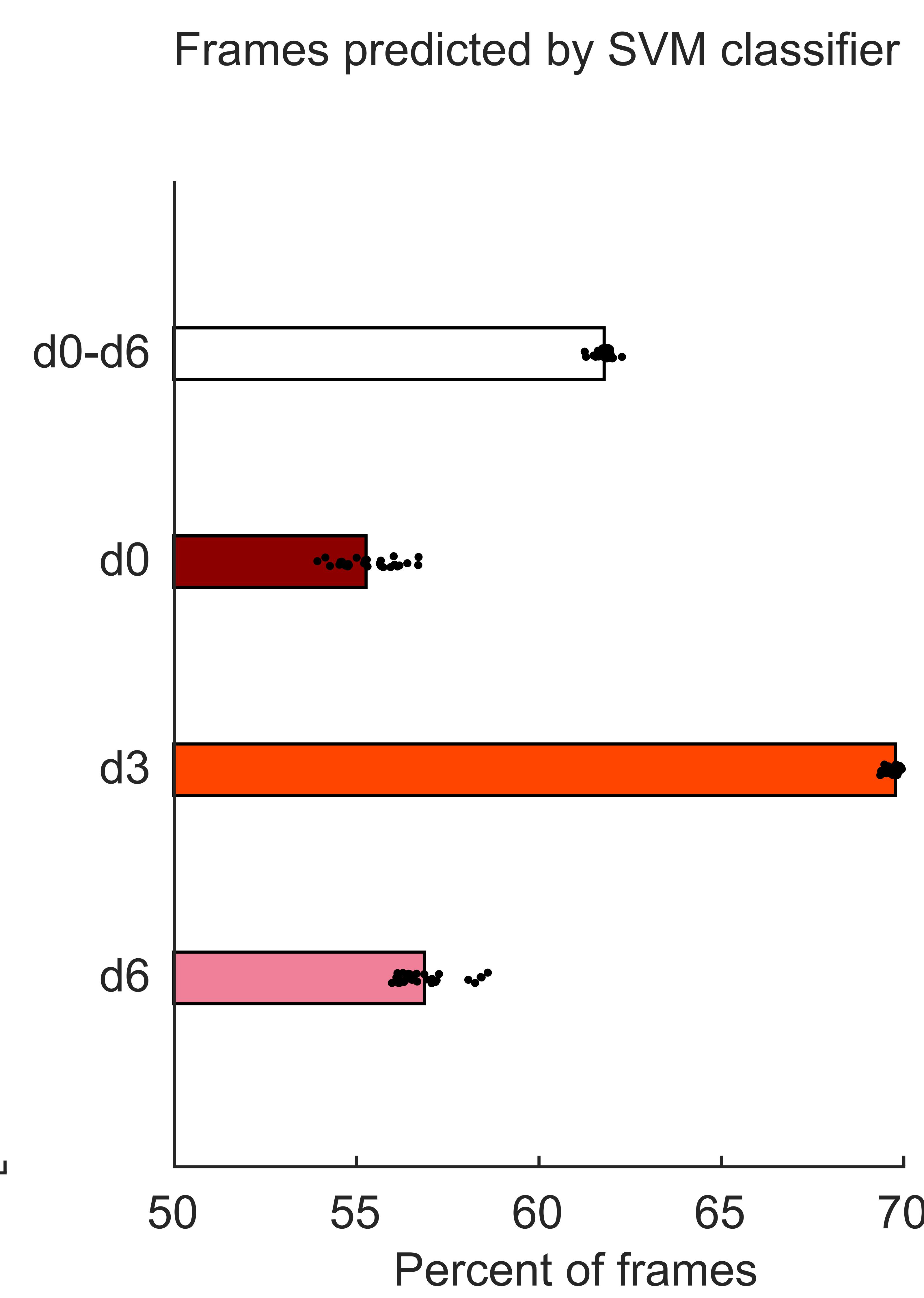
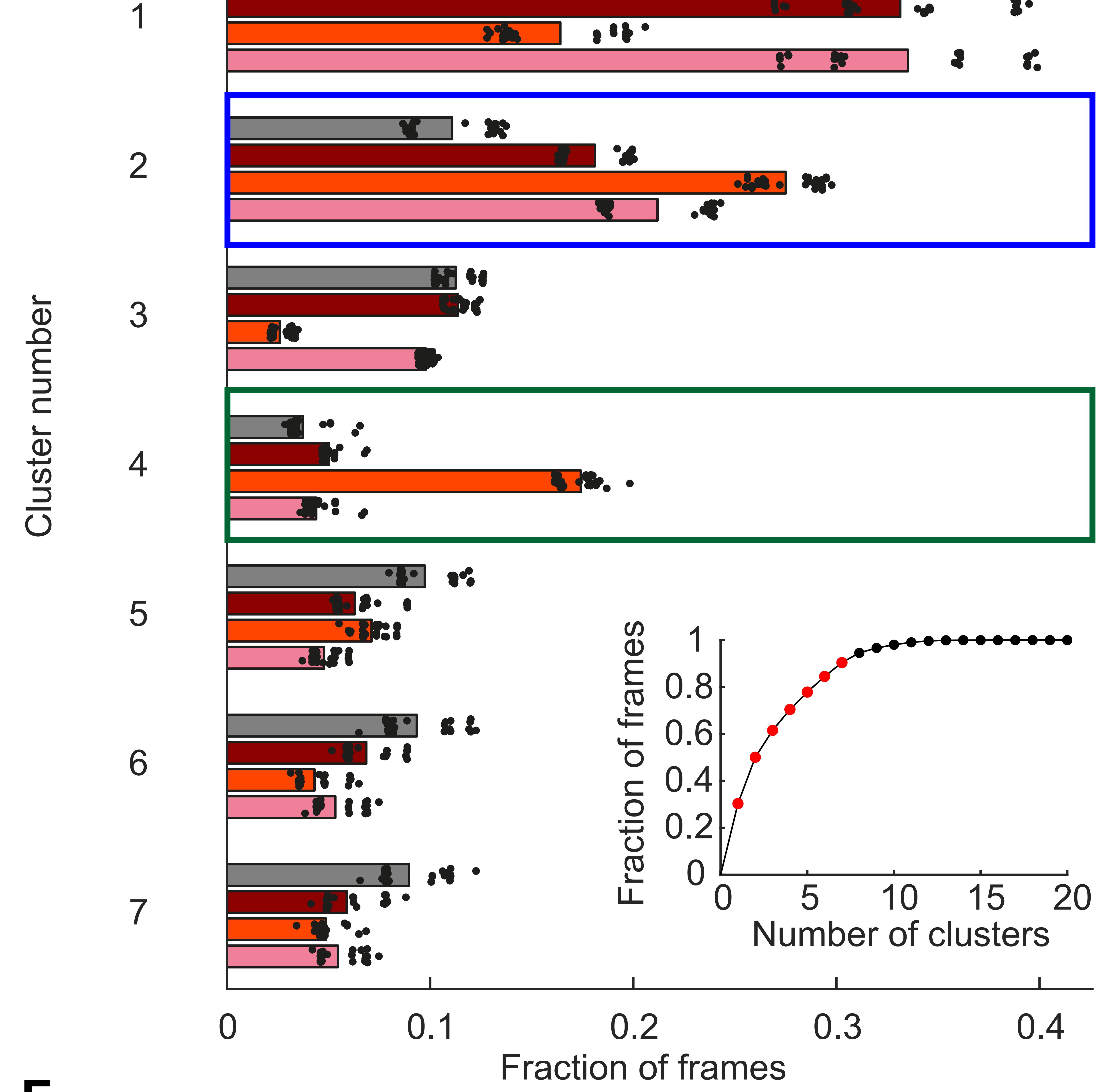
1694



A**B****C****D**

Female pC1-Int activated

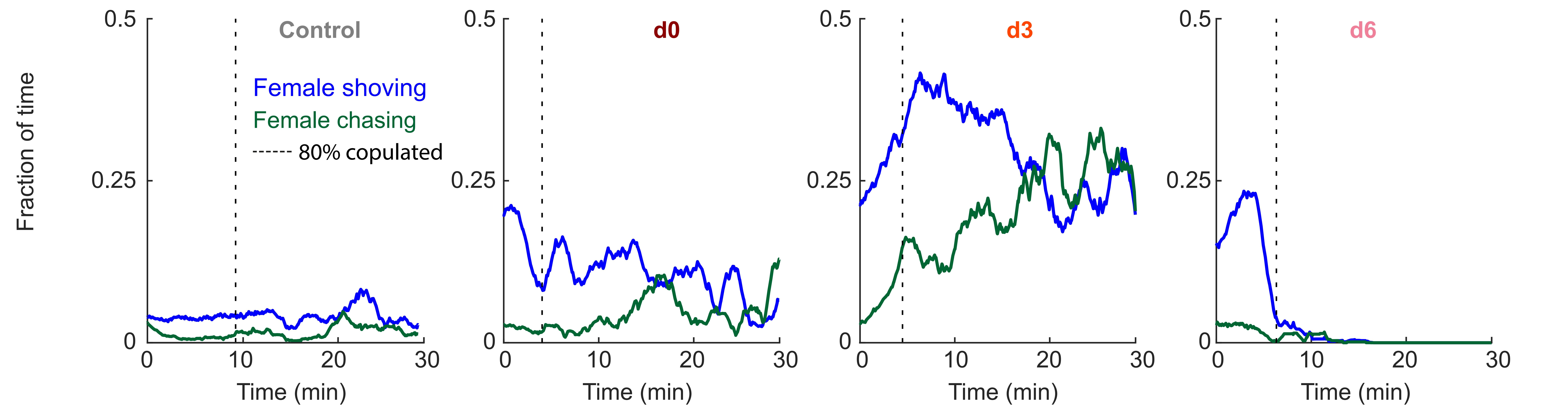


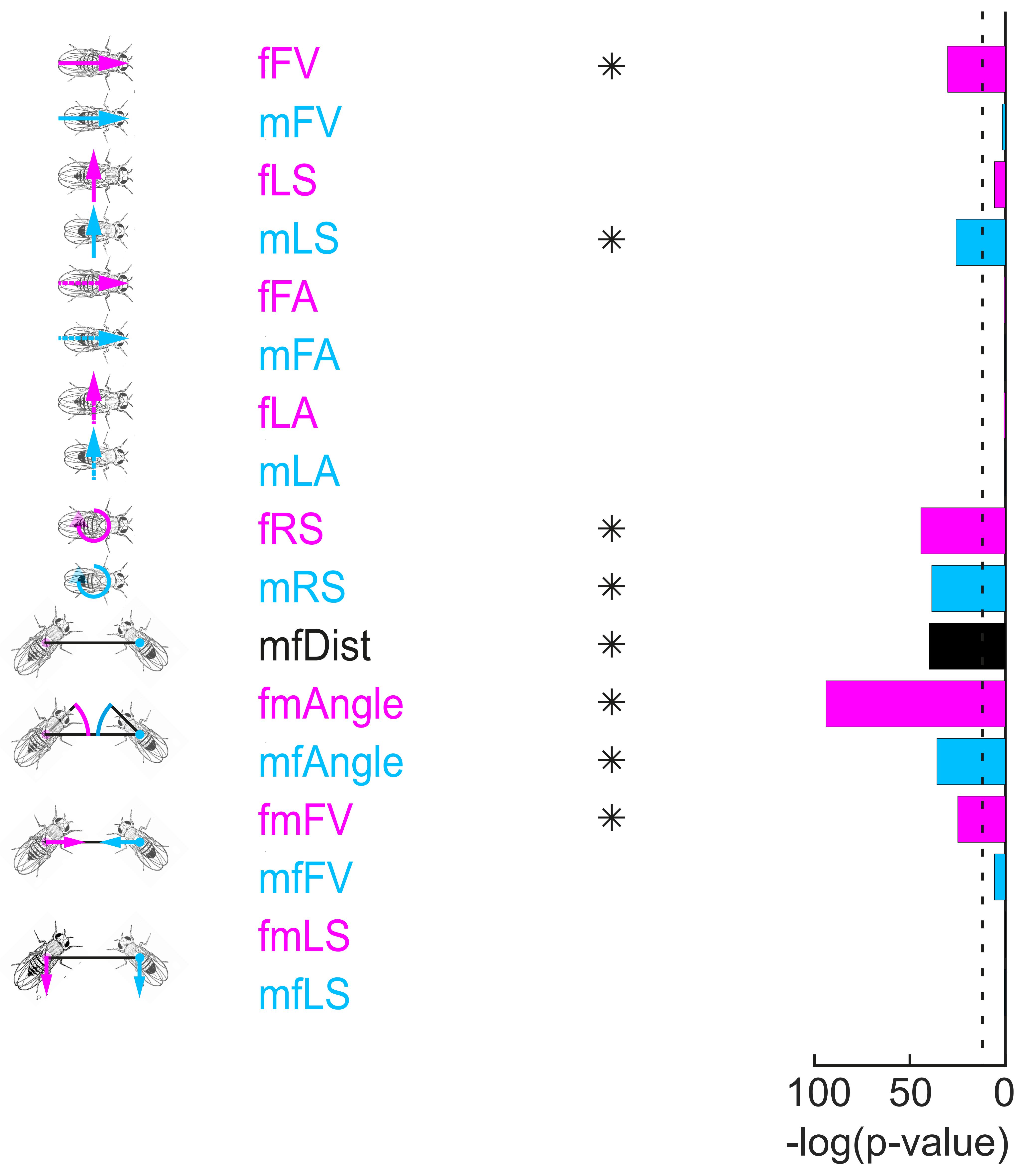
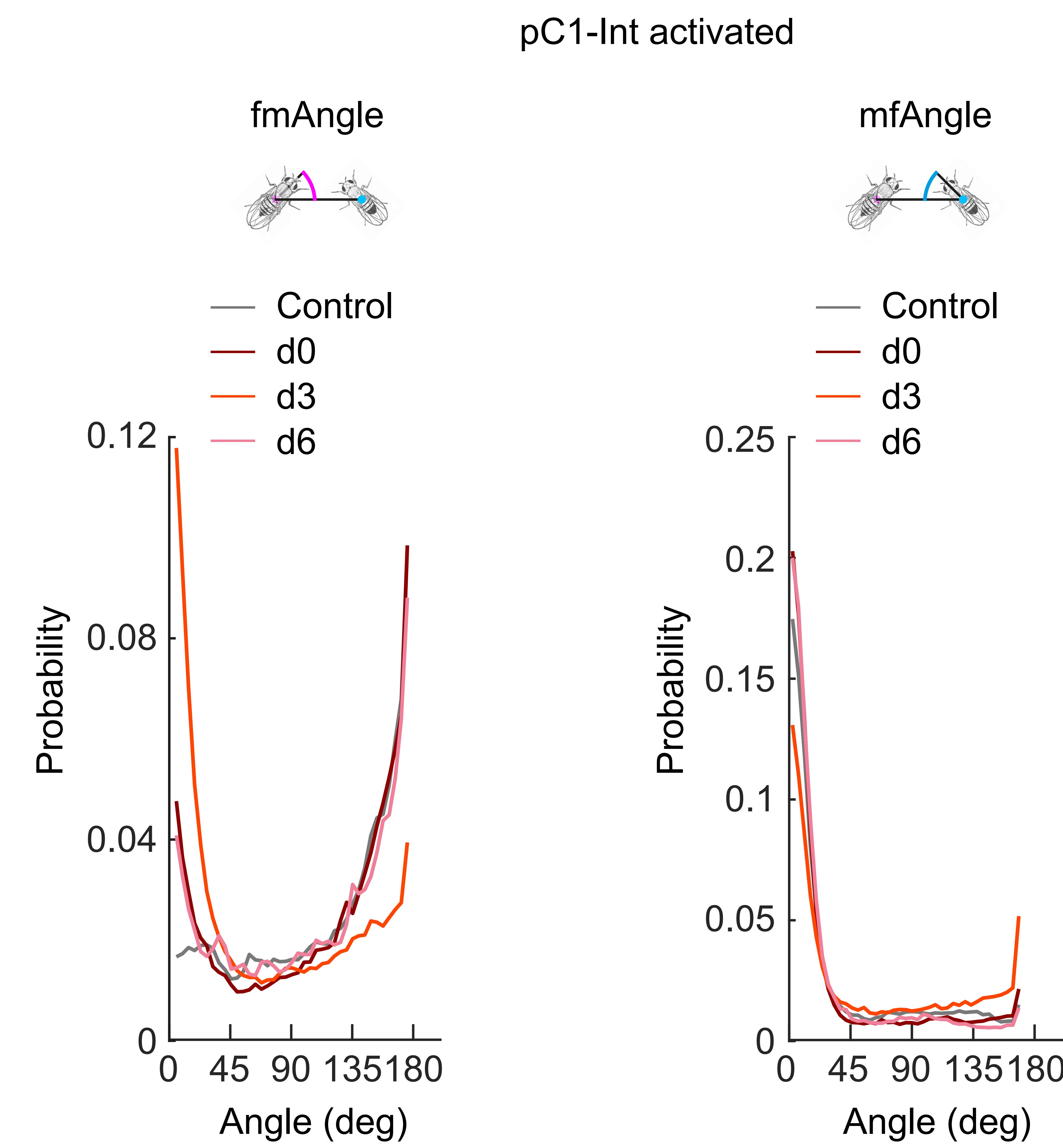
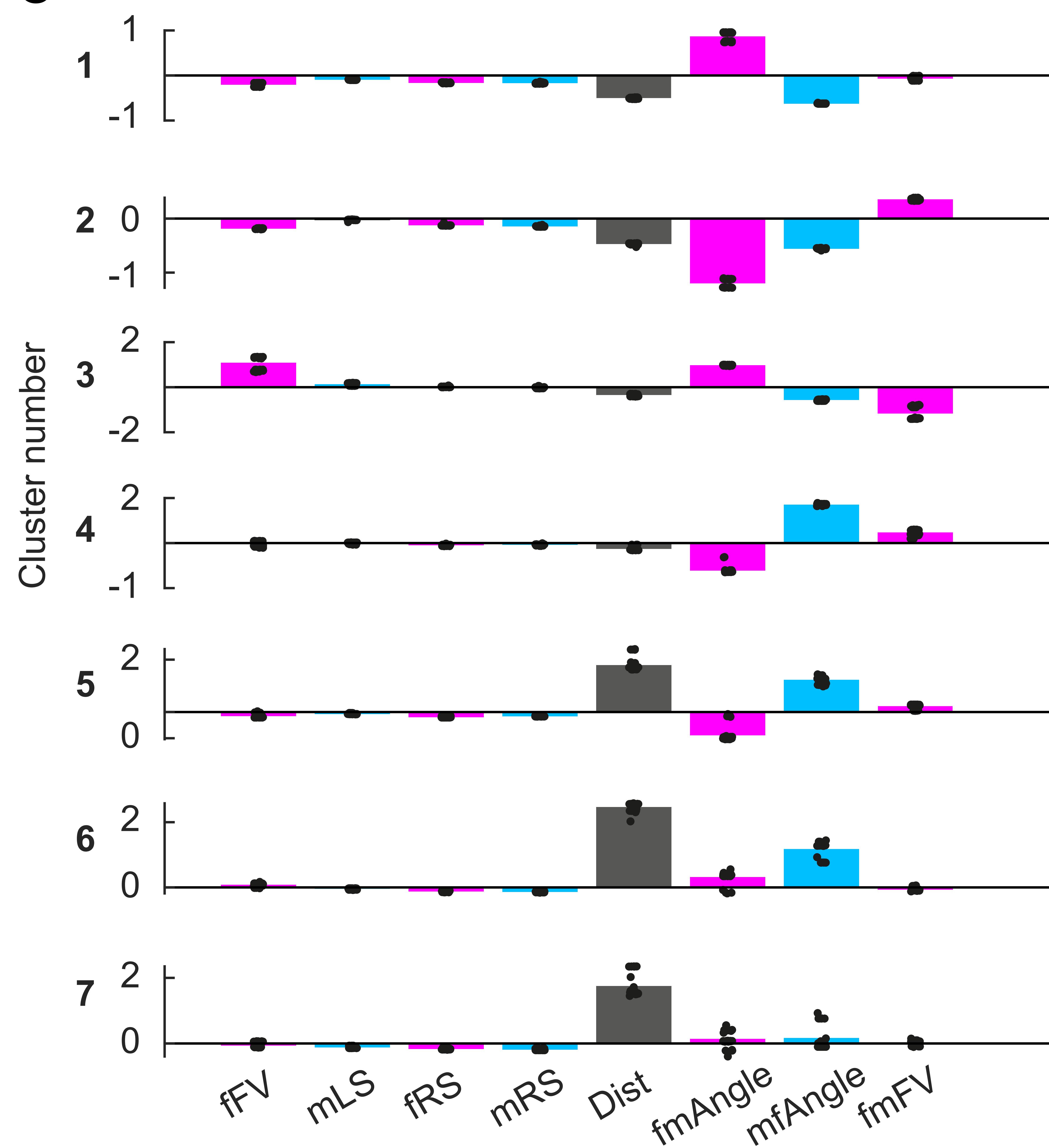
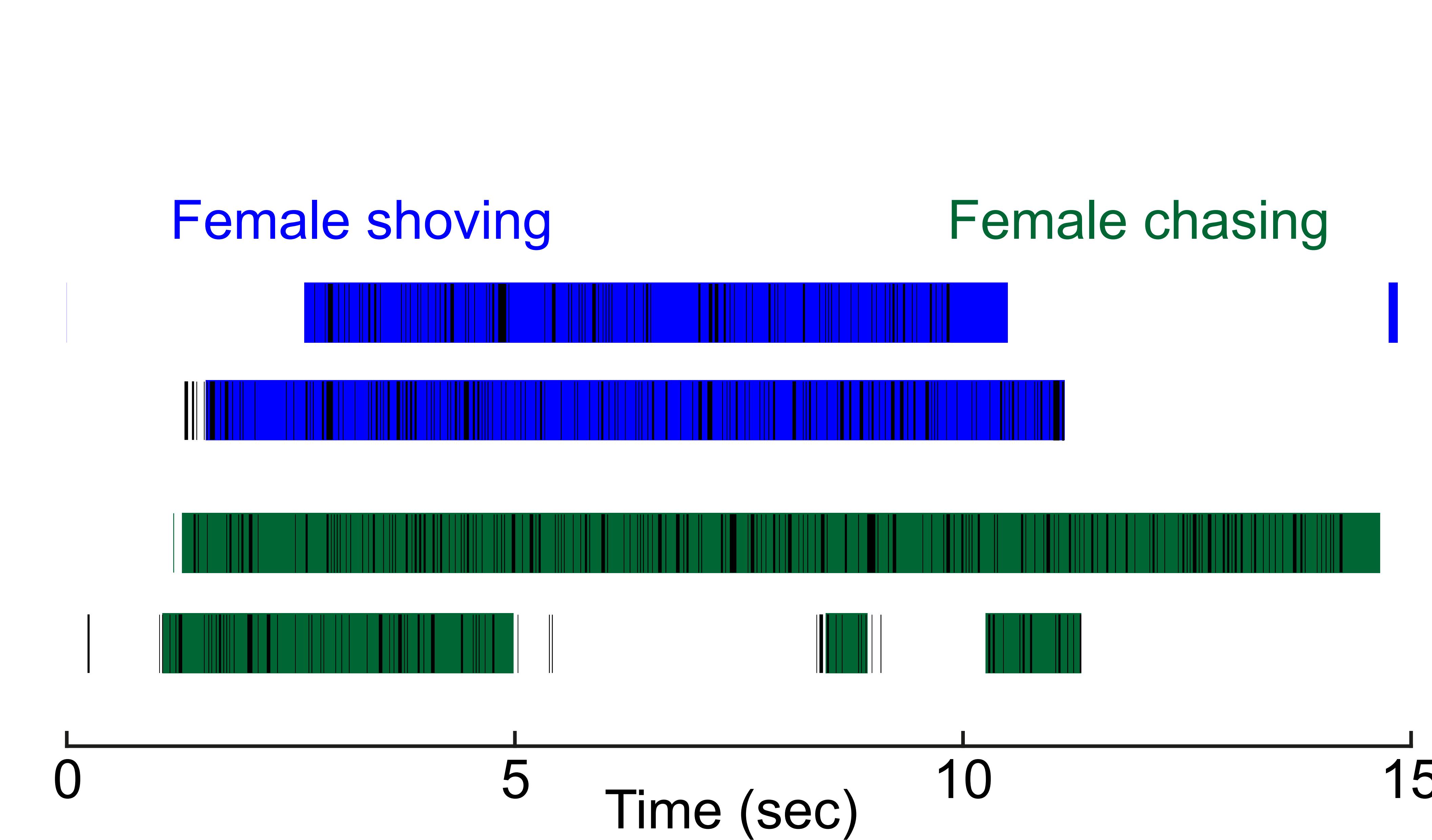
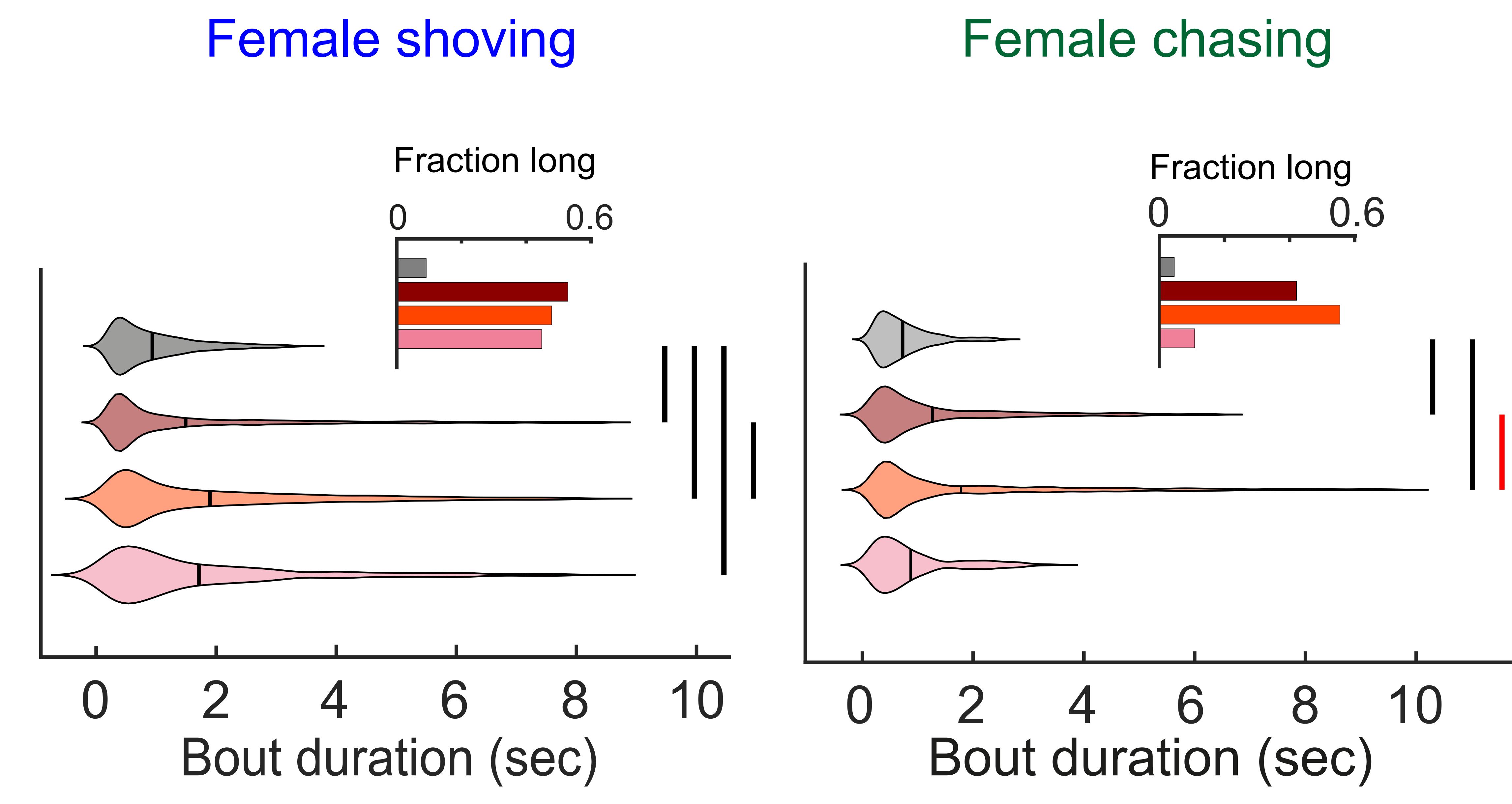
A**B****C****D**

Female shoving

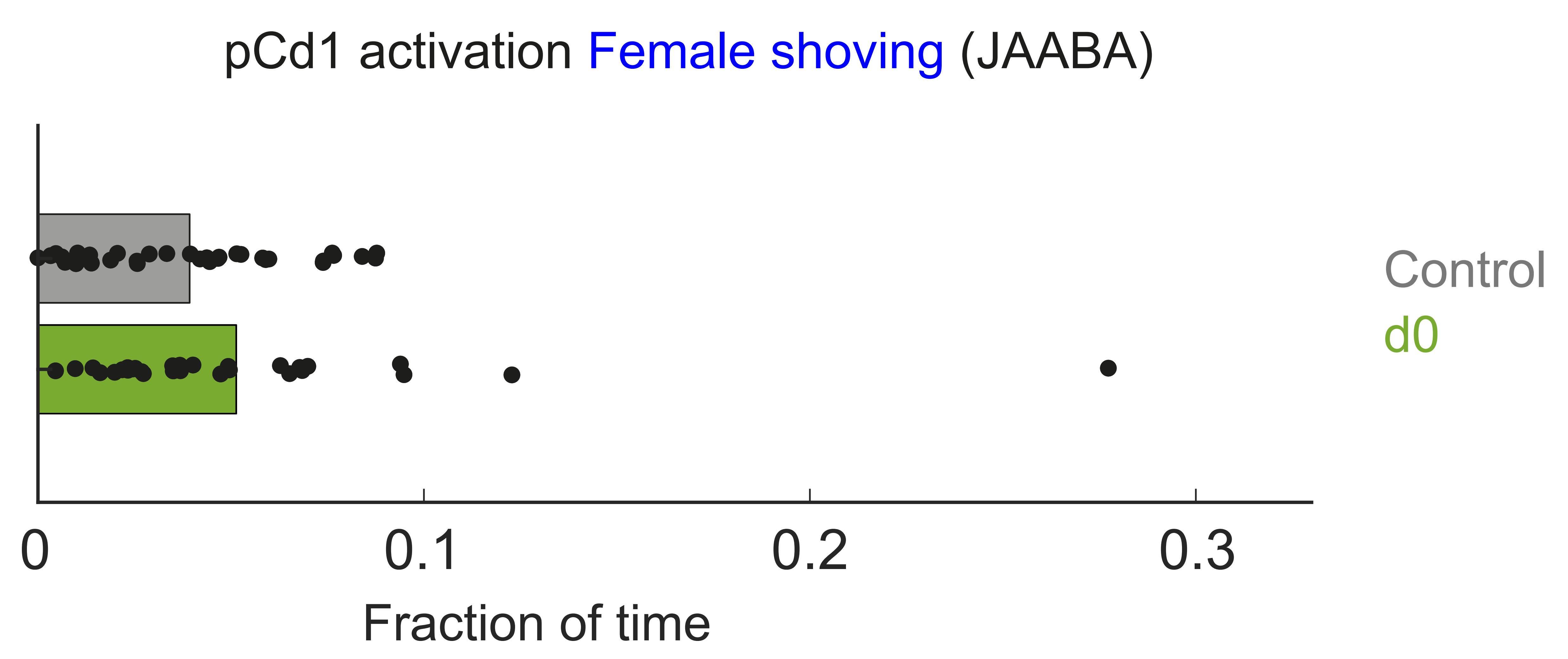
Female shoving

Female chasing

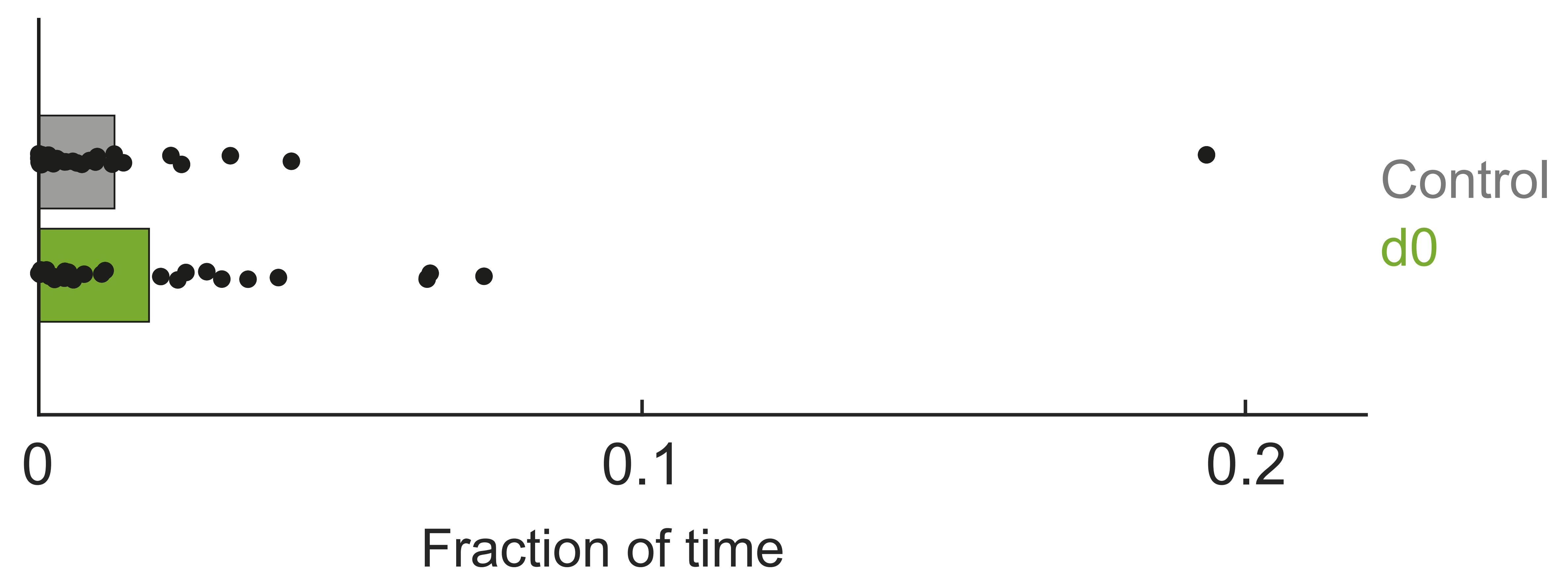
F

A**B****C****D****E**

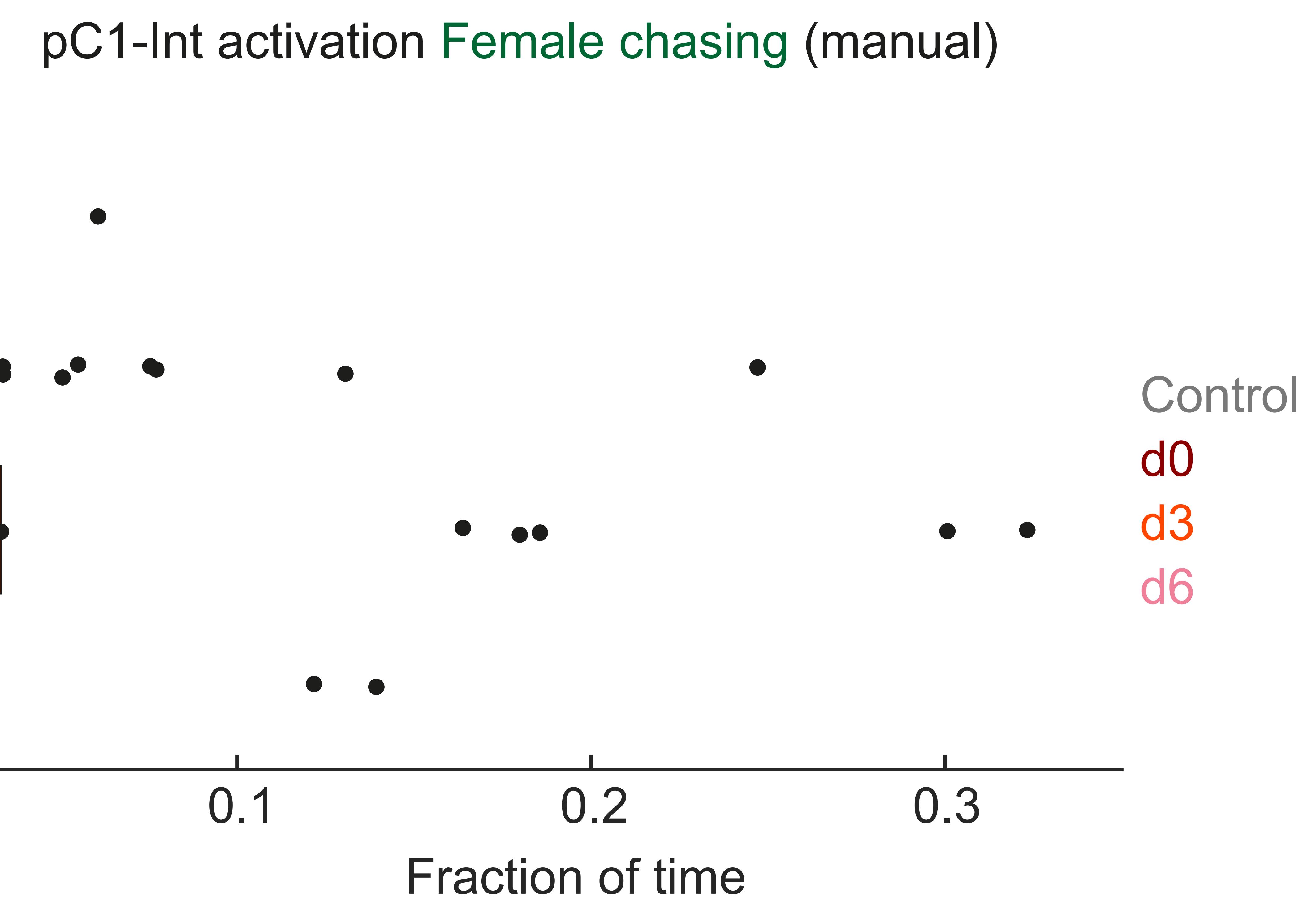
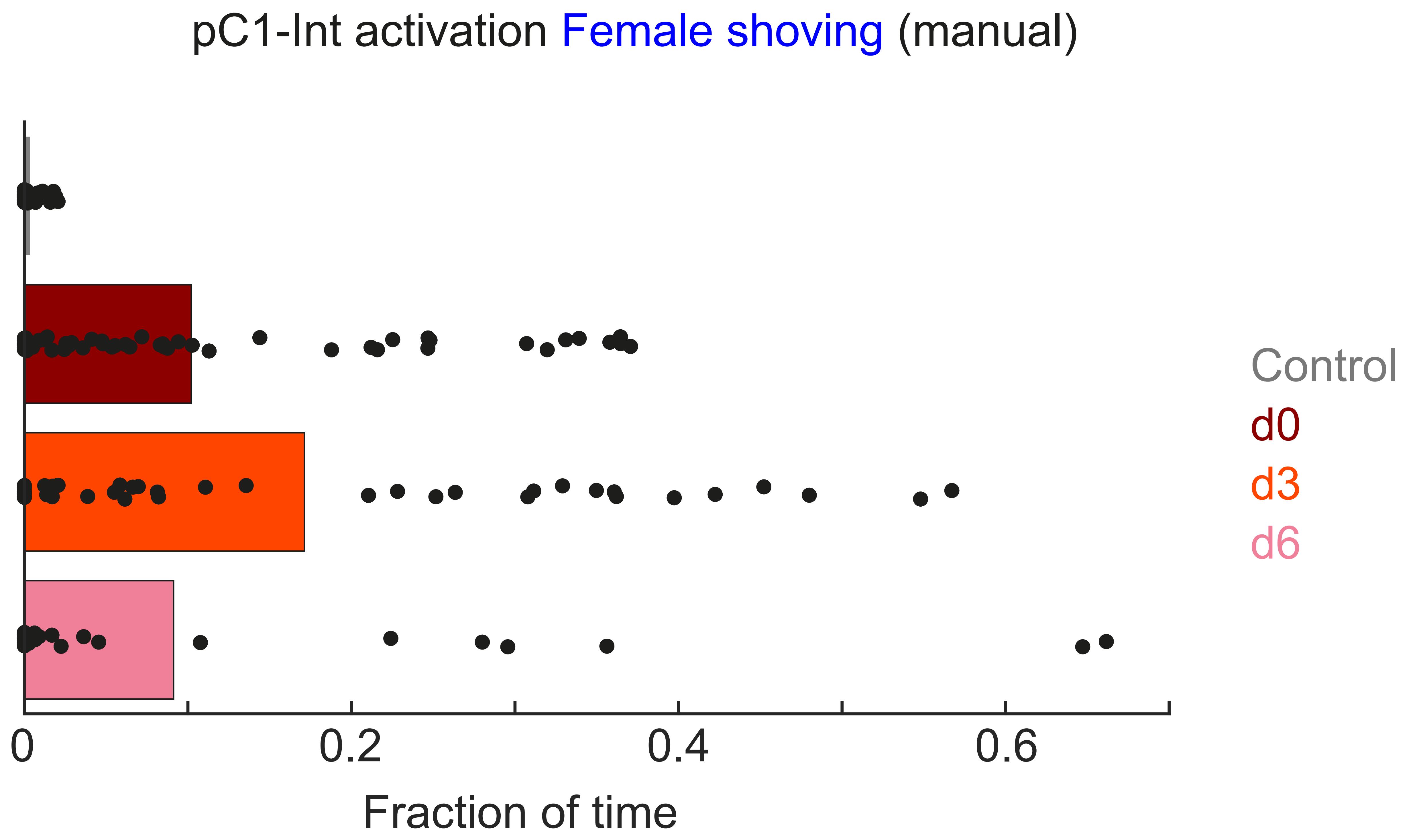
A



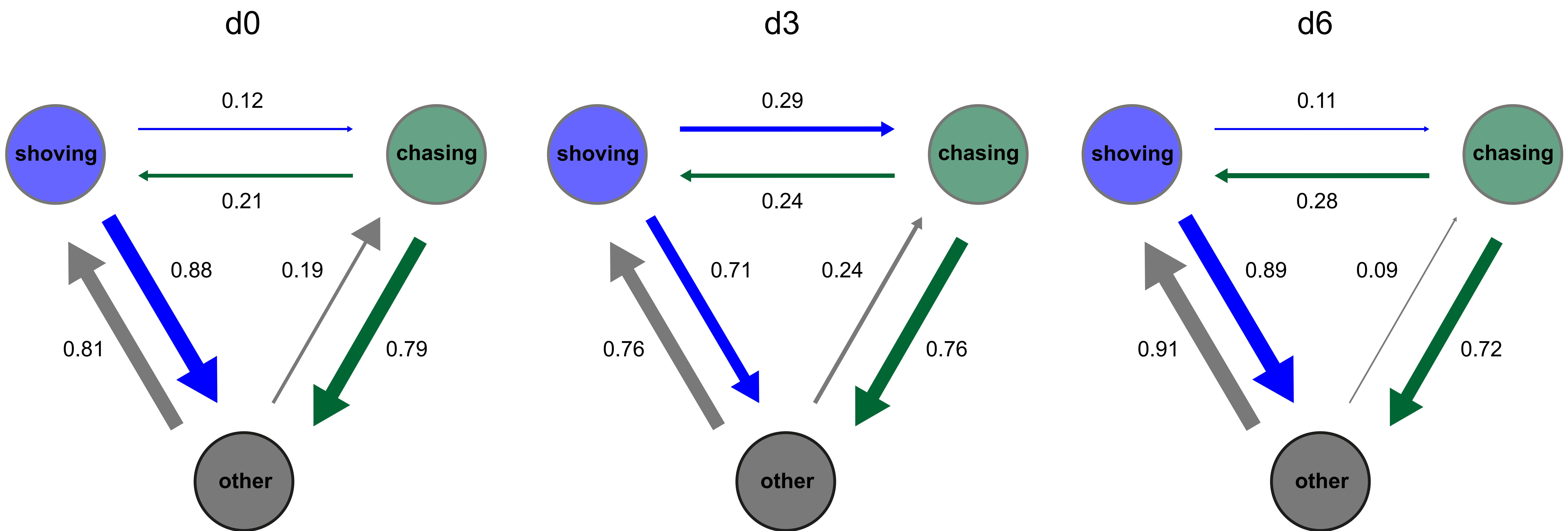
pCd1 activation Female chasing (JAABA)

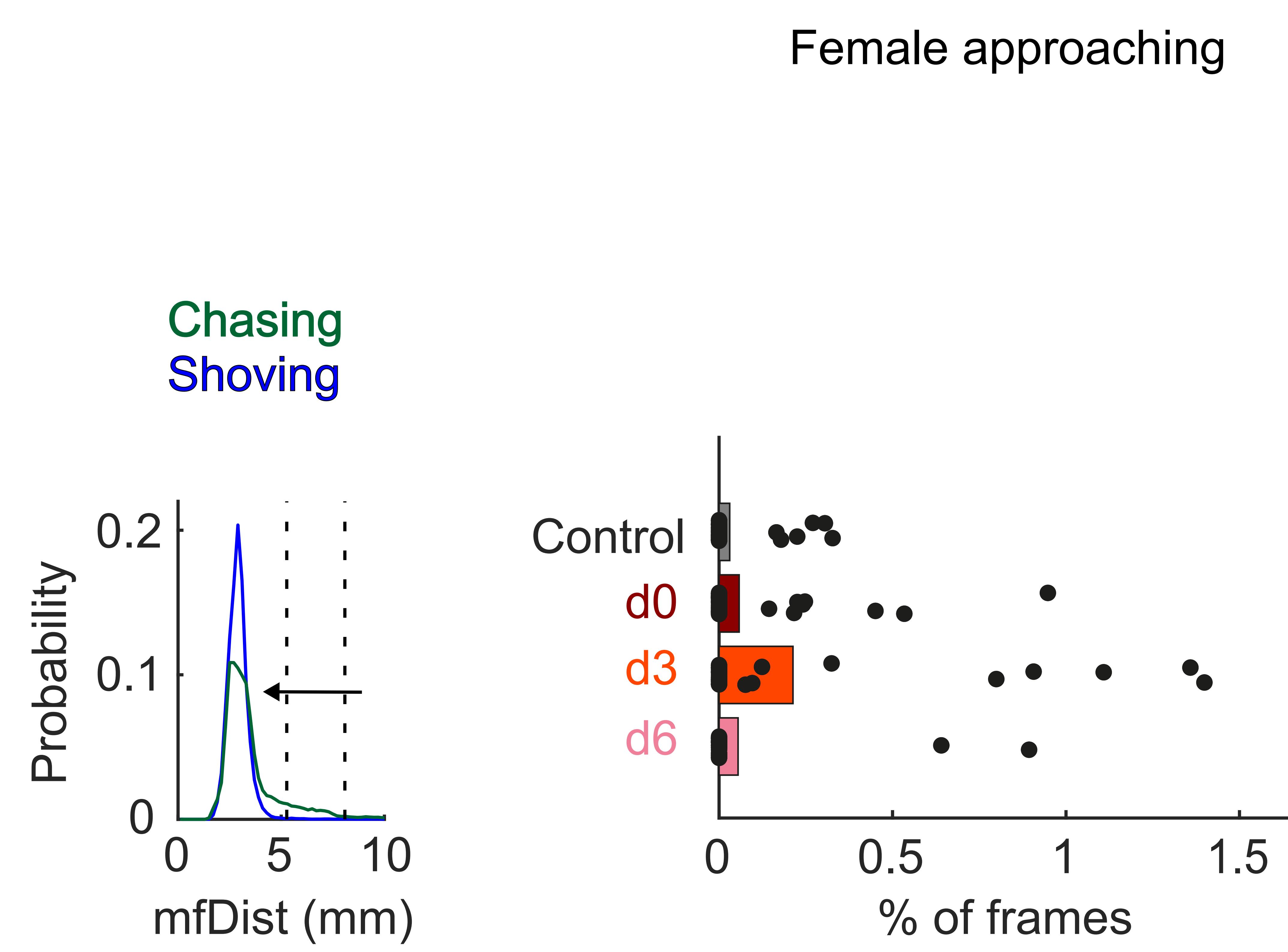
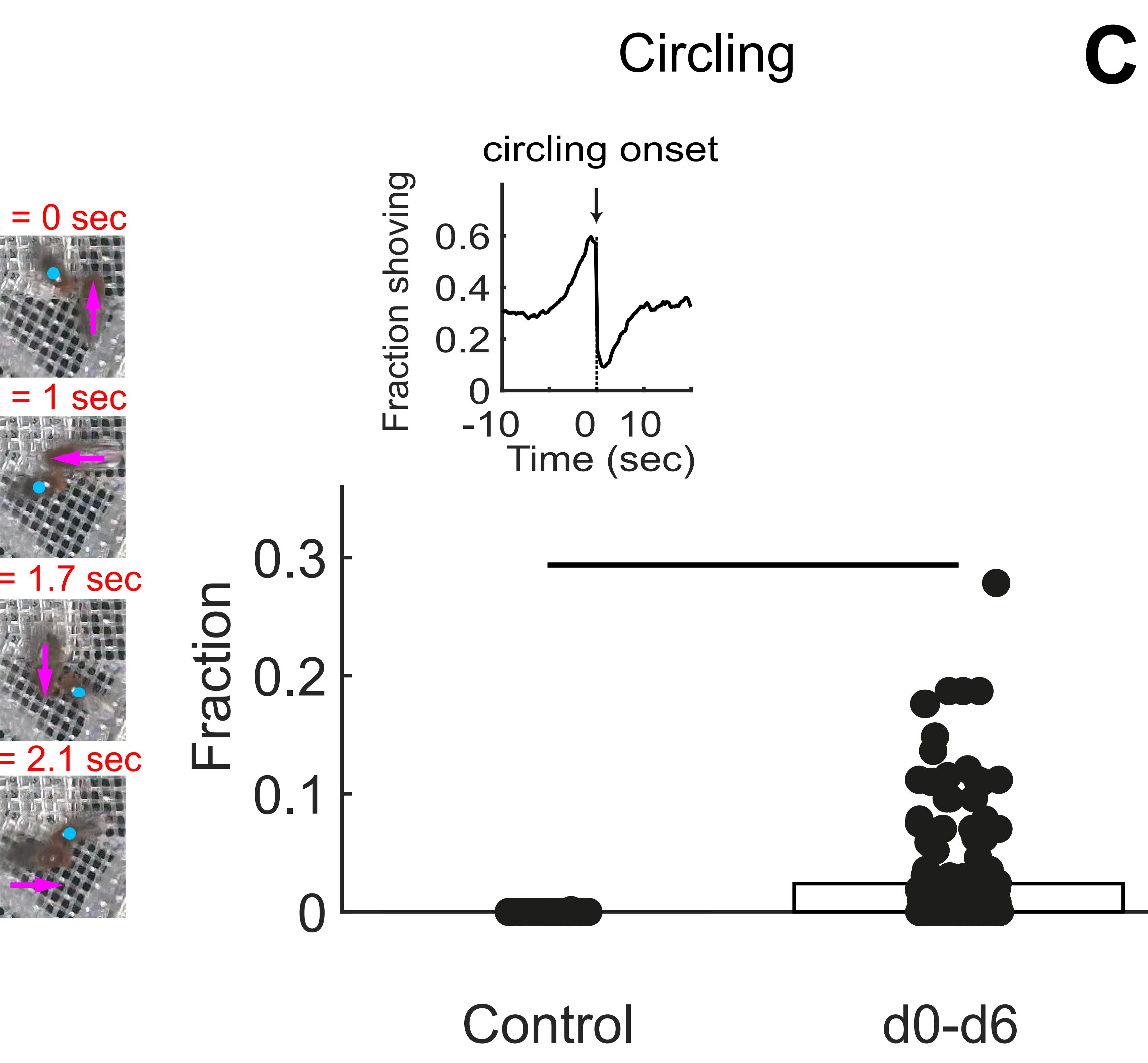
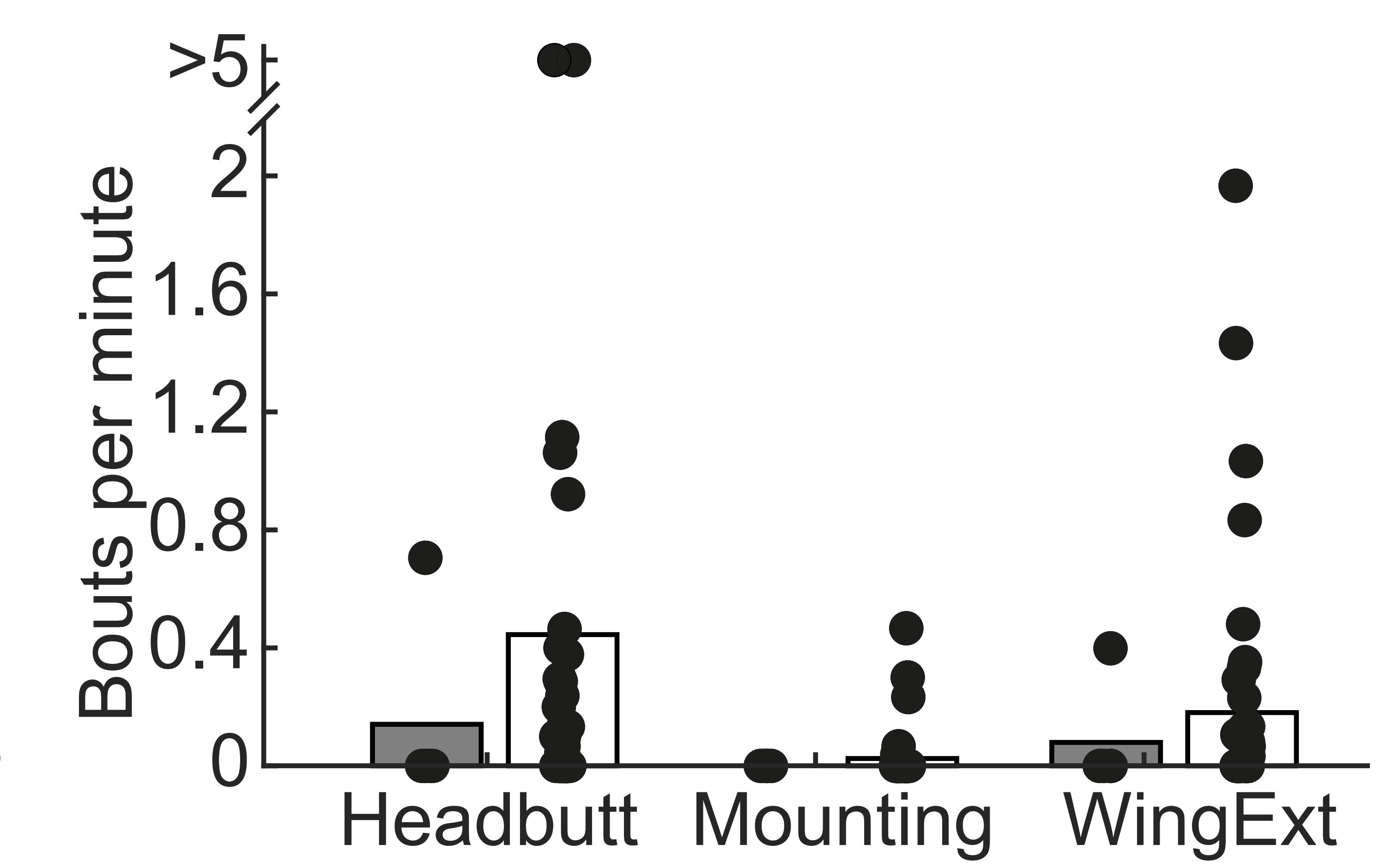
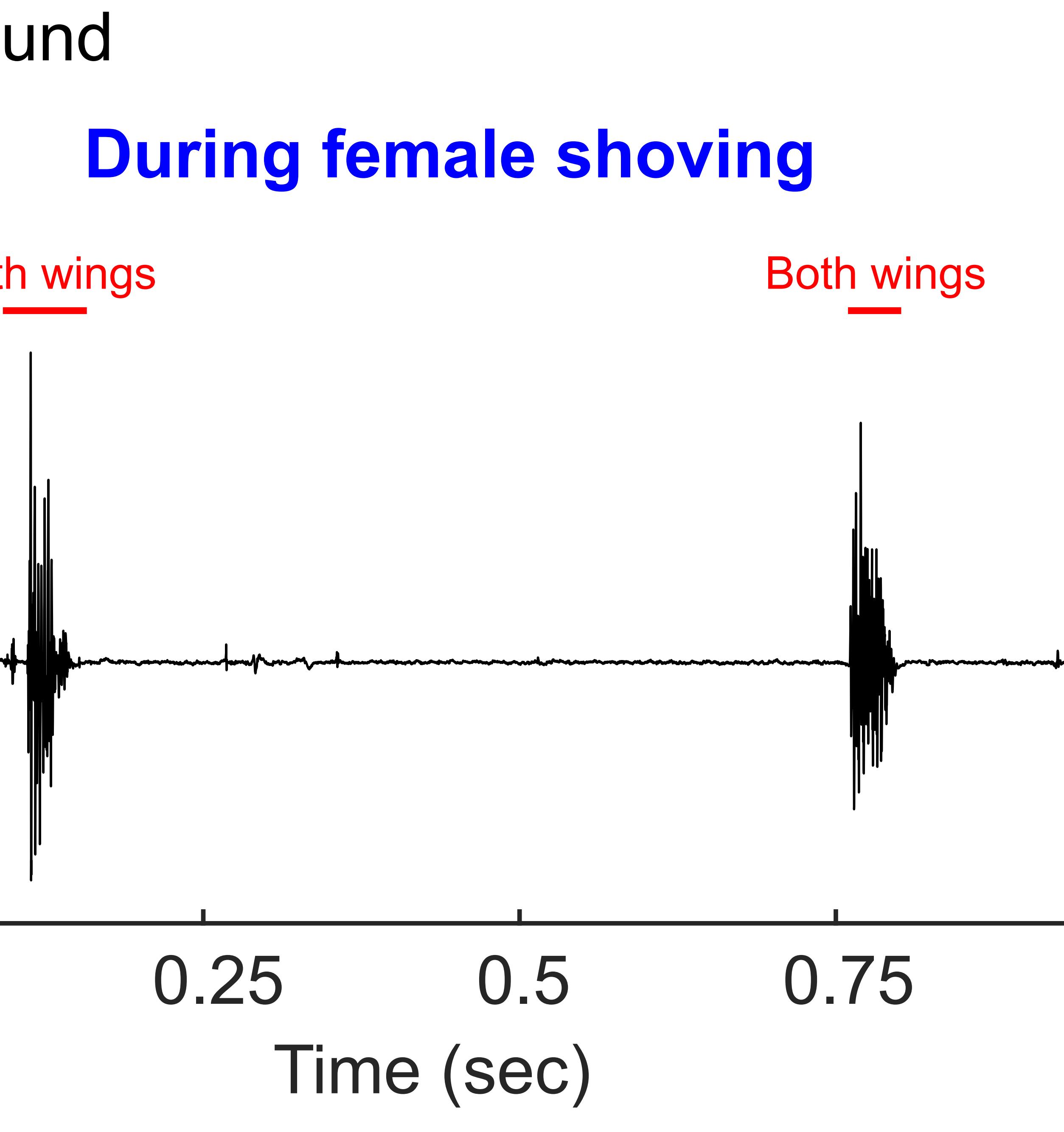
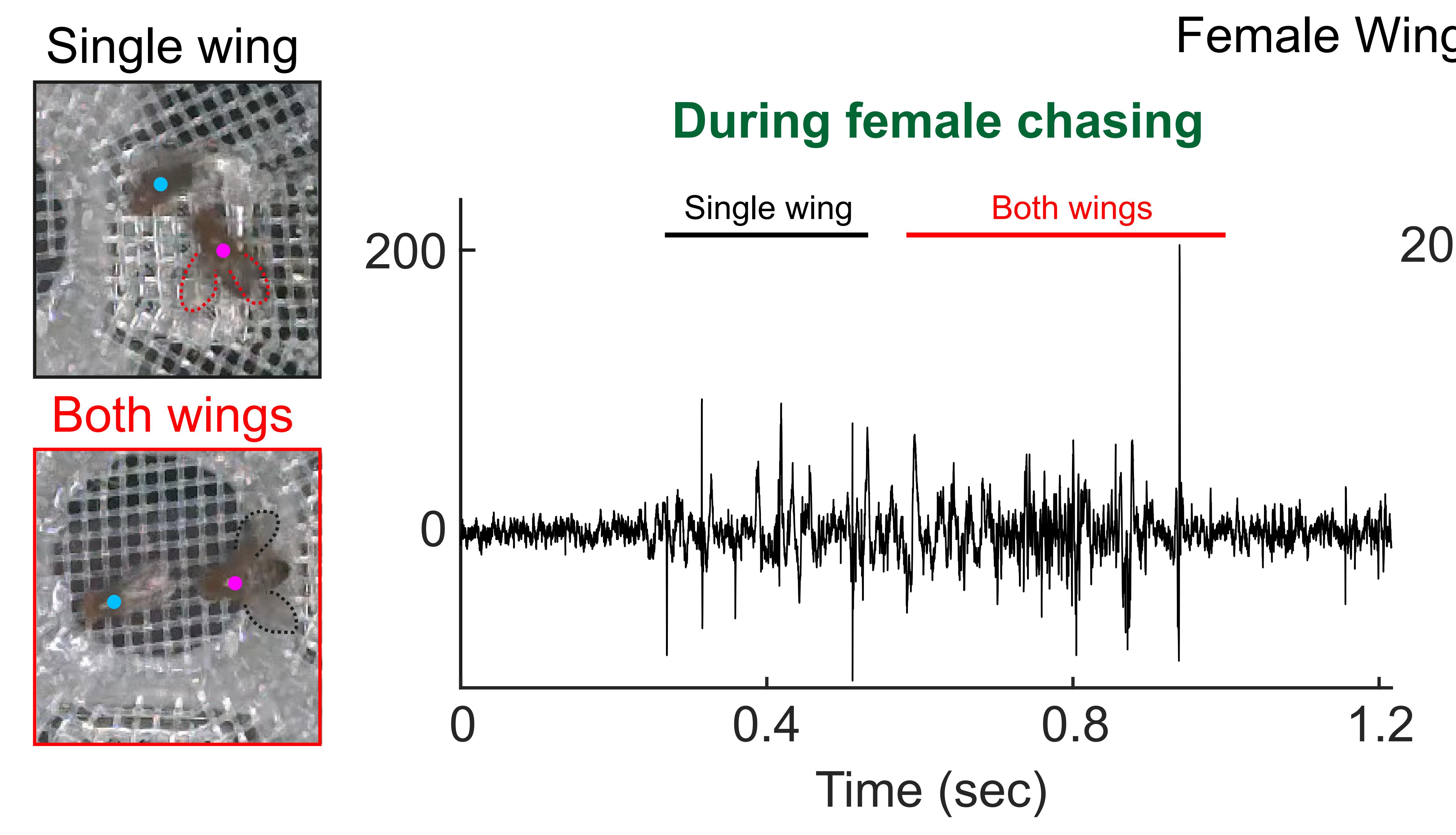
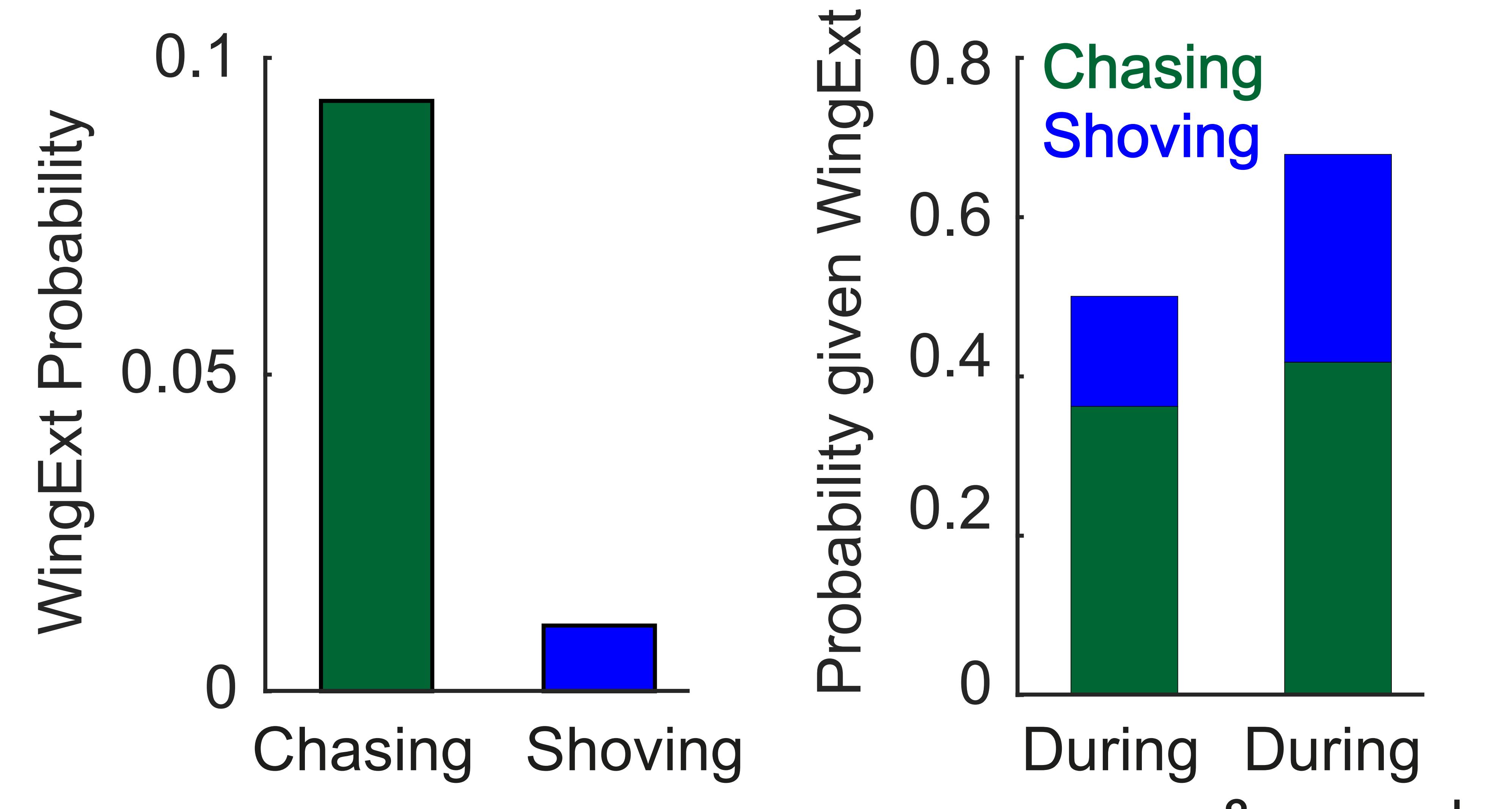


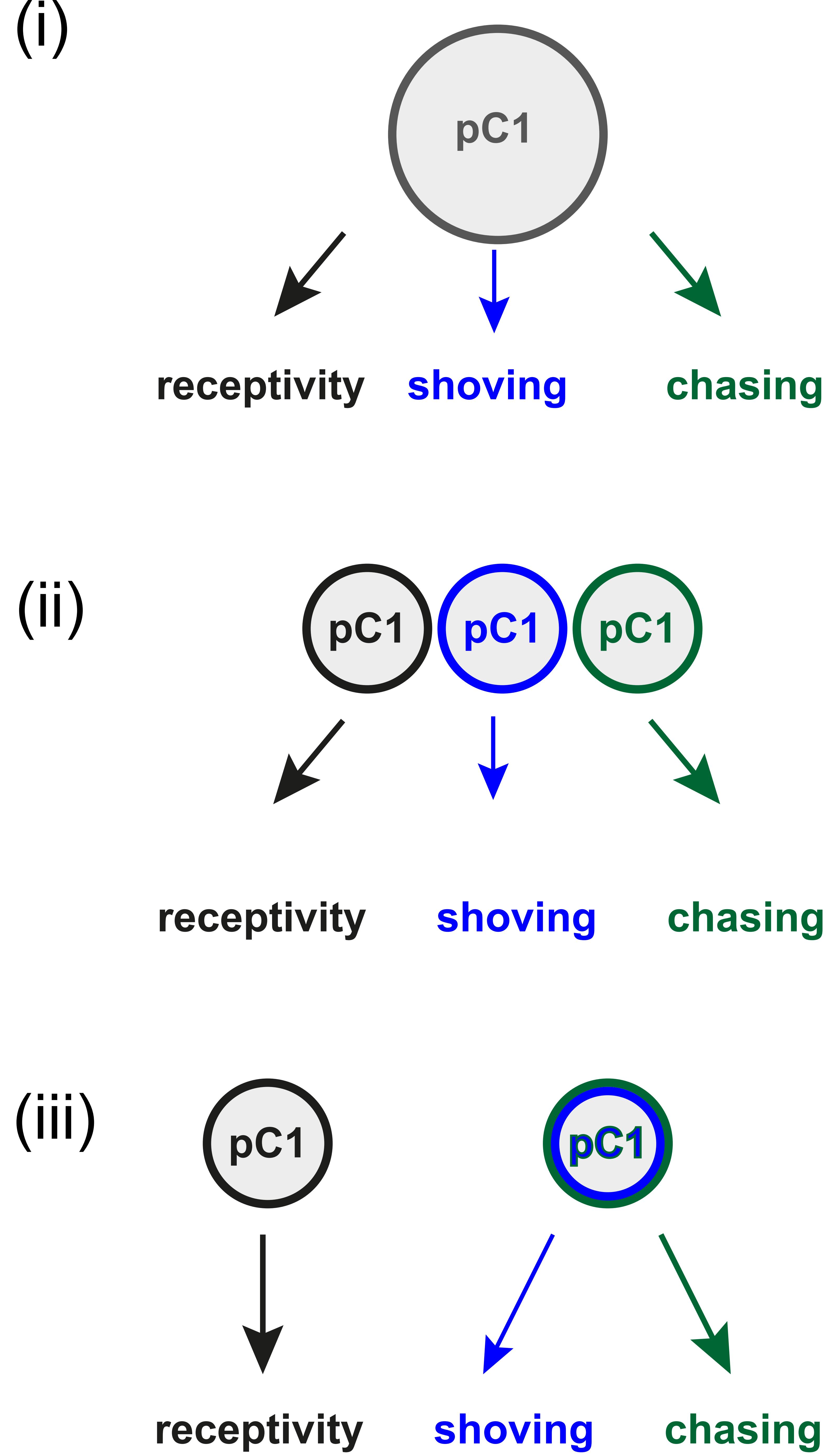
B



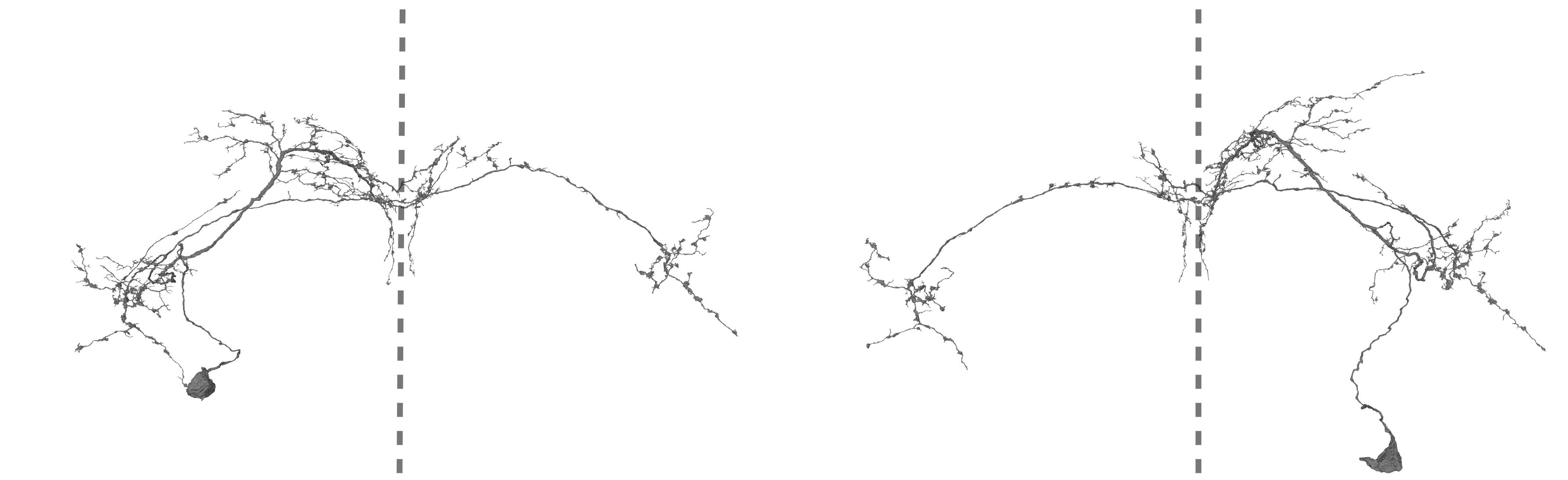
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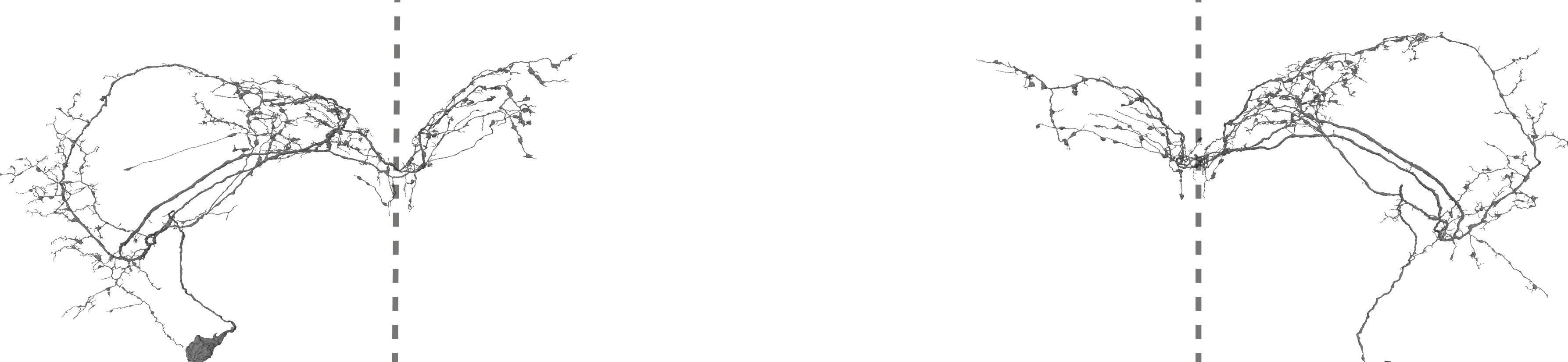
A**B****C****D****E**

A**C**

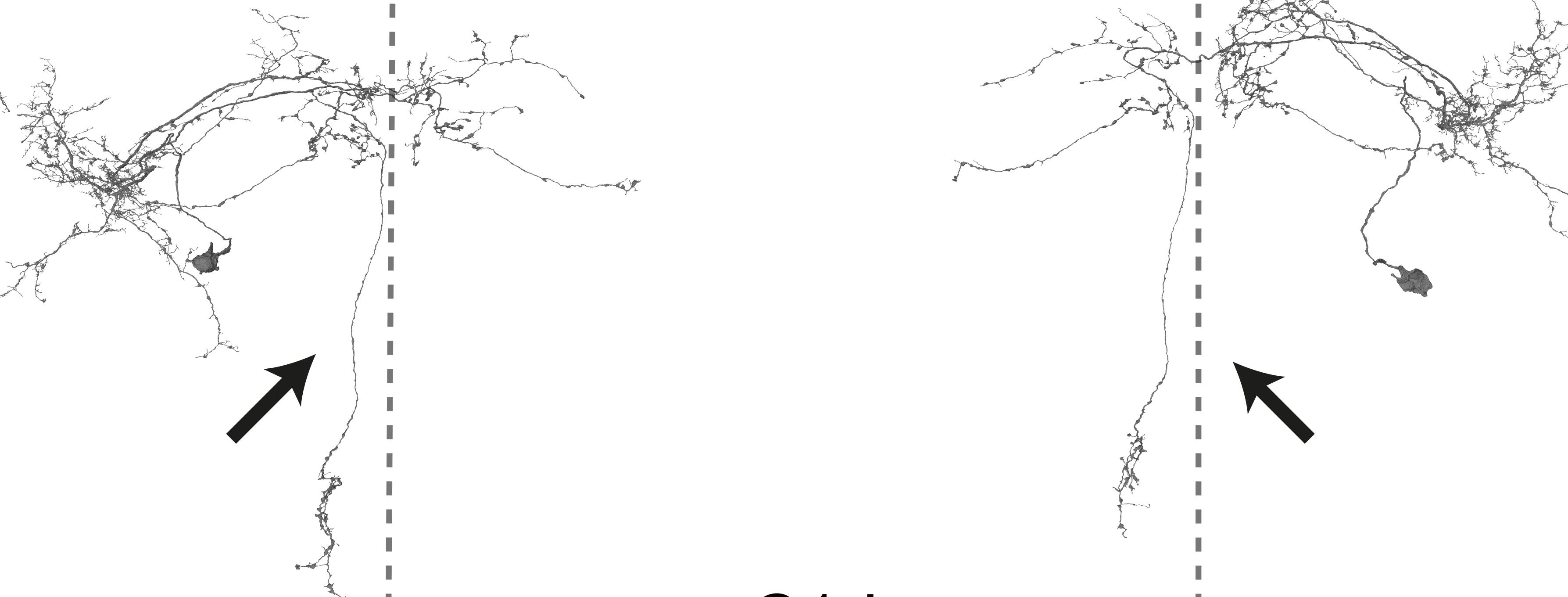
pC1a



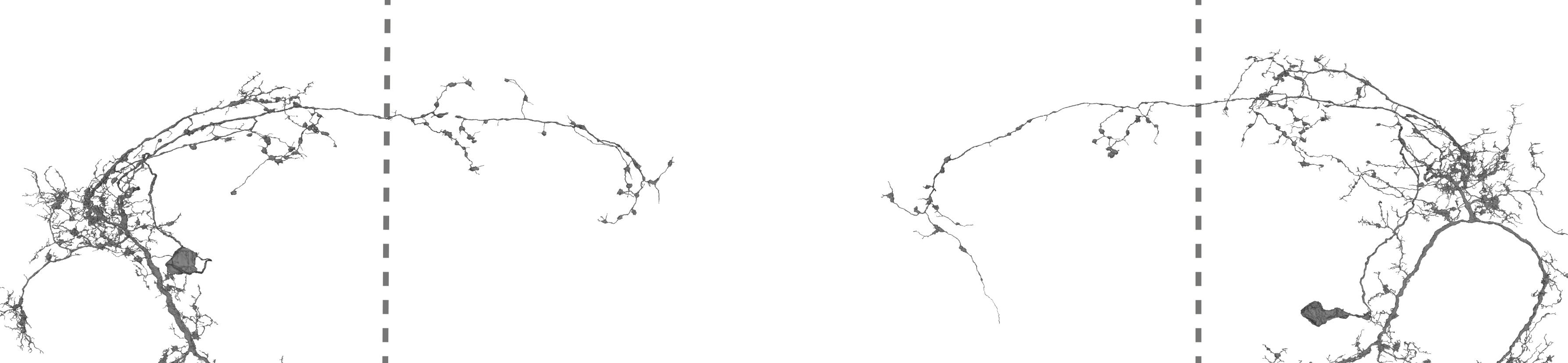
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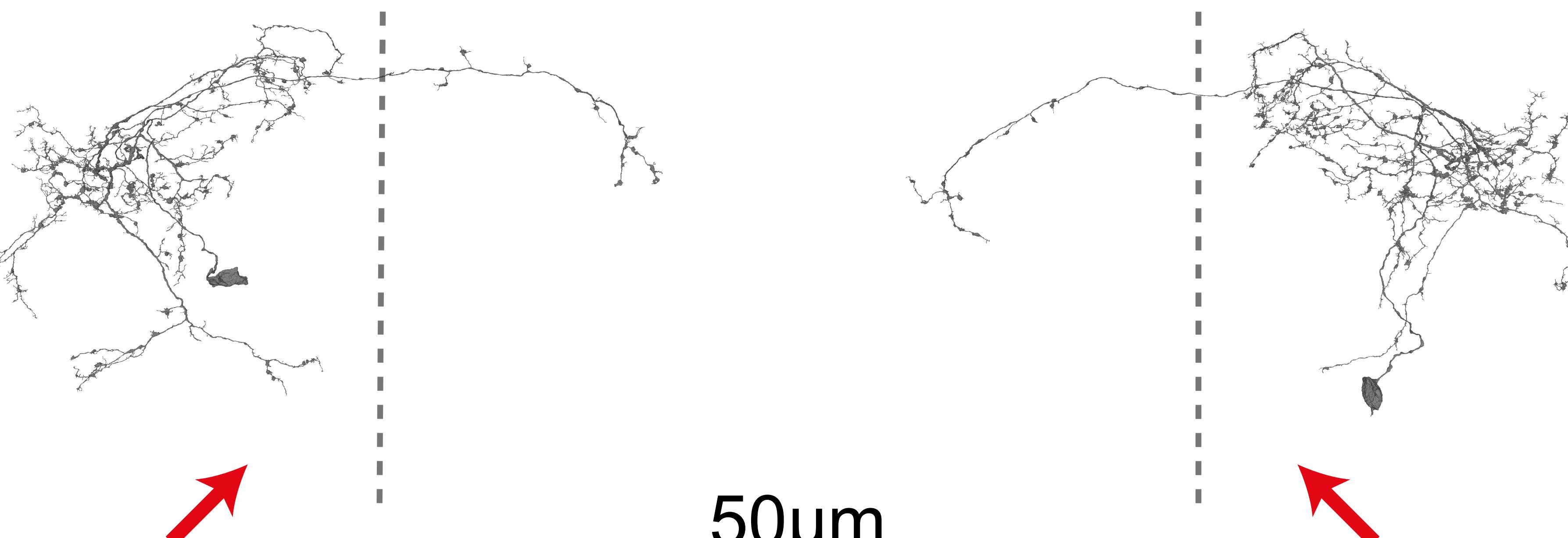
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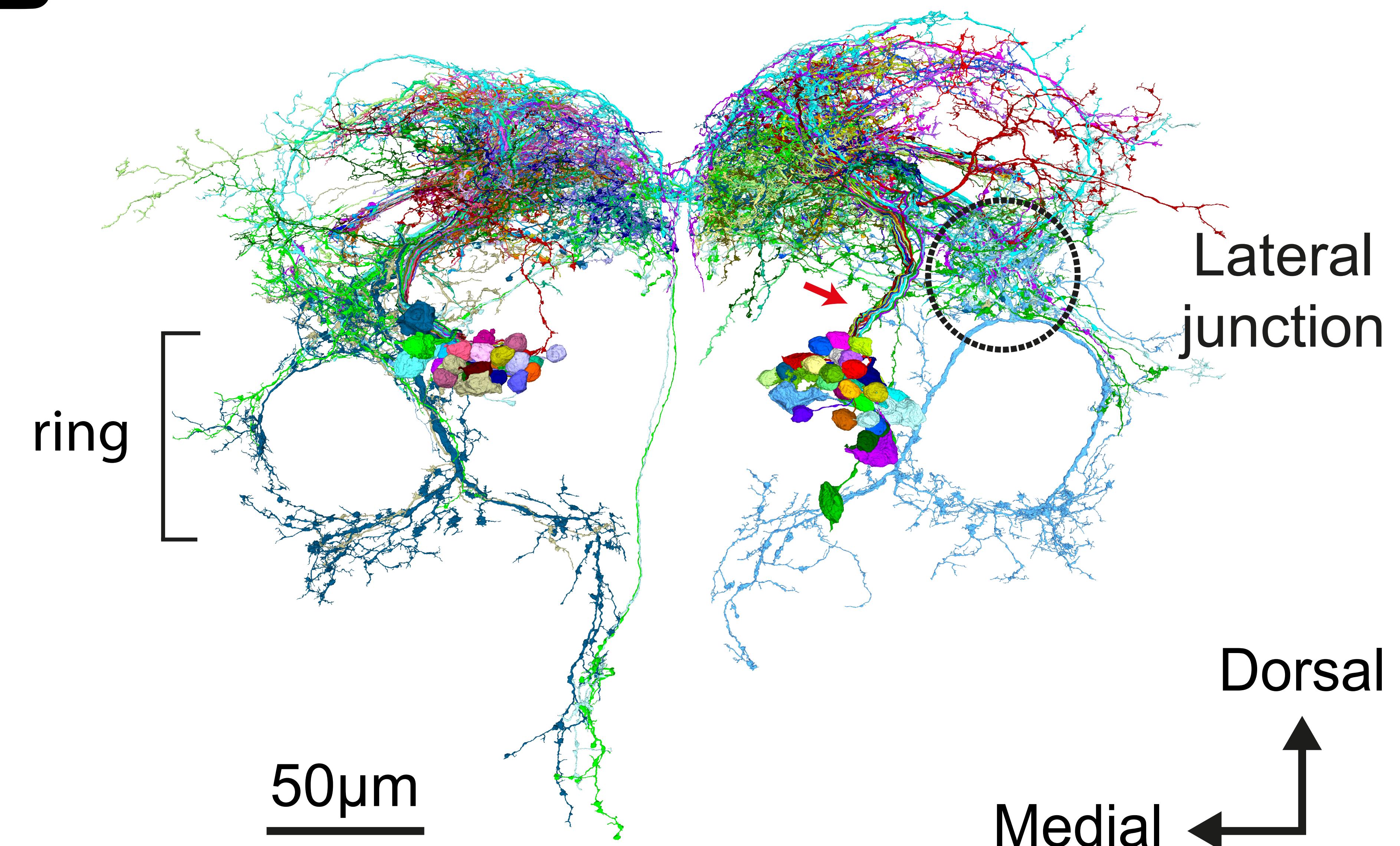
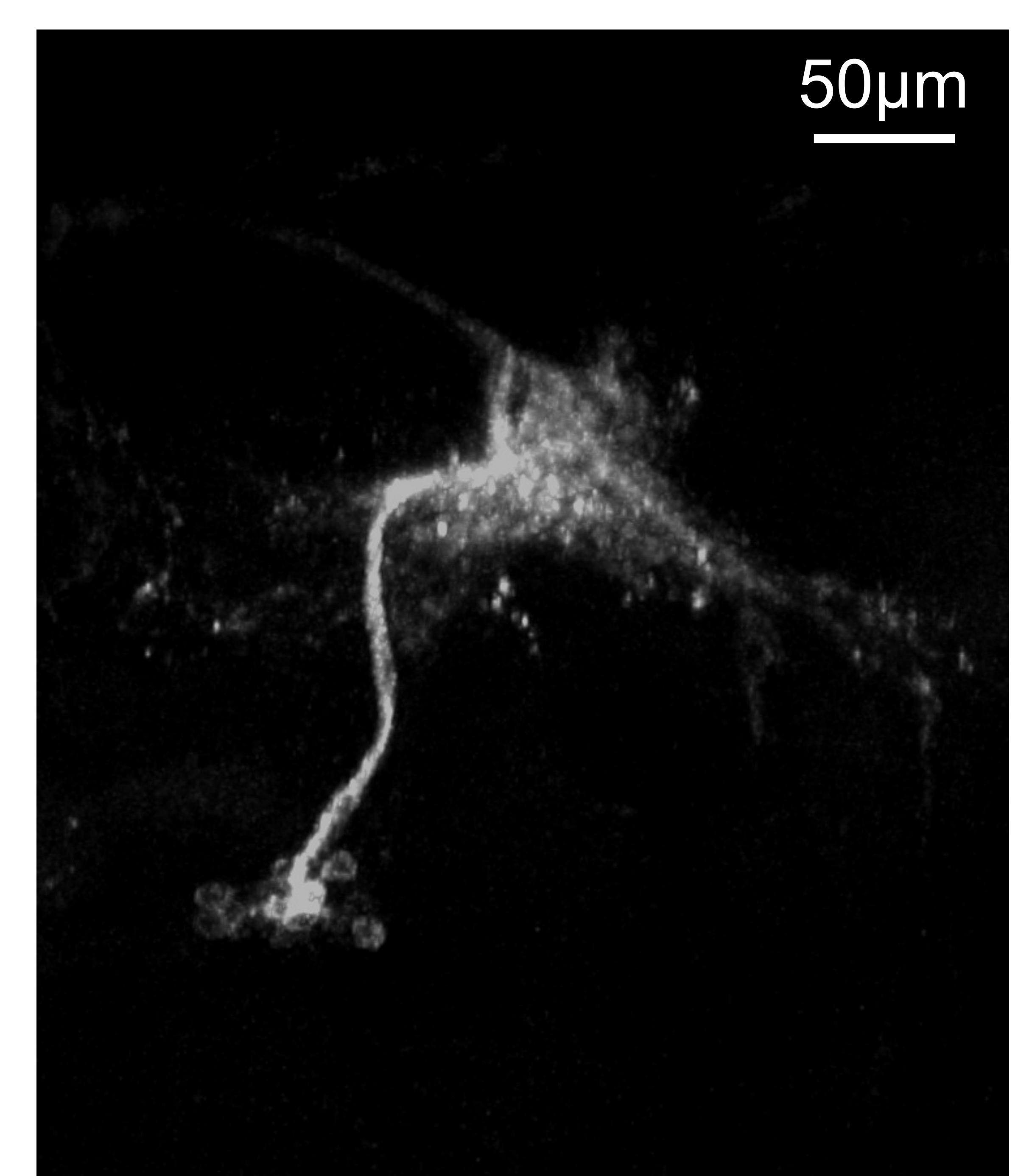
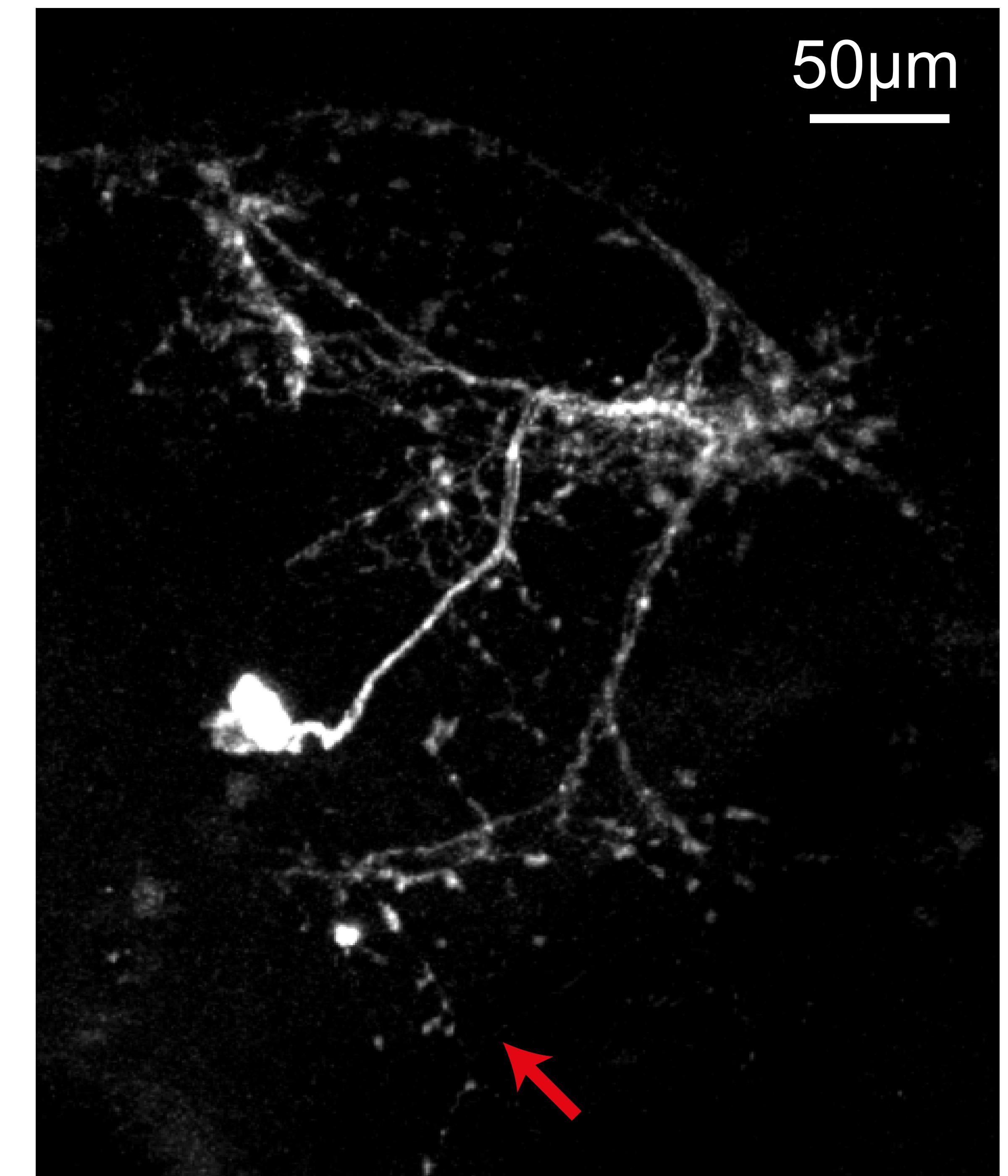
pC1d



pC1e

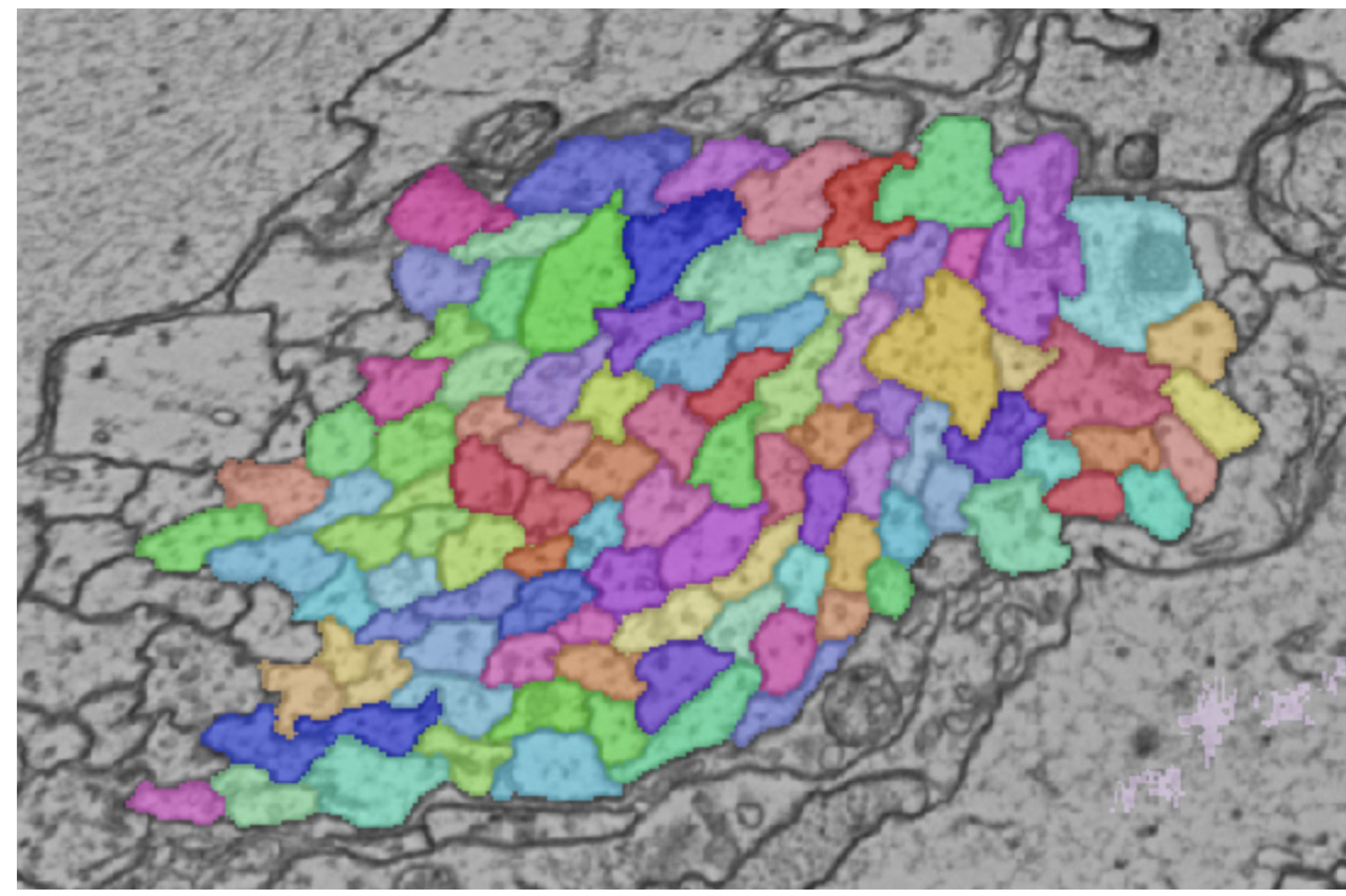


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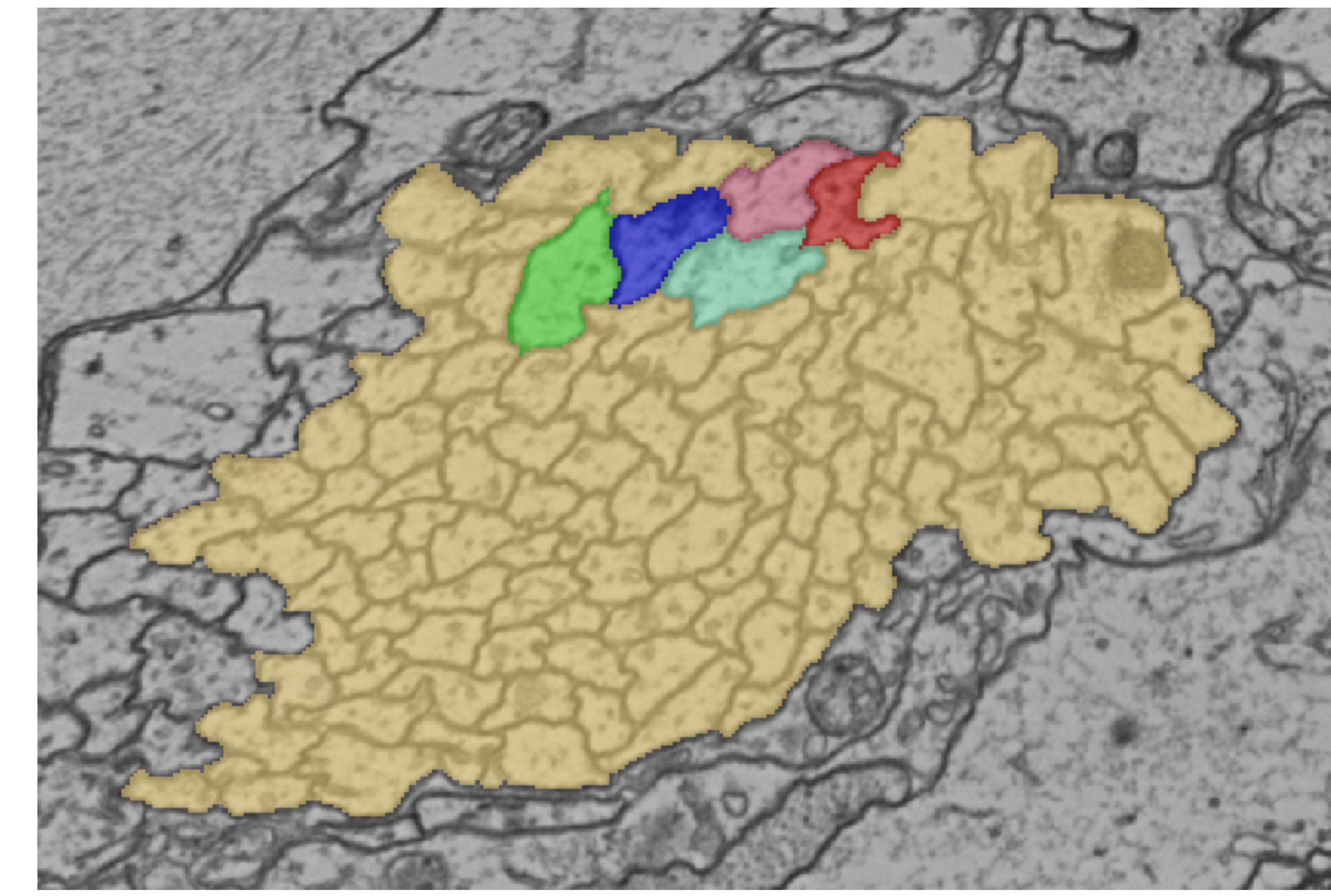
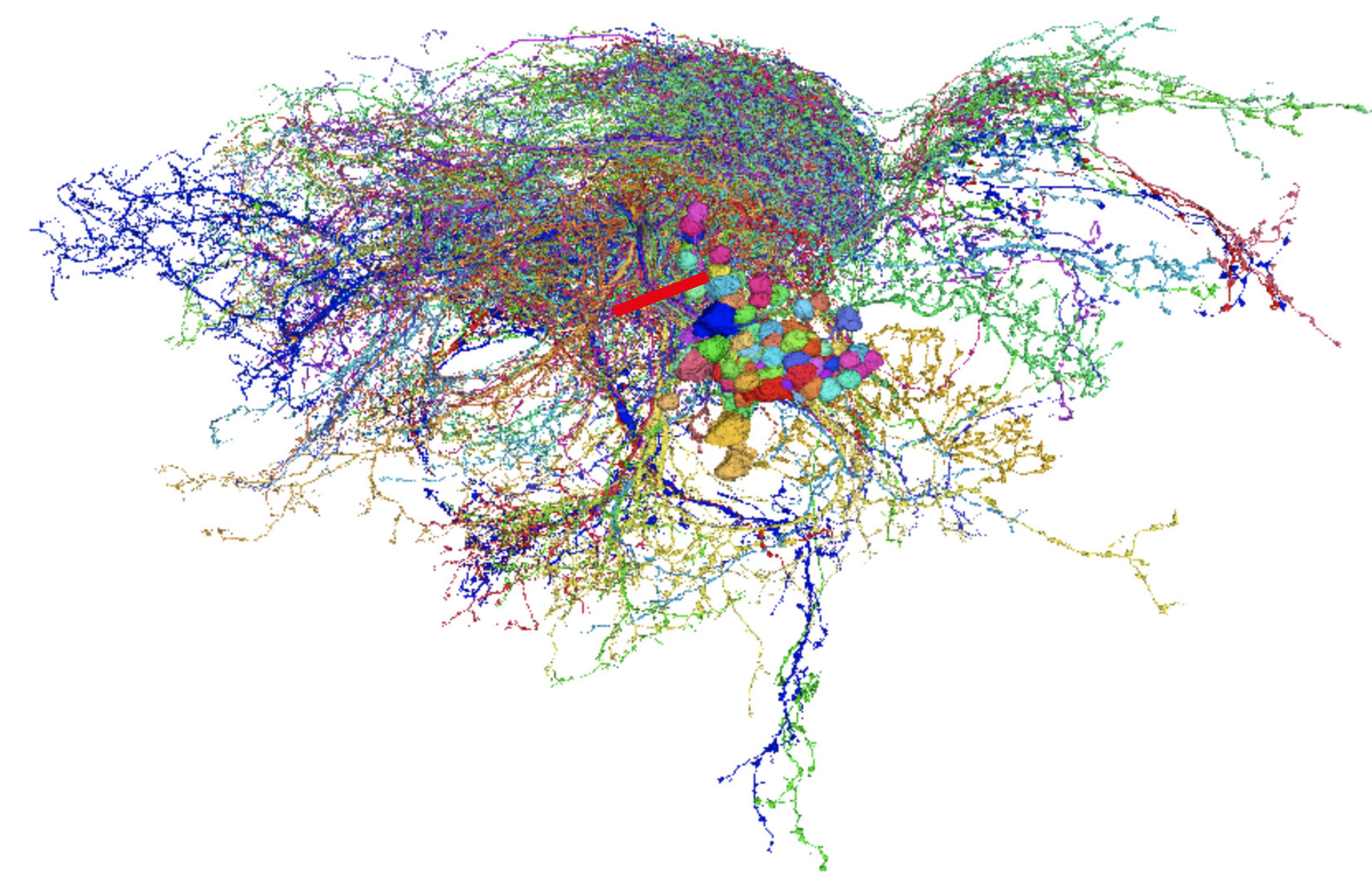
B**D**pC1-S
R71G01.AD;DSX.DBDpC1-A
VT25602.AD;VT2064.DBD

A

pC1-bundle
FlyWire coordinates: 148463, 39668, 4044

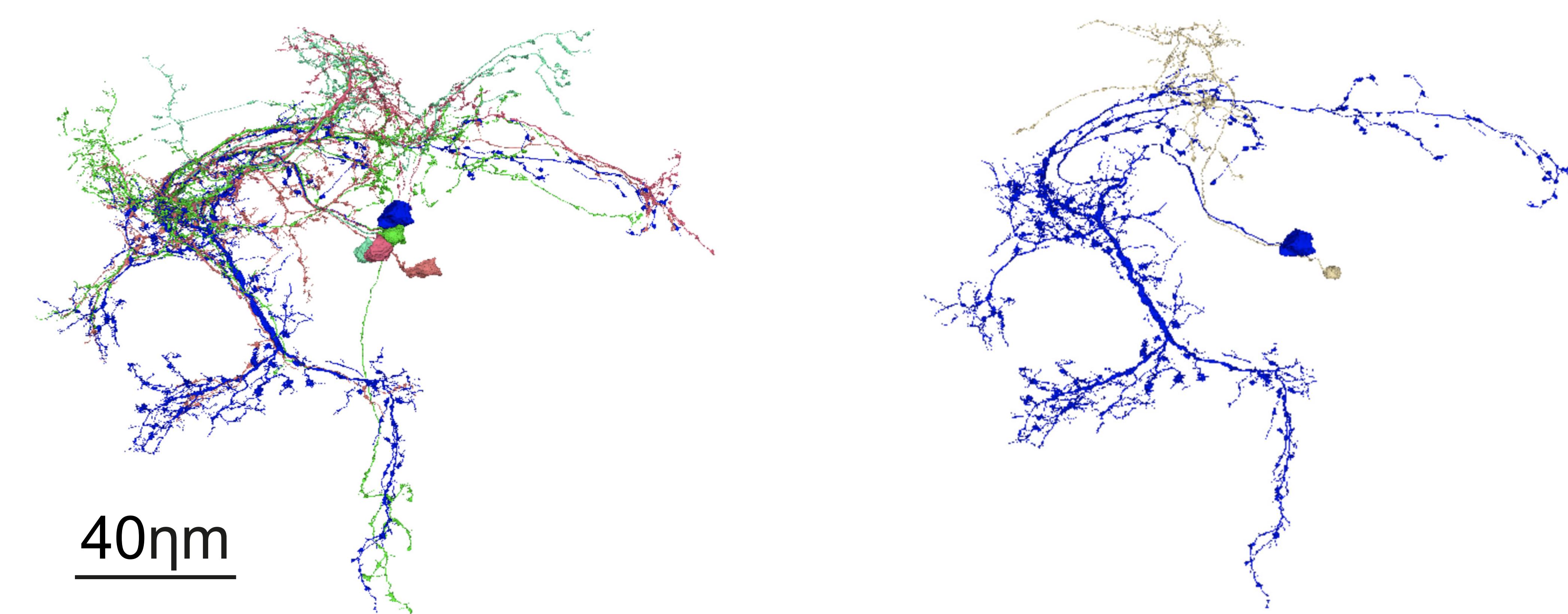
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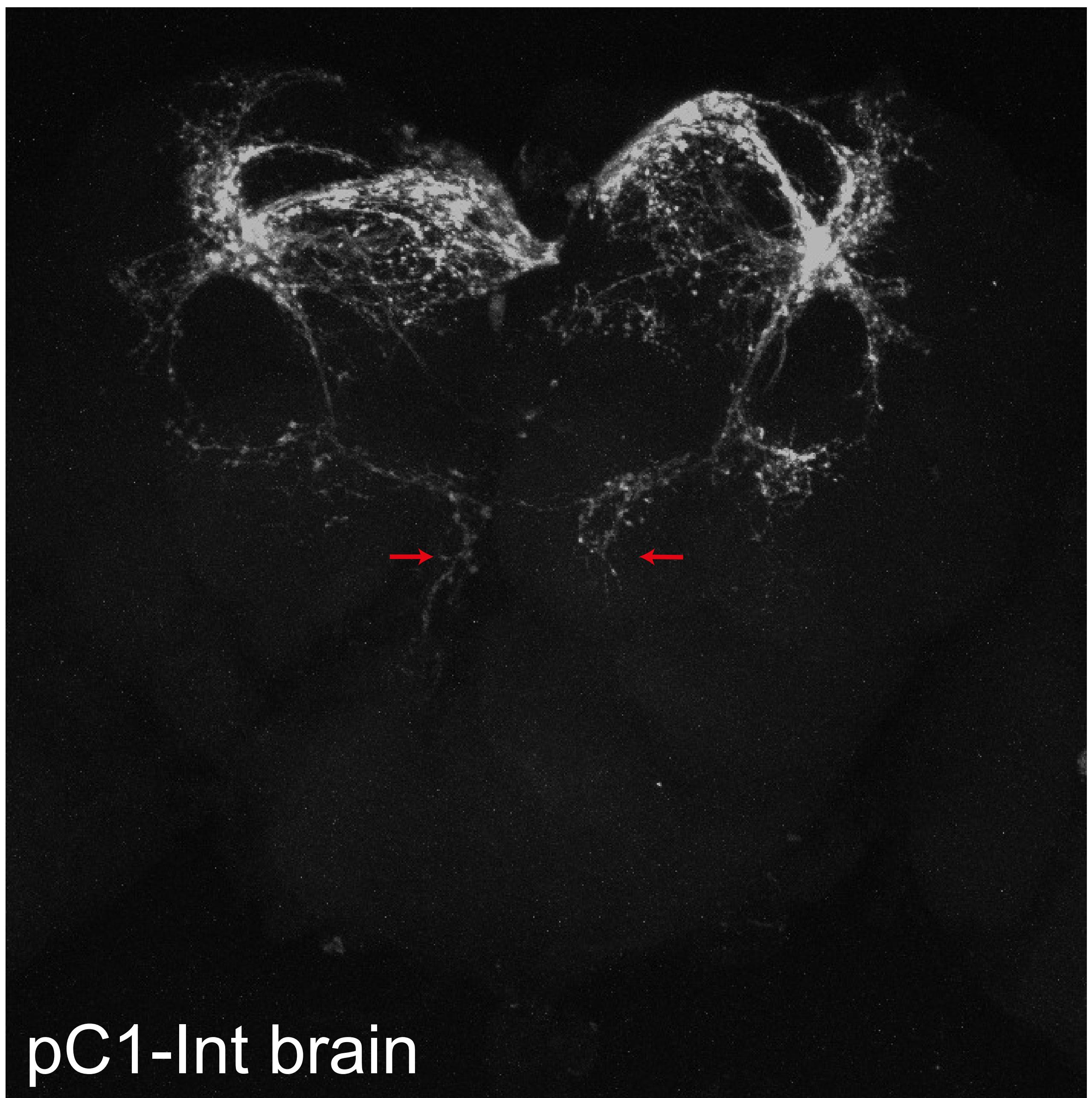
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**C****D**

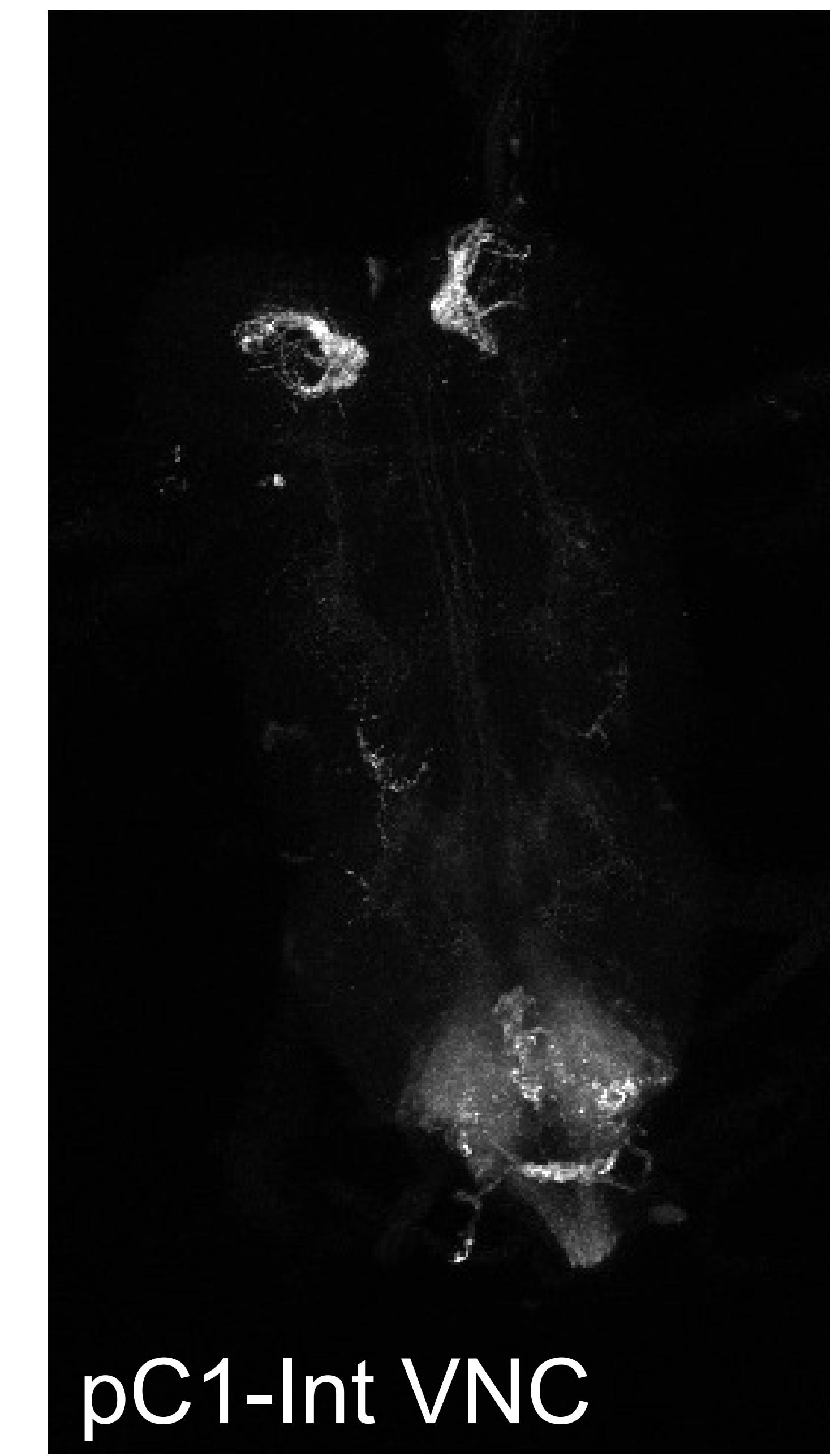
pC1a-e

pC1d +
non-pC1 example

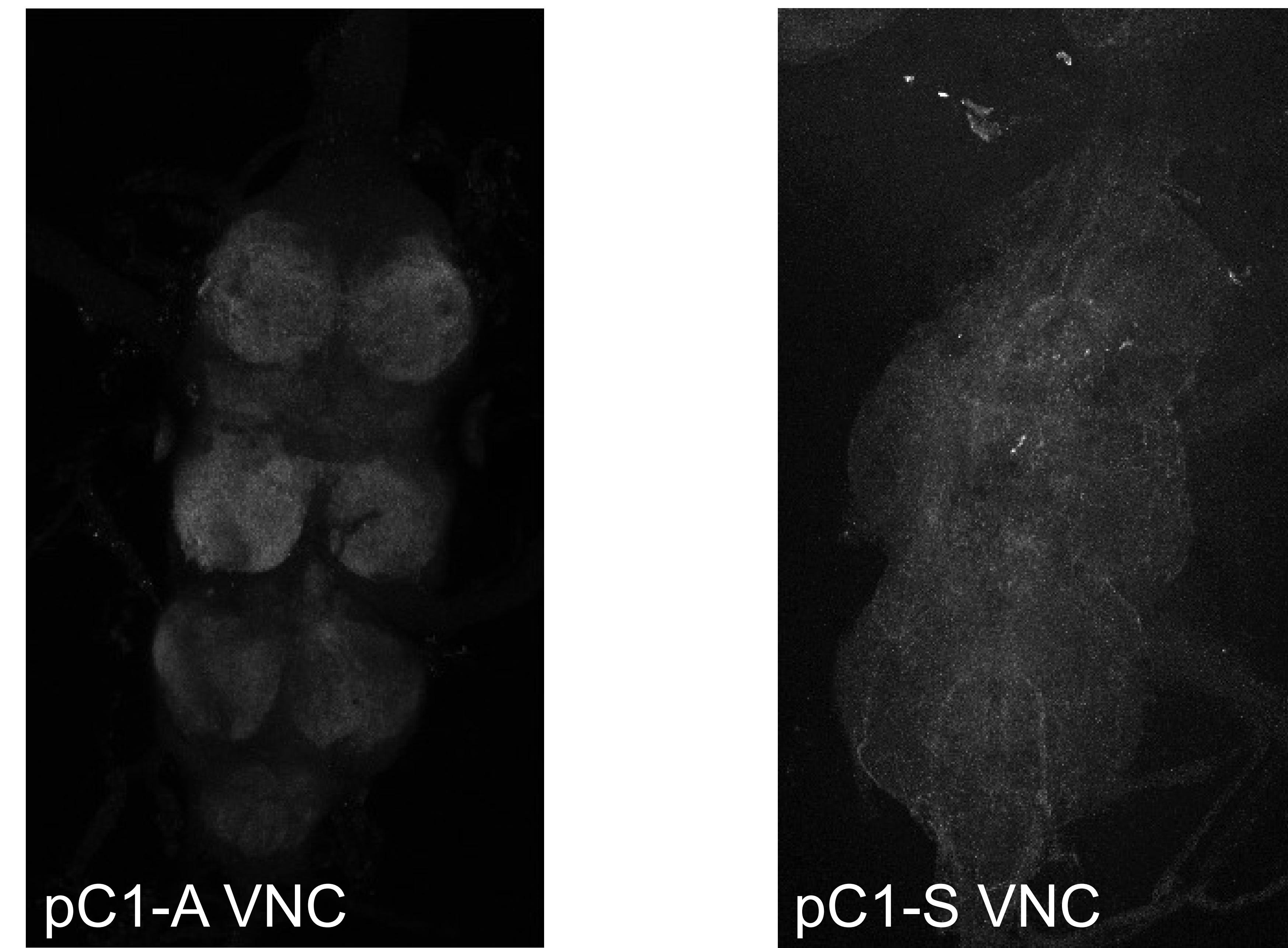


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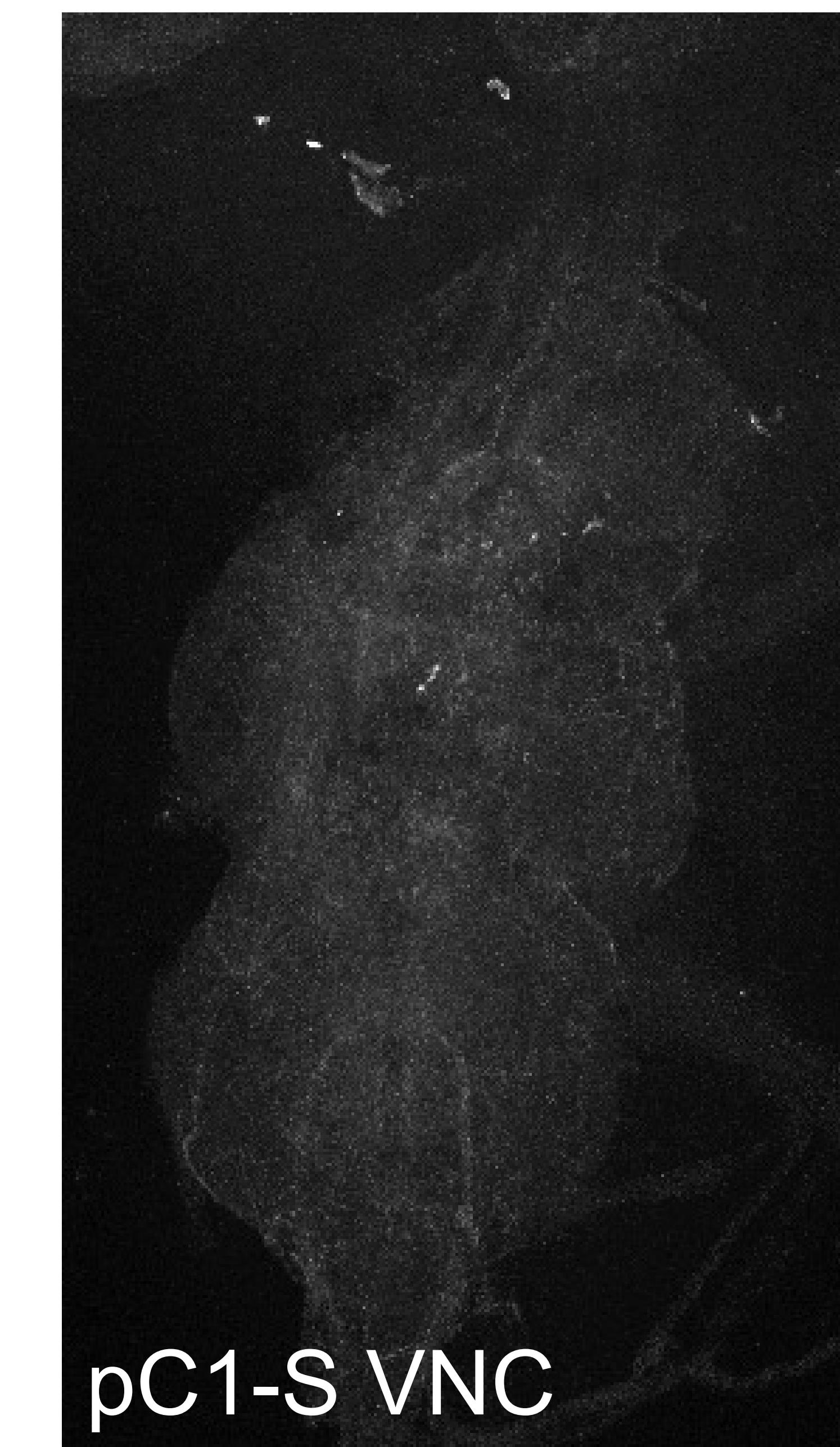
pC1-Int brain

B

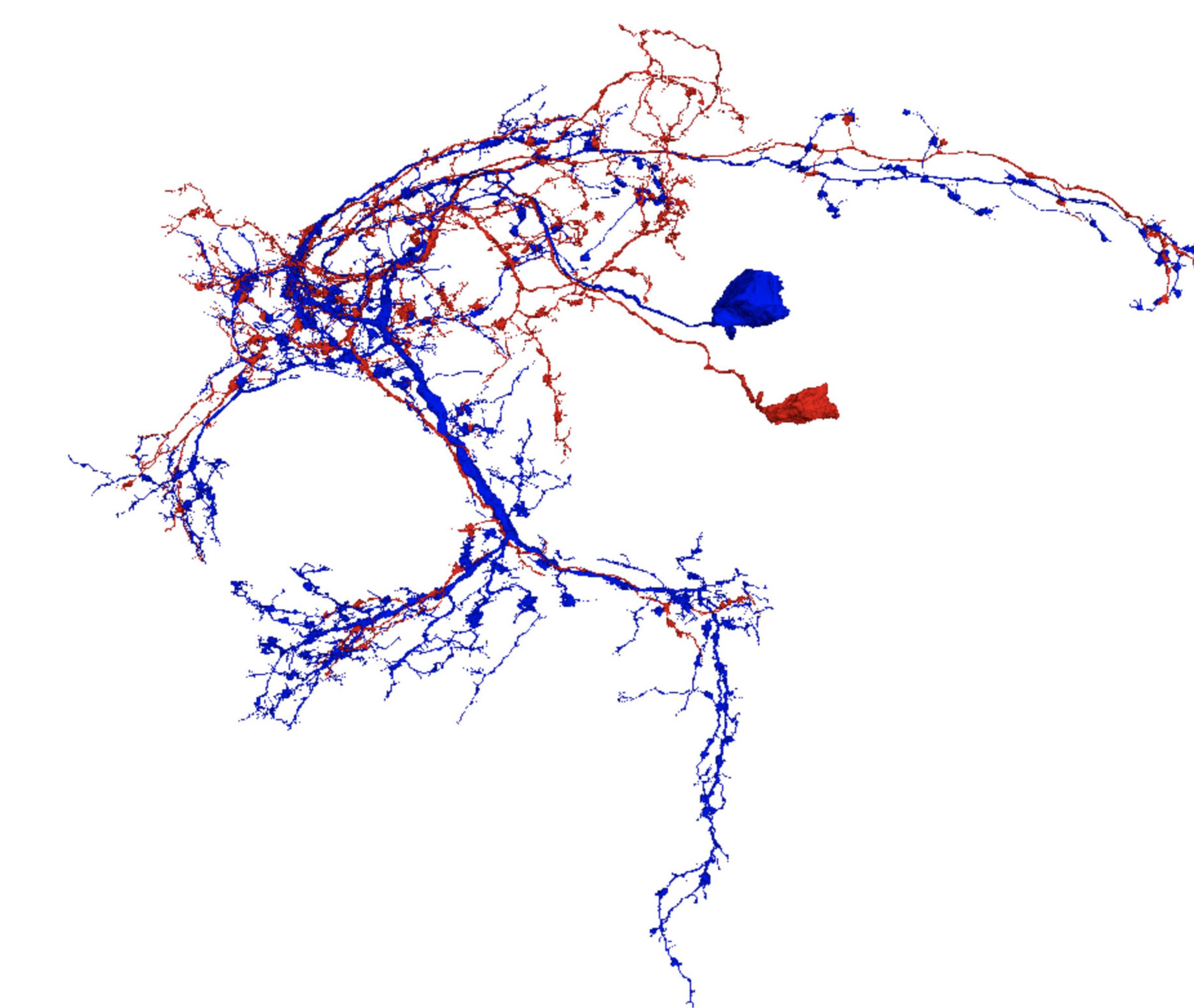
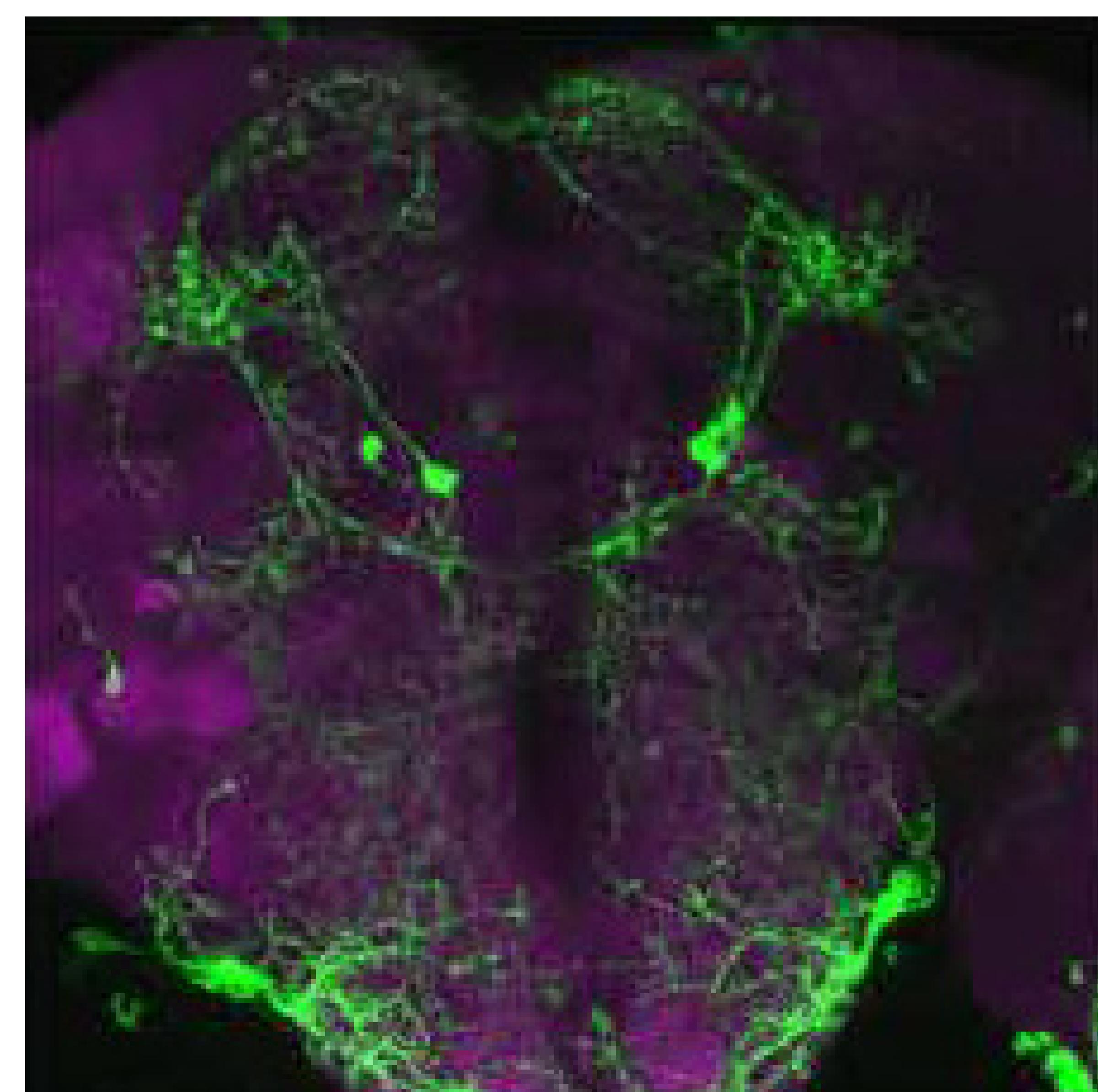
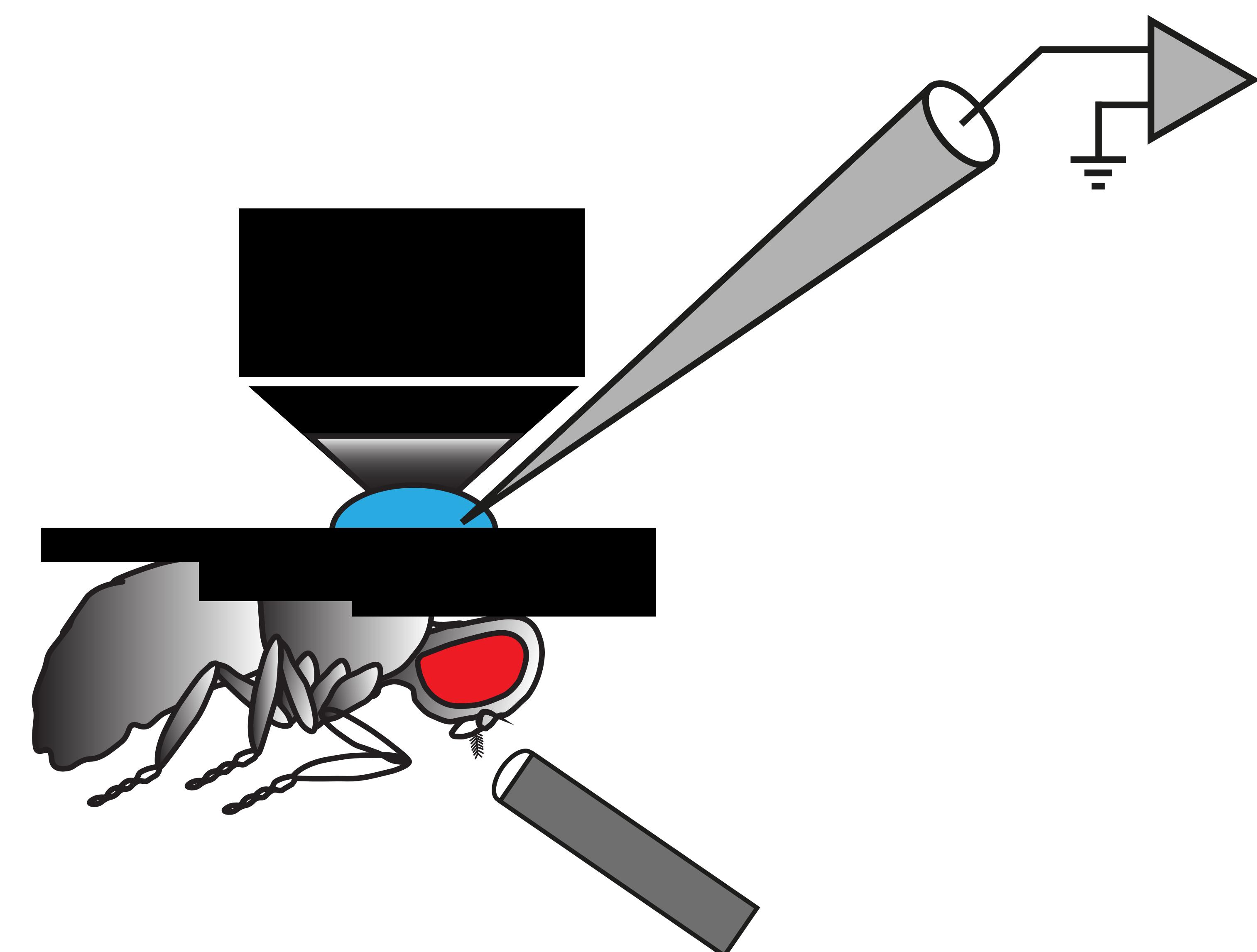
pC1-Int VNC

C

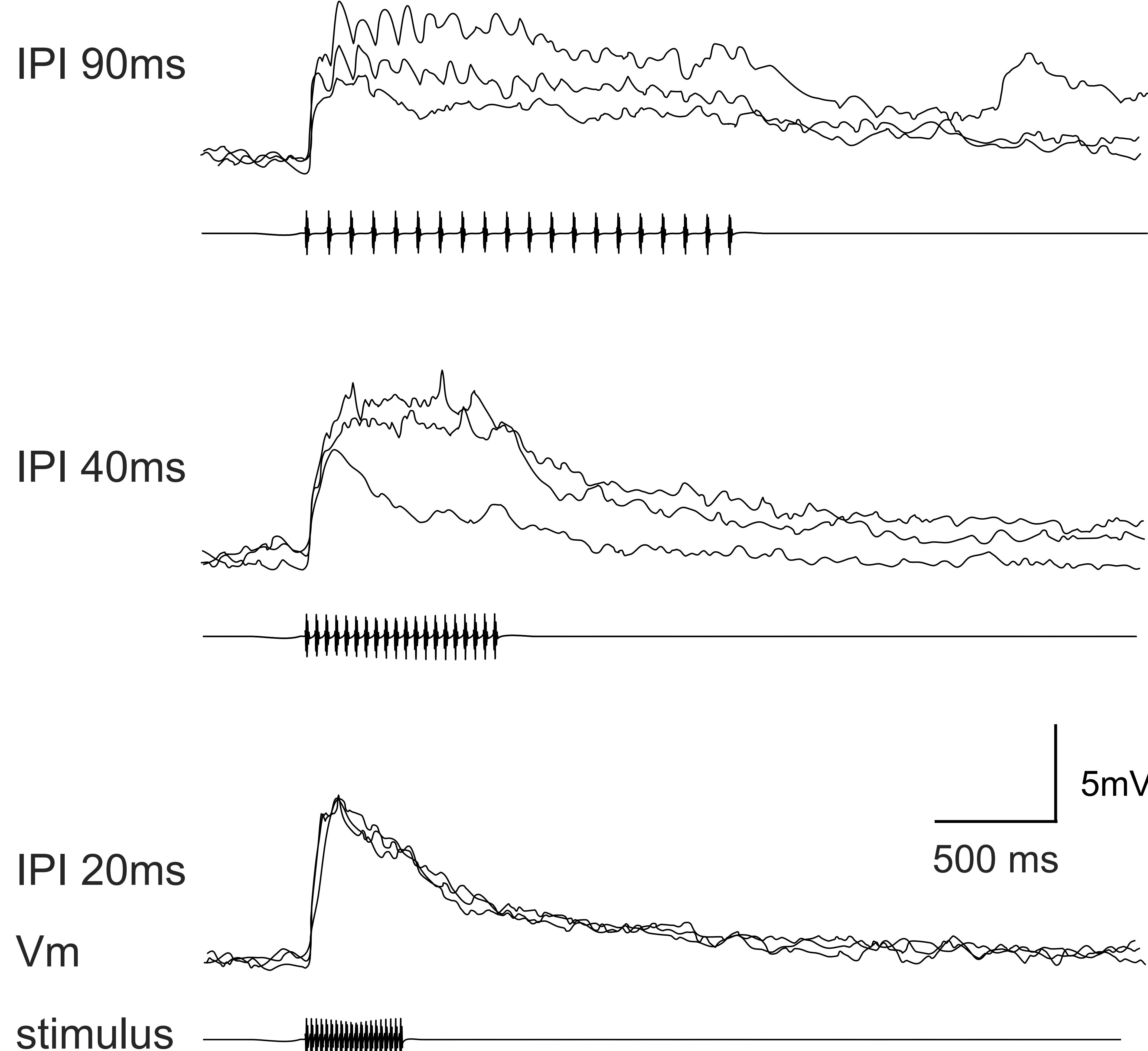
pC1-A VNC



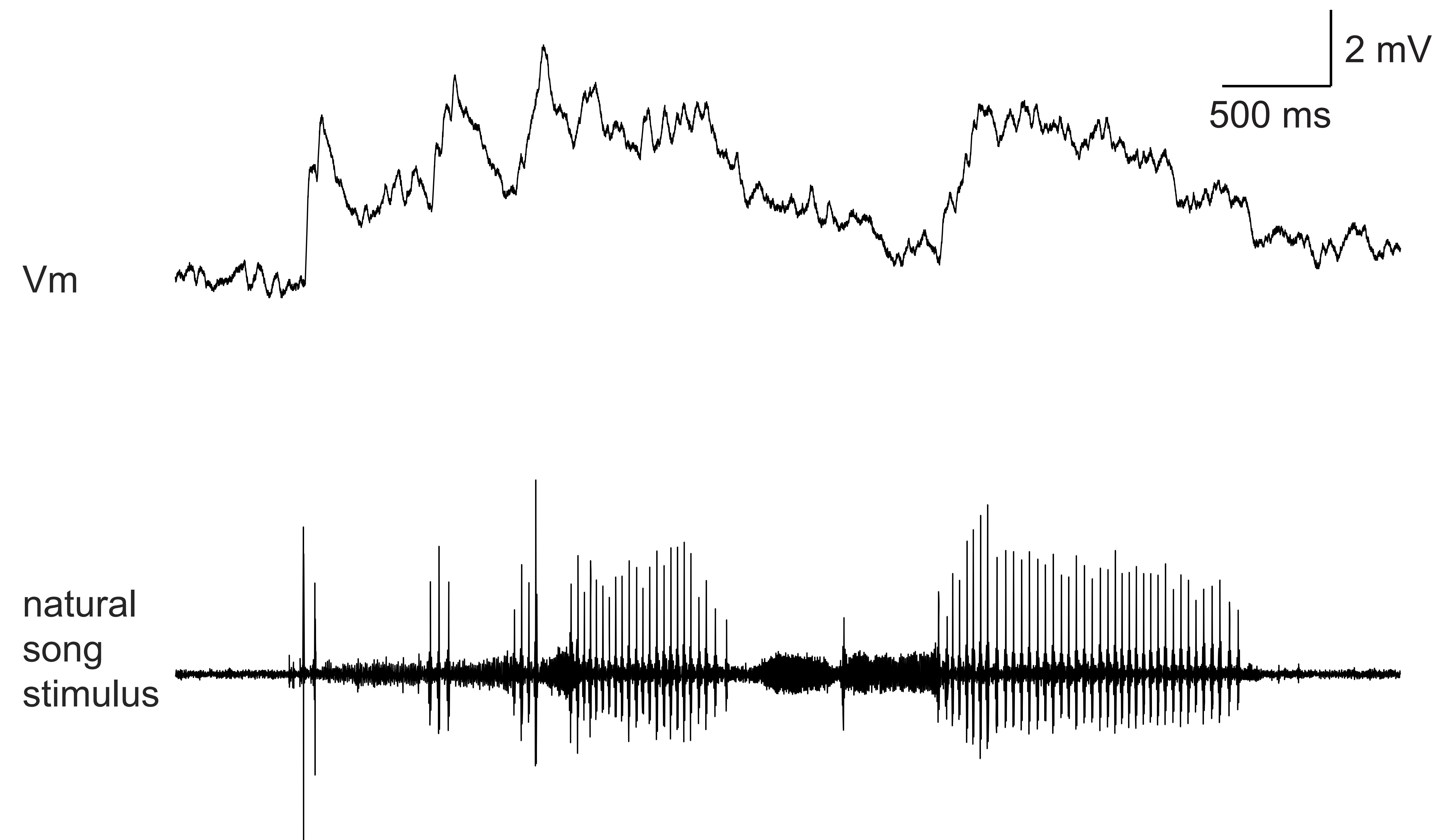
pC1-S VNC

A**B**

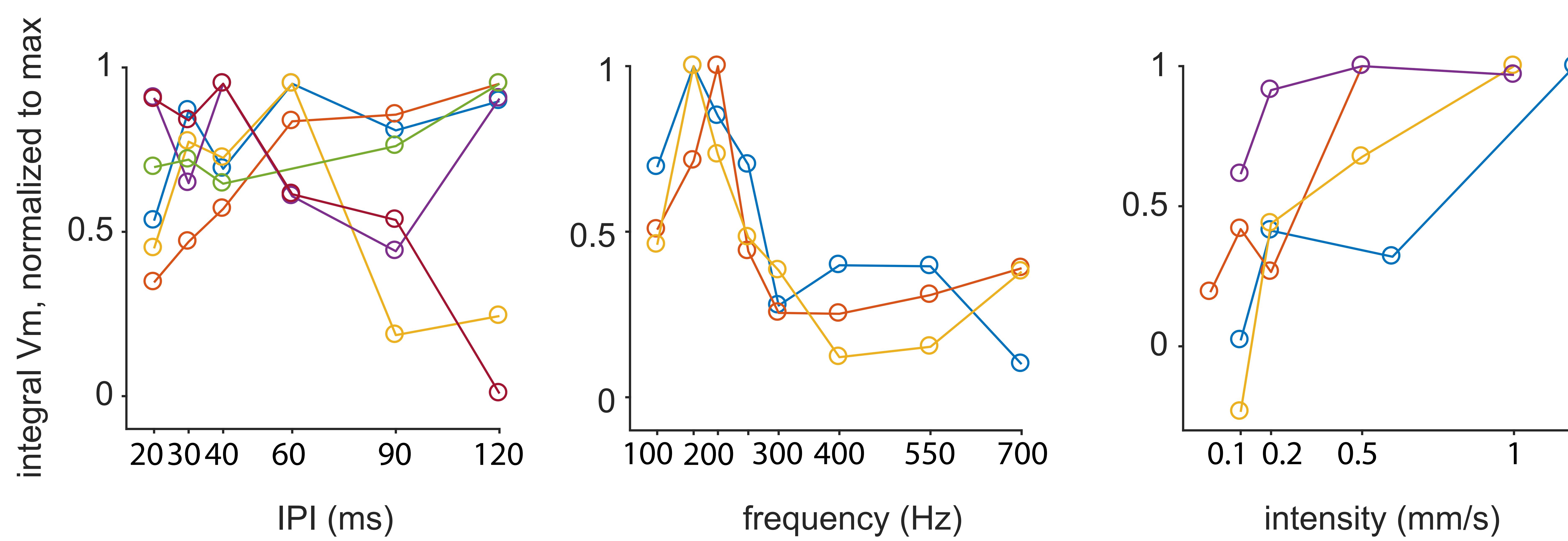
Example responses to artificial pulse trains

**C**

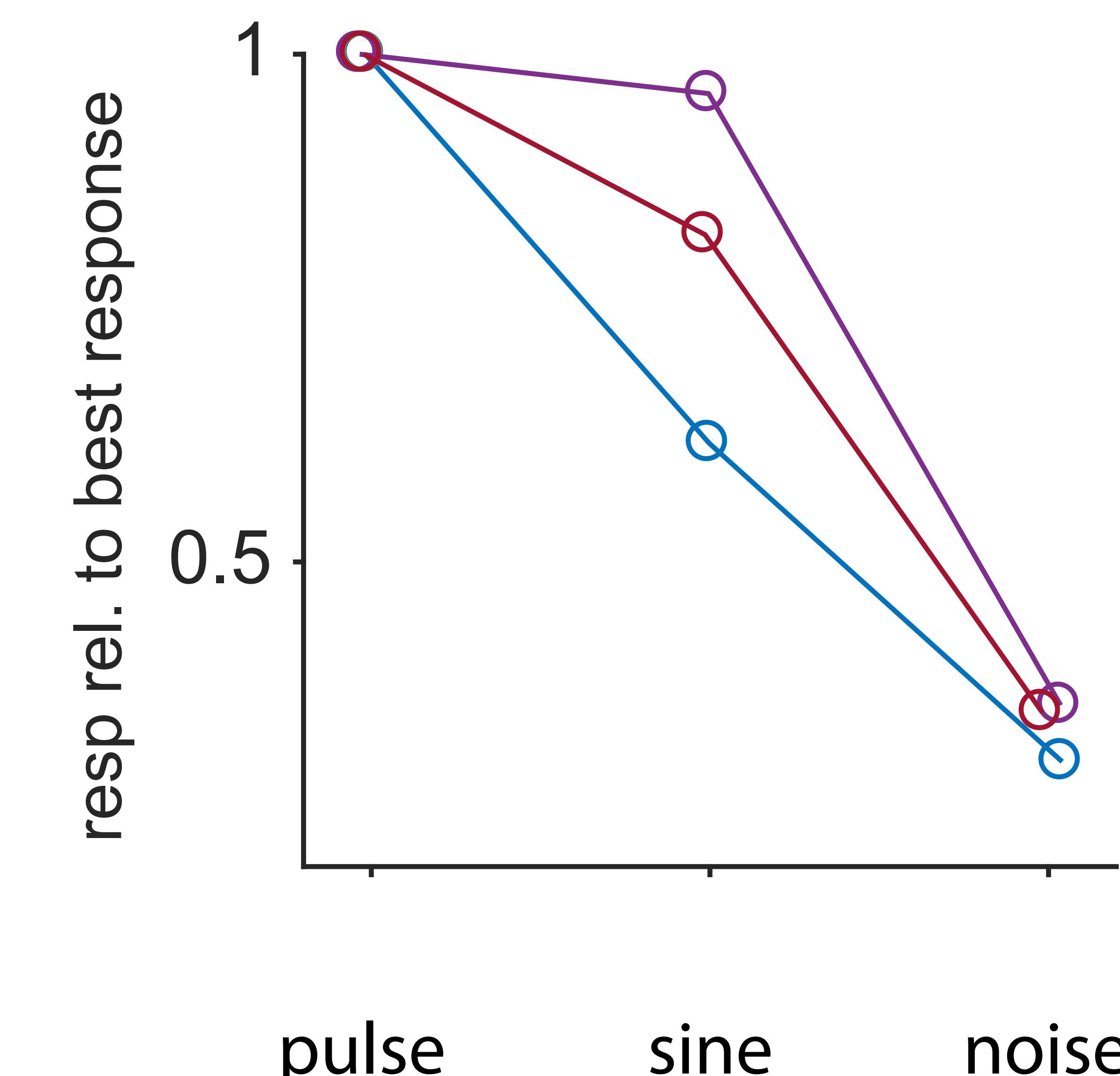
Example responses to natural courtship song

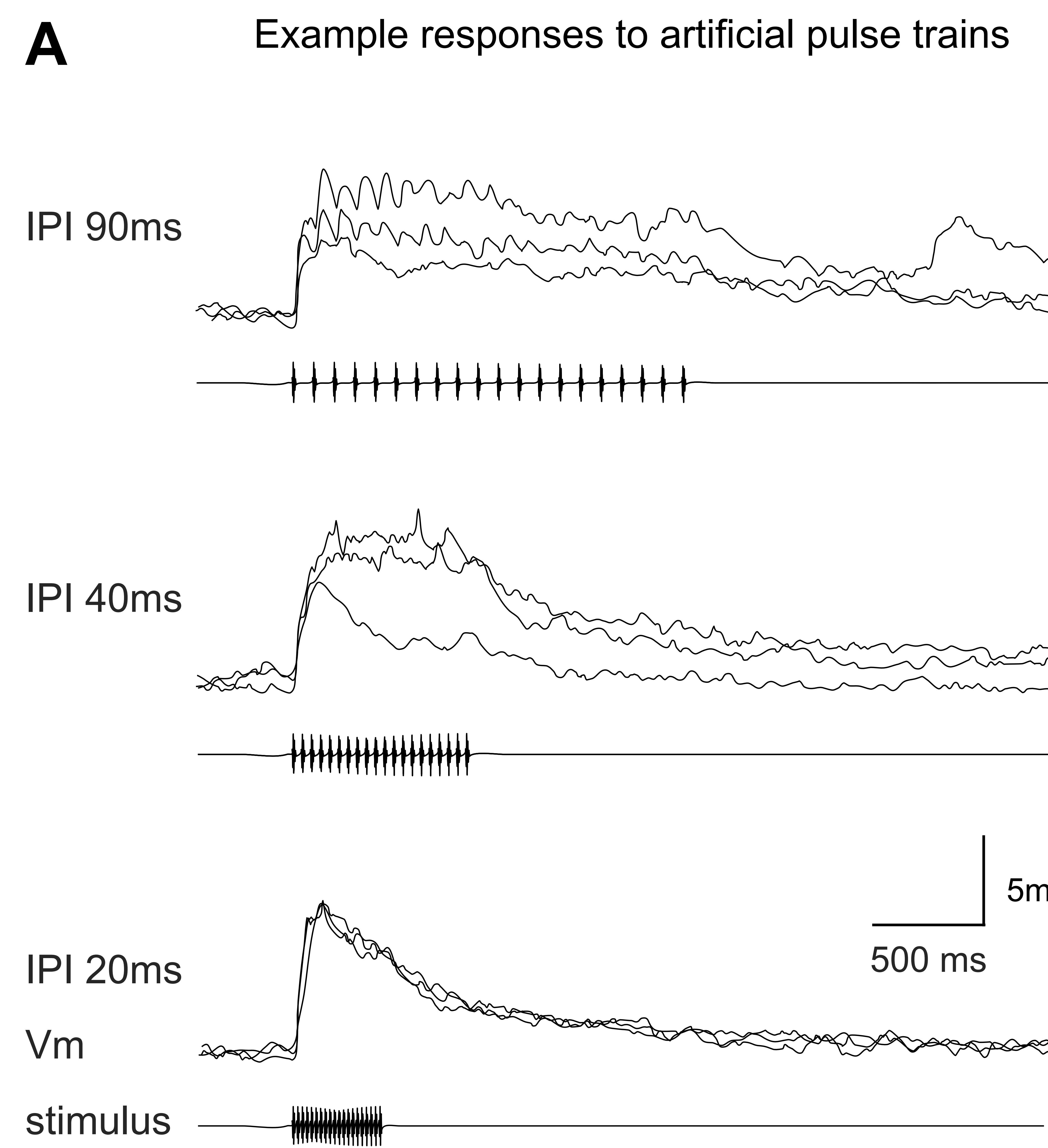
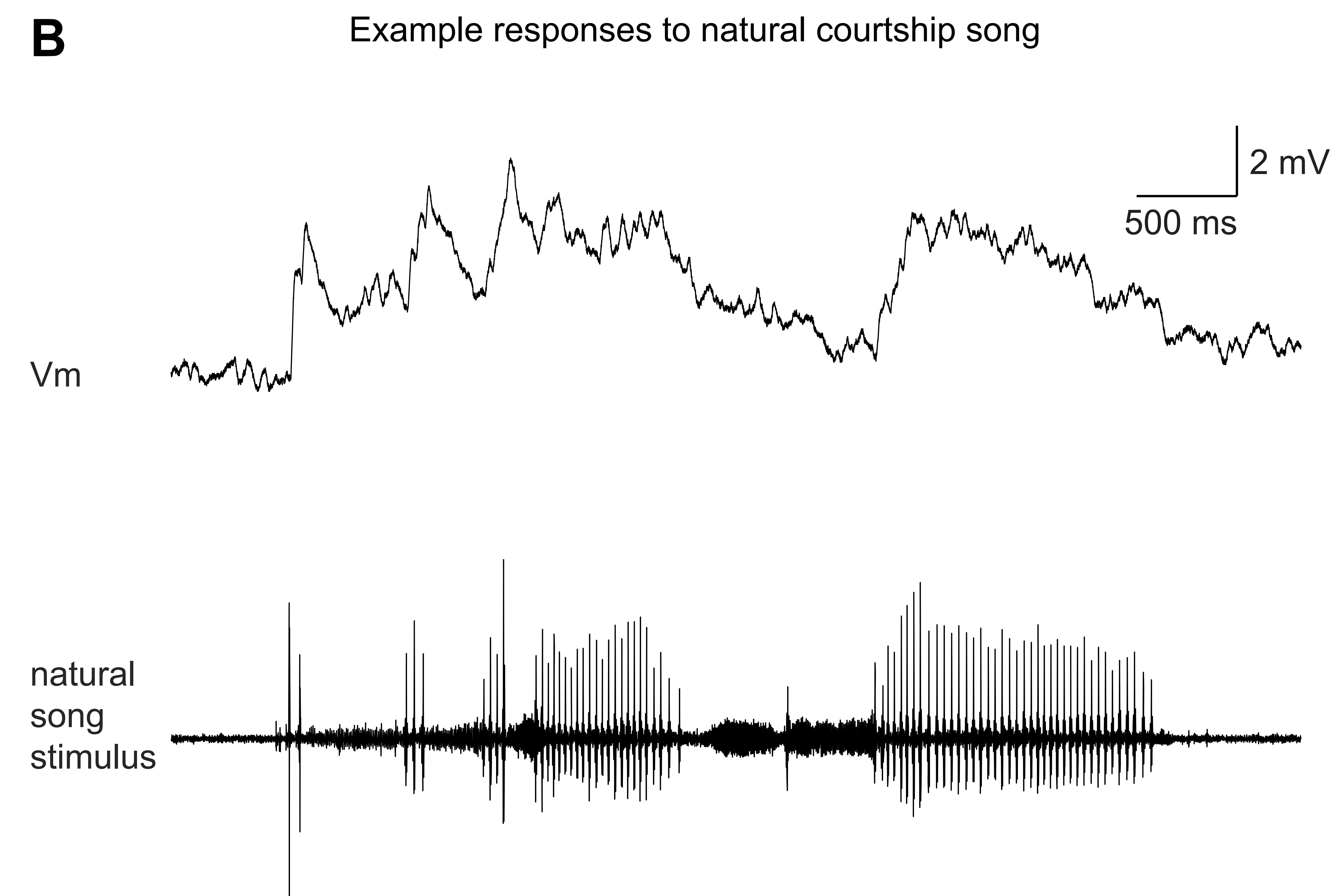
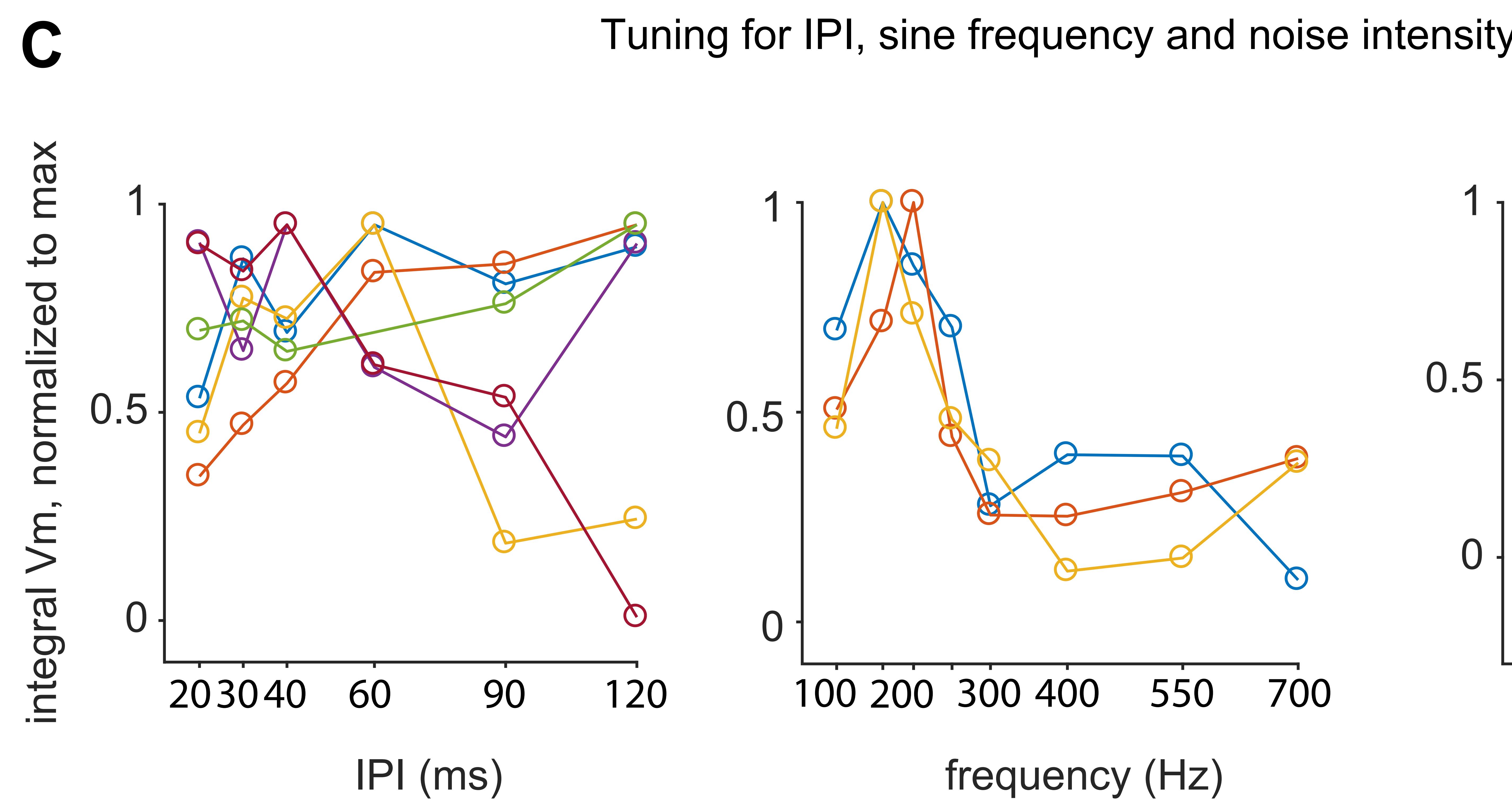
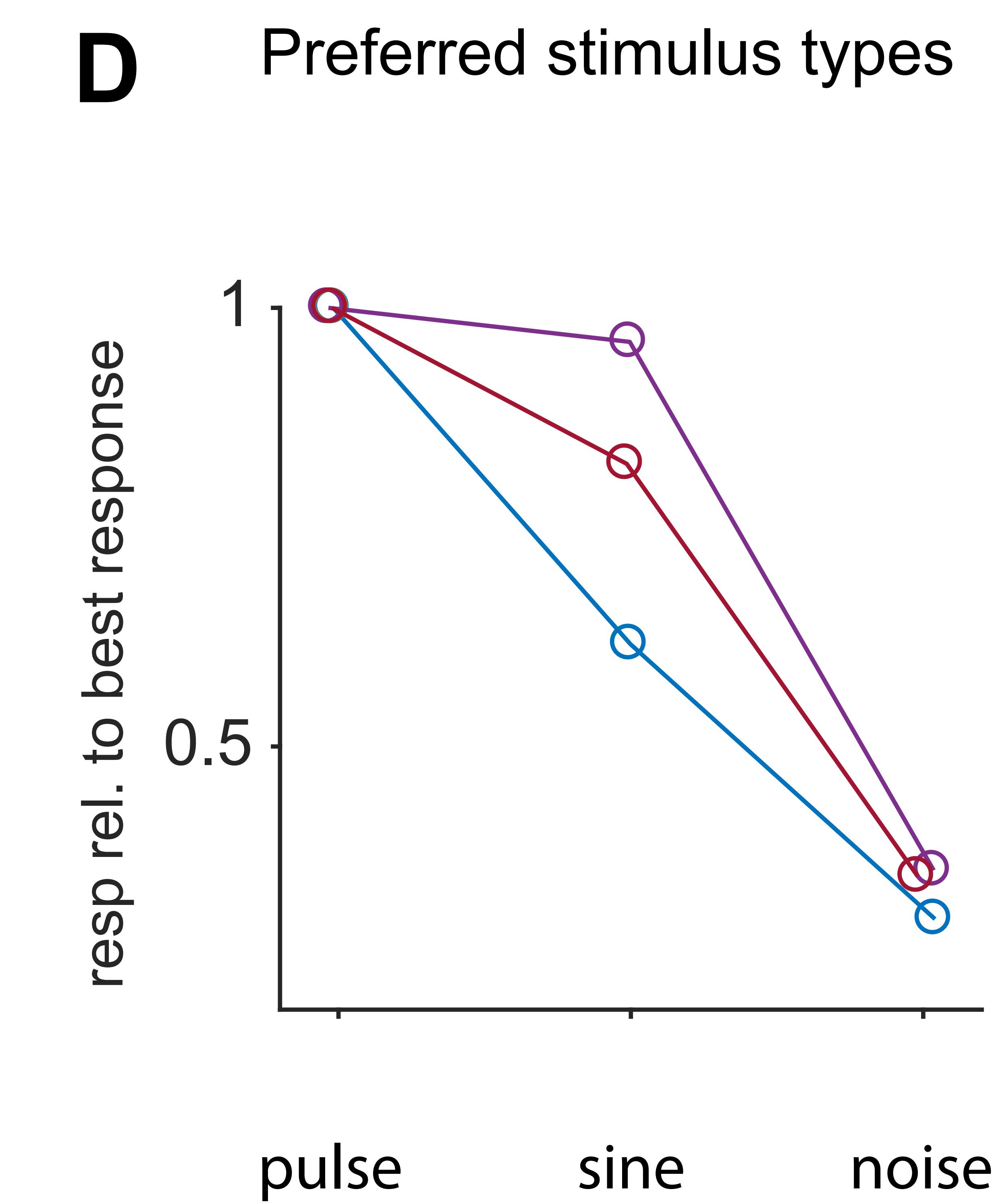
**D**

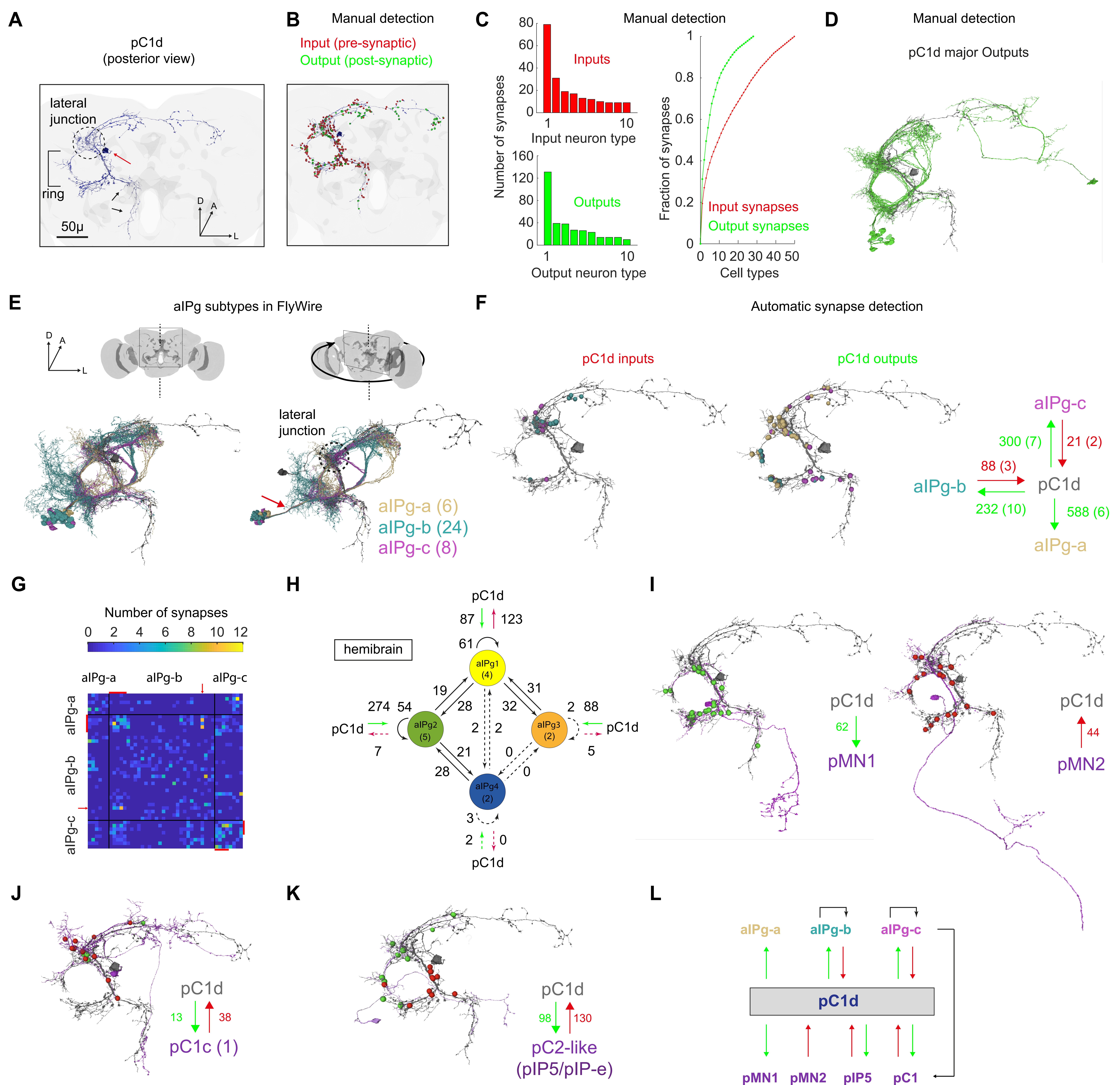
Tuning for IPI, sine frequency and noise intensity



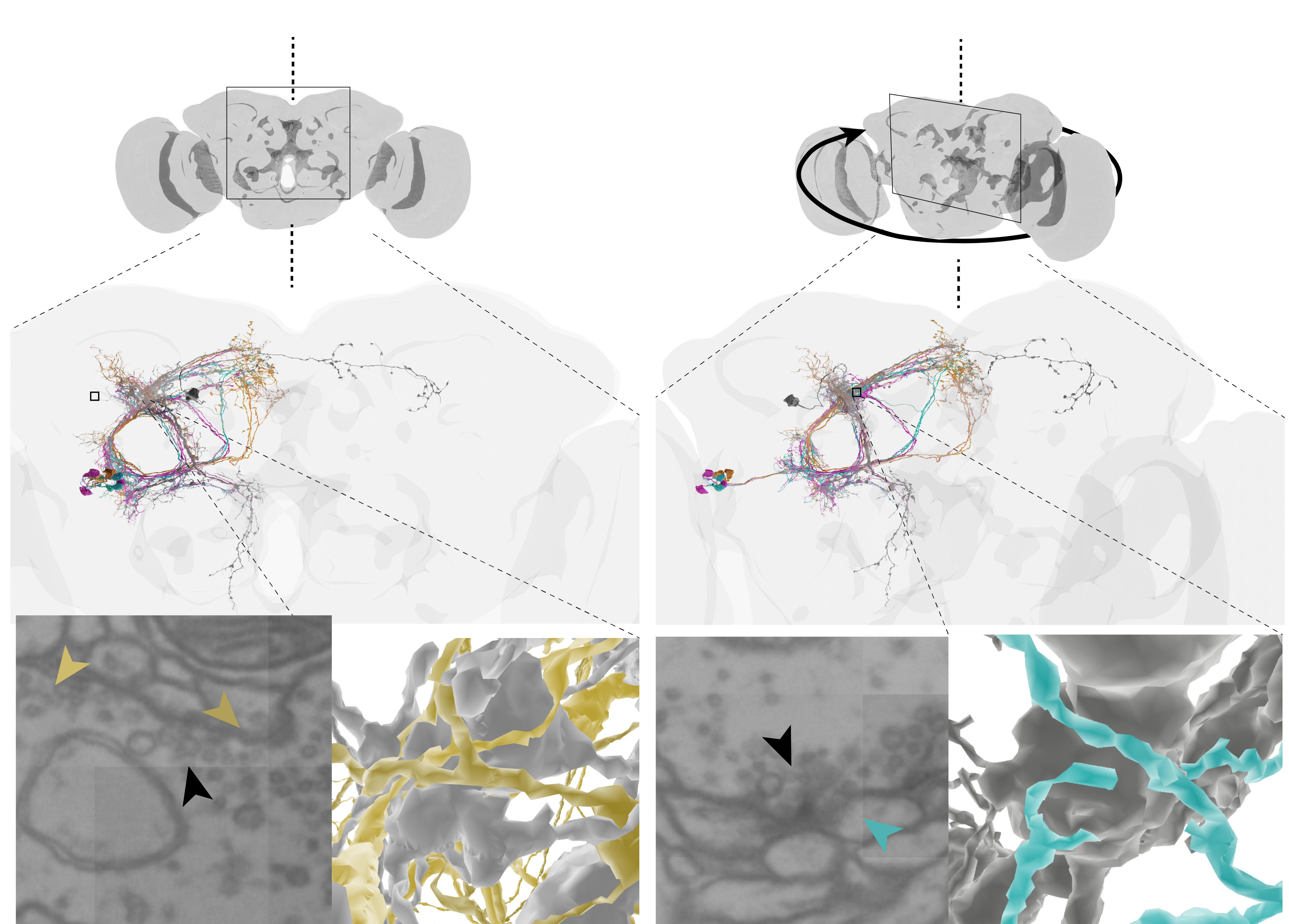
Preferred stimulus types



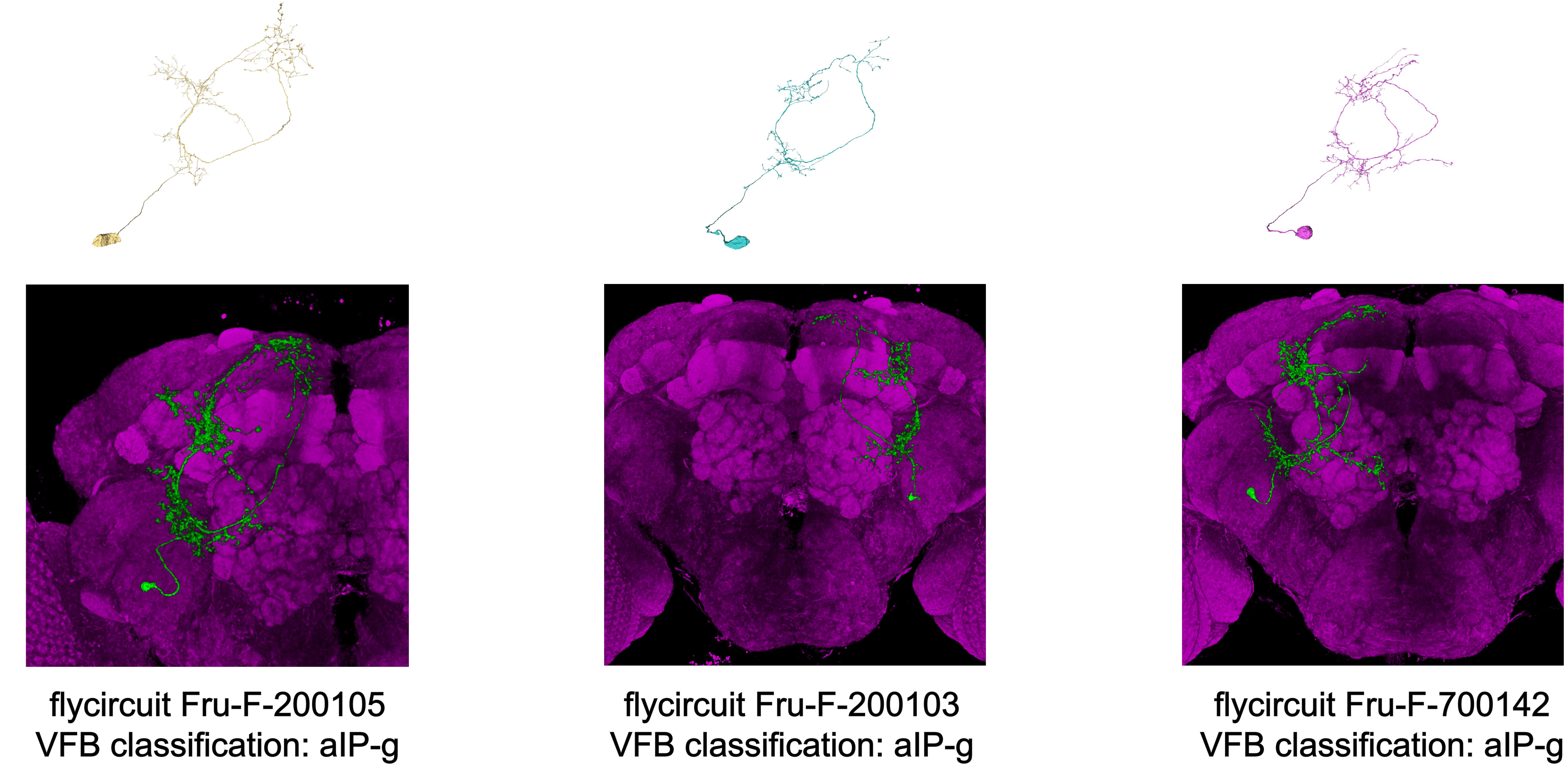
A**B****C****D**



A pC1d alPg-a alPg-b alPg-c



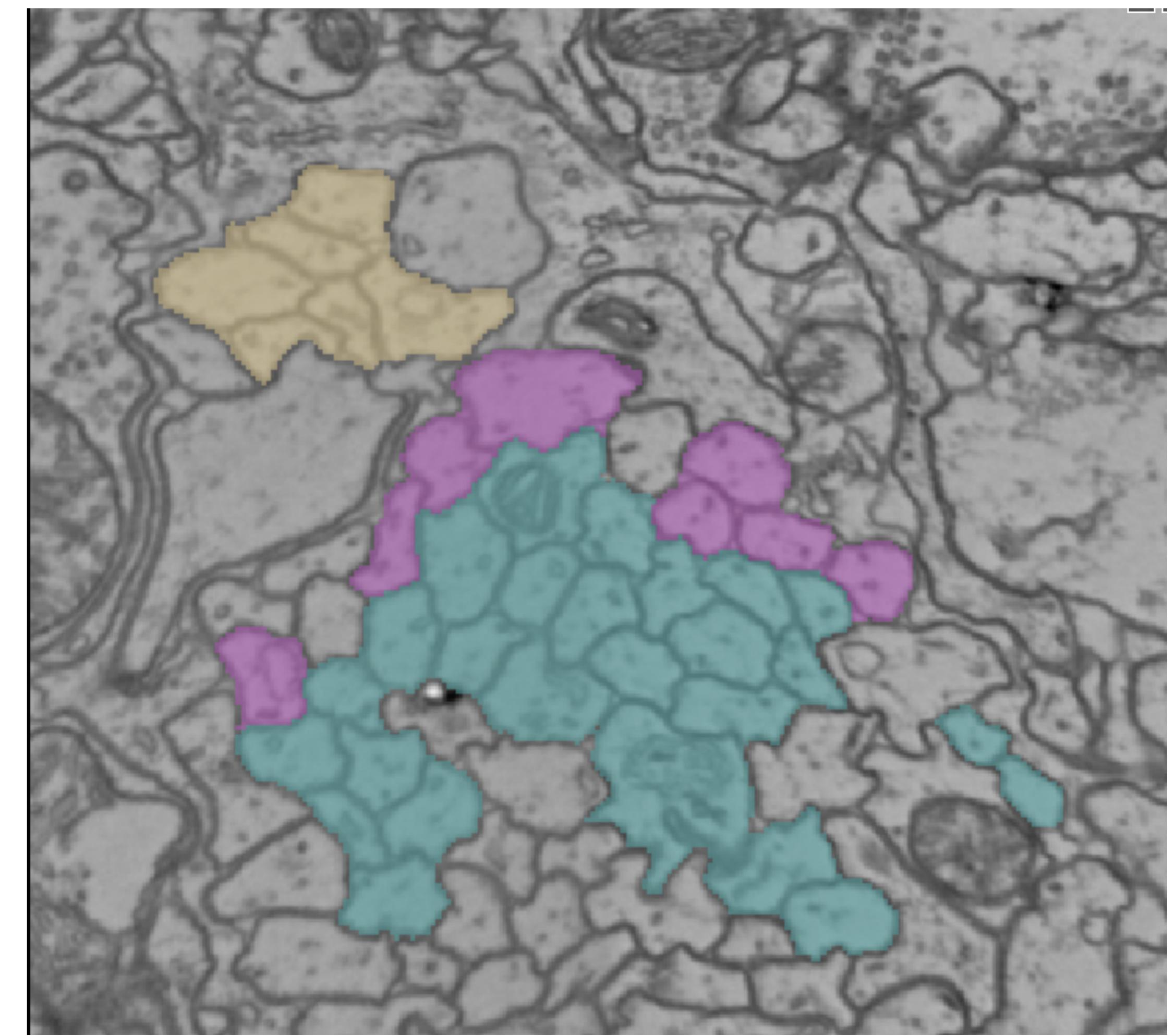
B alPg-a alPg-b alPg-c



C

Anterior view

FlyWire: 161410, 55014, 3940



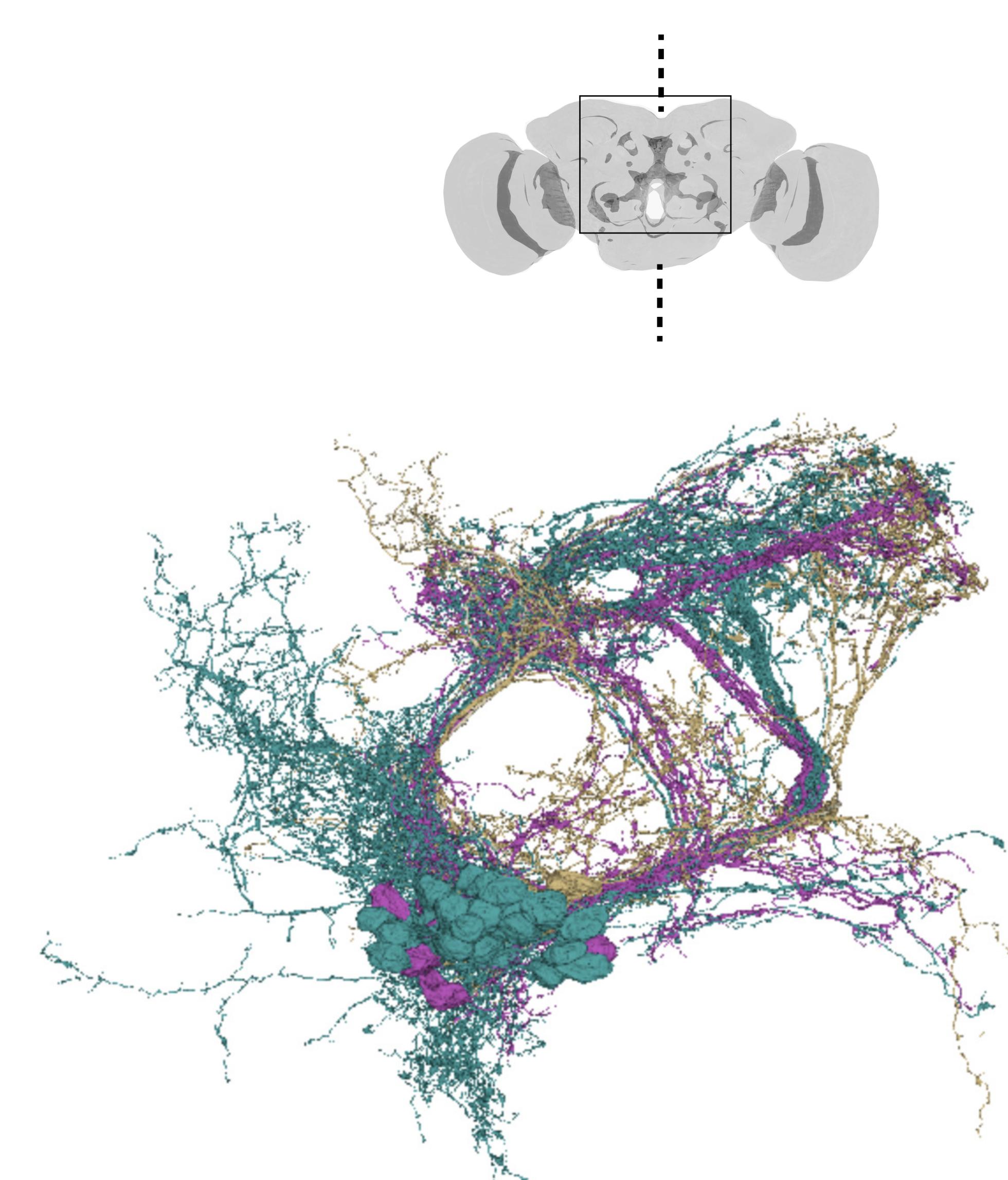
alPg-a ($N = 6$)
alPg-b ($N = 24$)
alPg-c ($N = 8$)

Posterior view

(i)

(ii)

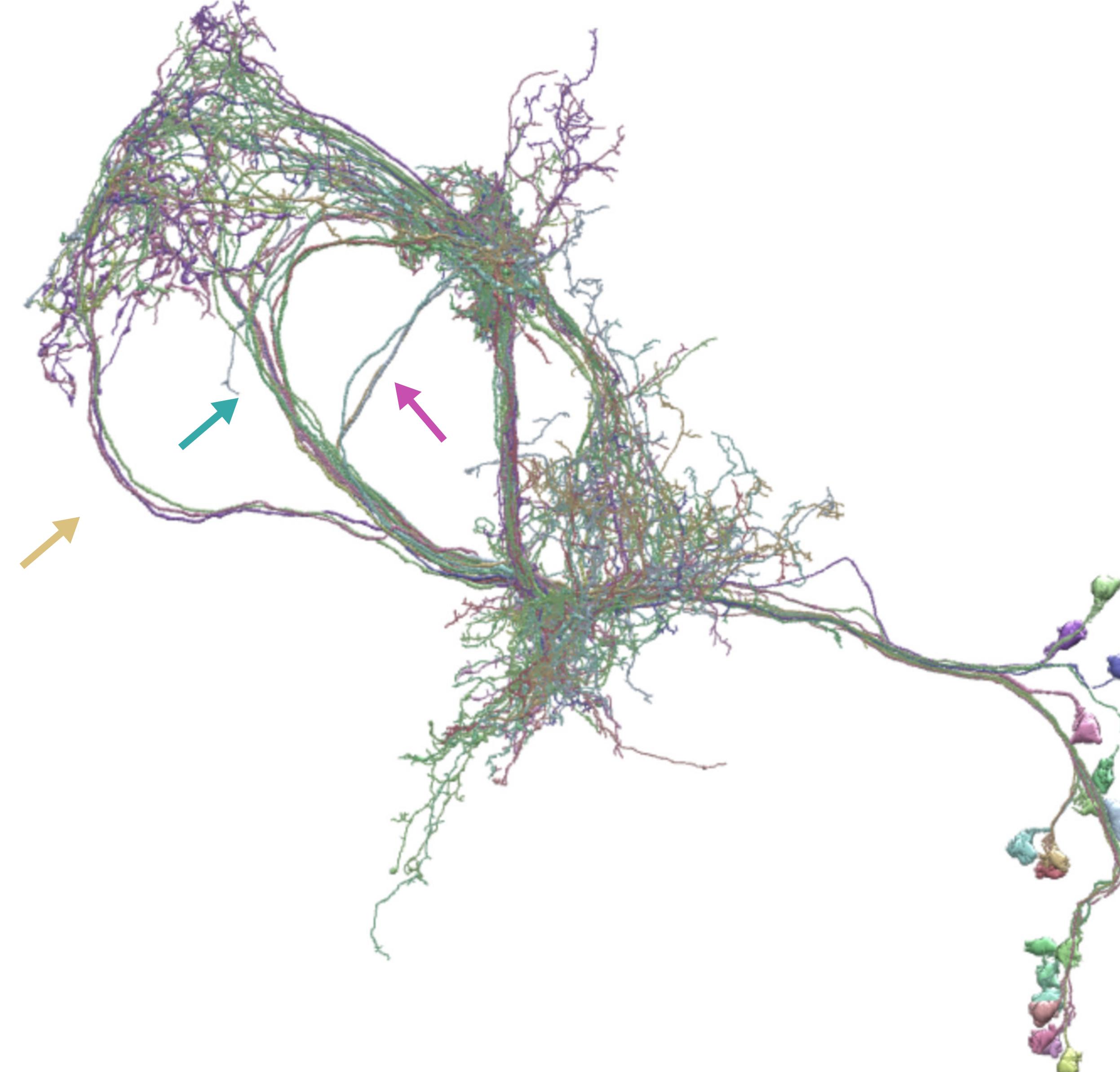
(iii)



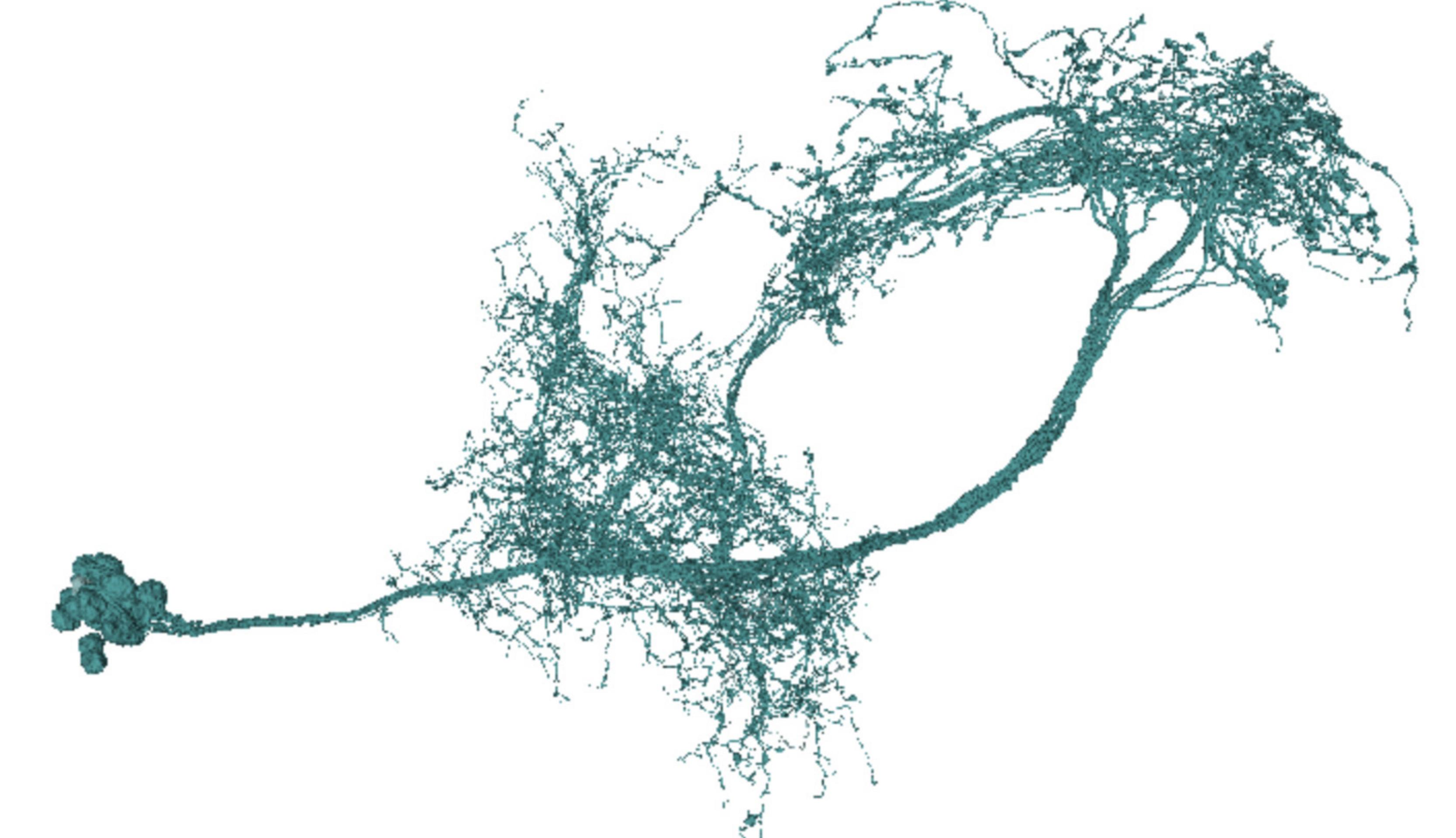
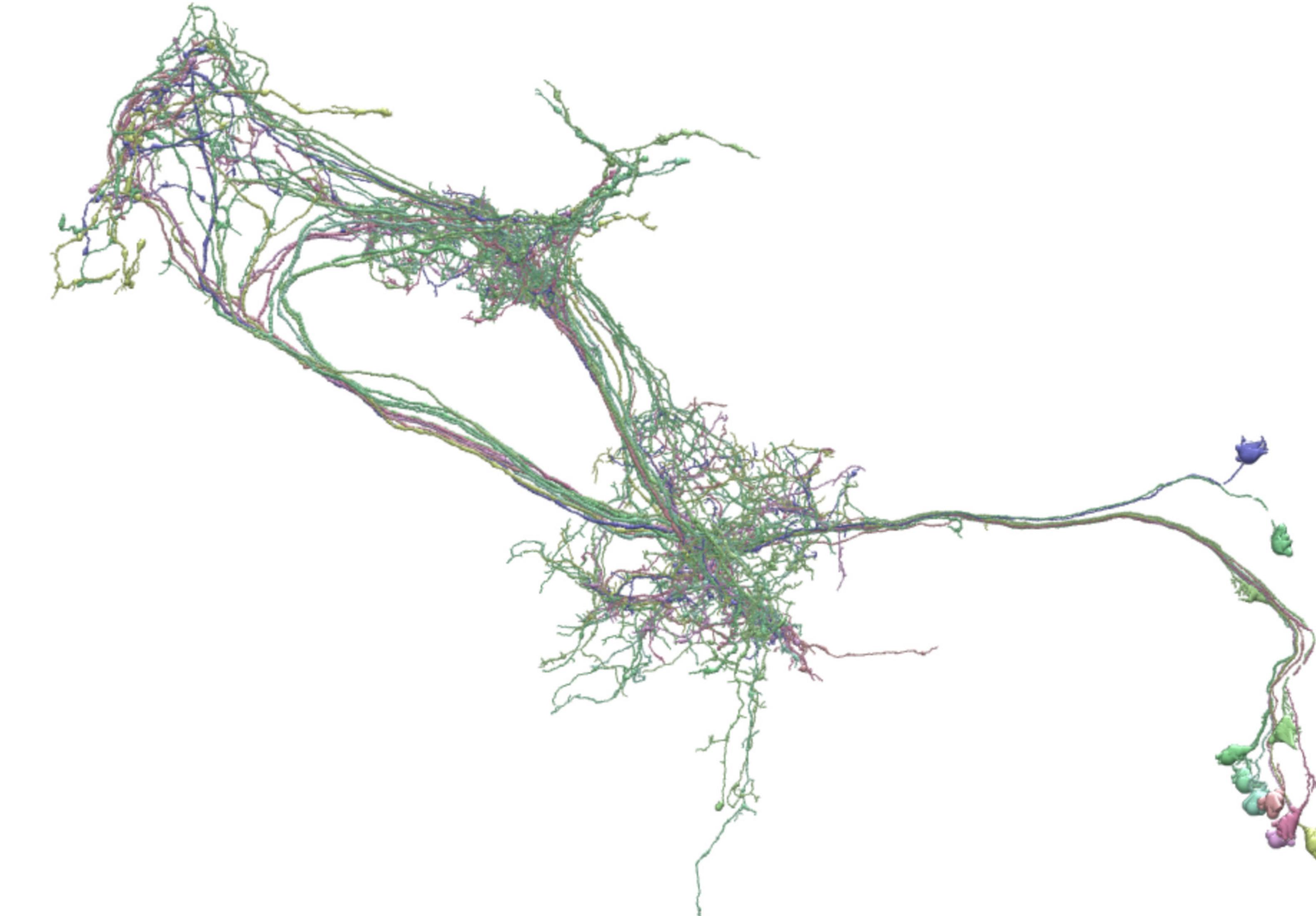
FlyWire alPg-b

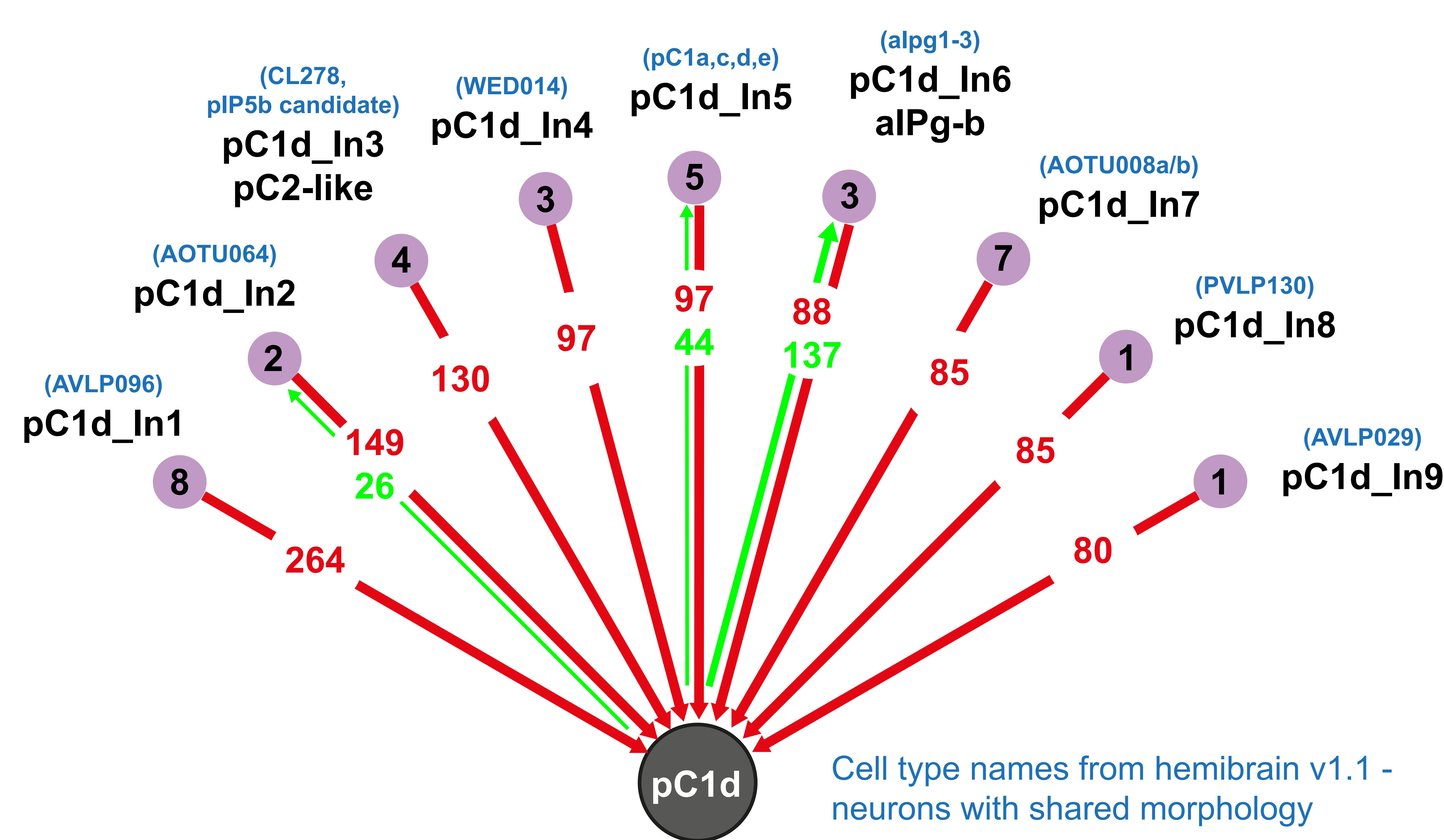
D

hemibrain
Posterior view

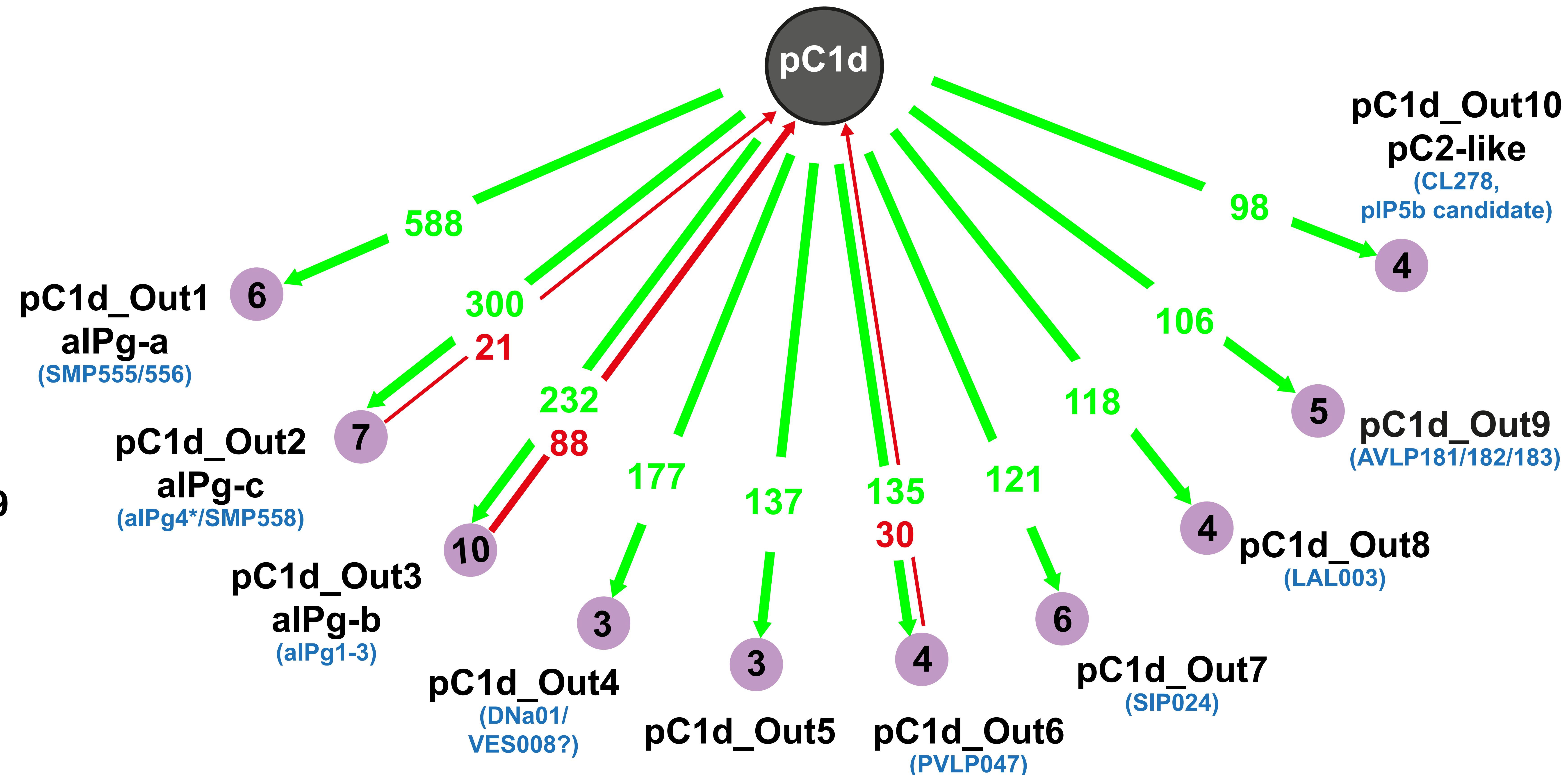
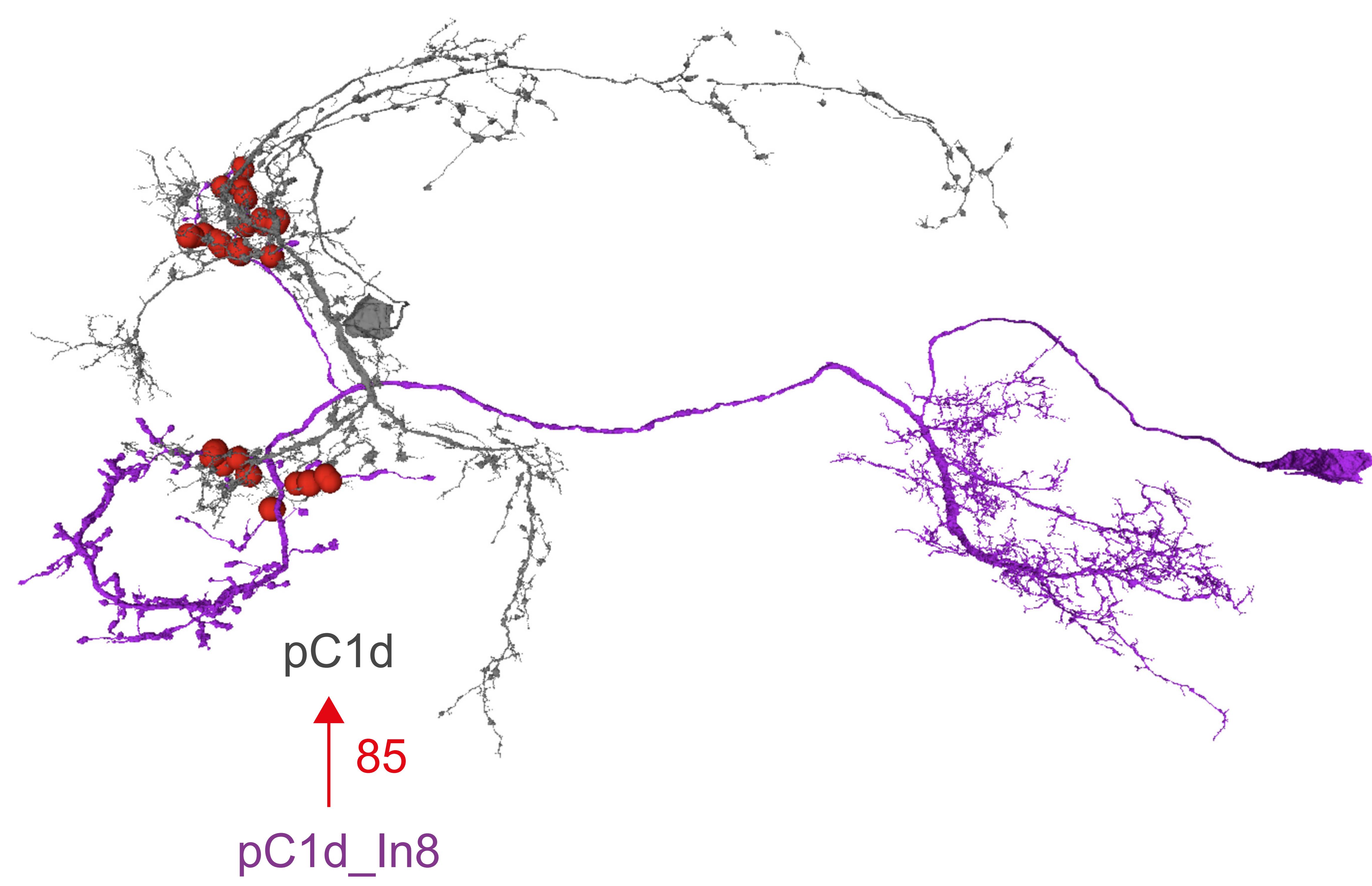


hemibrain alPg1-3

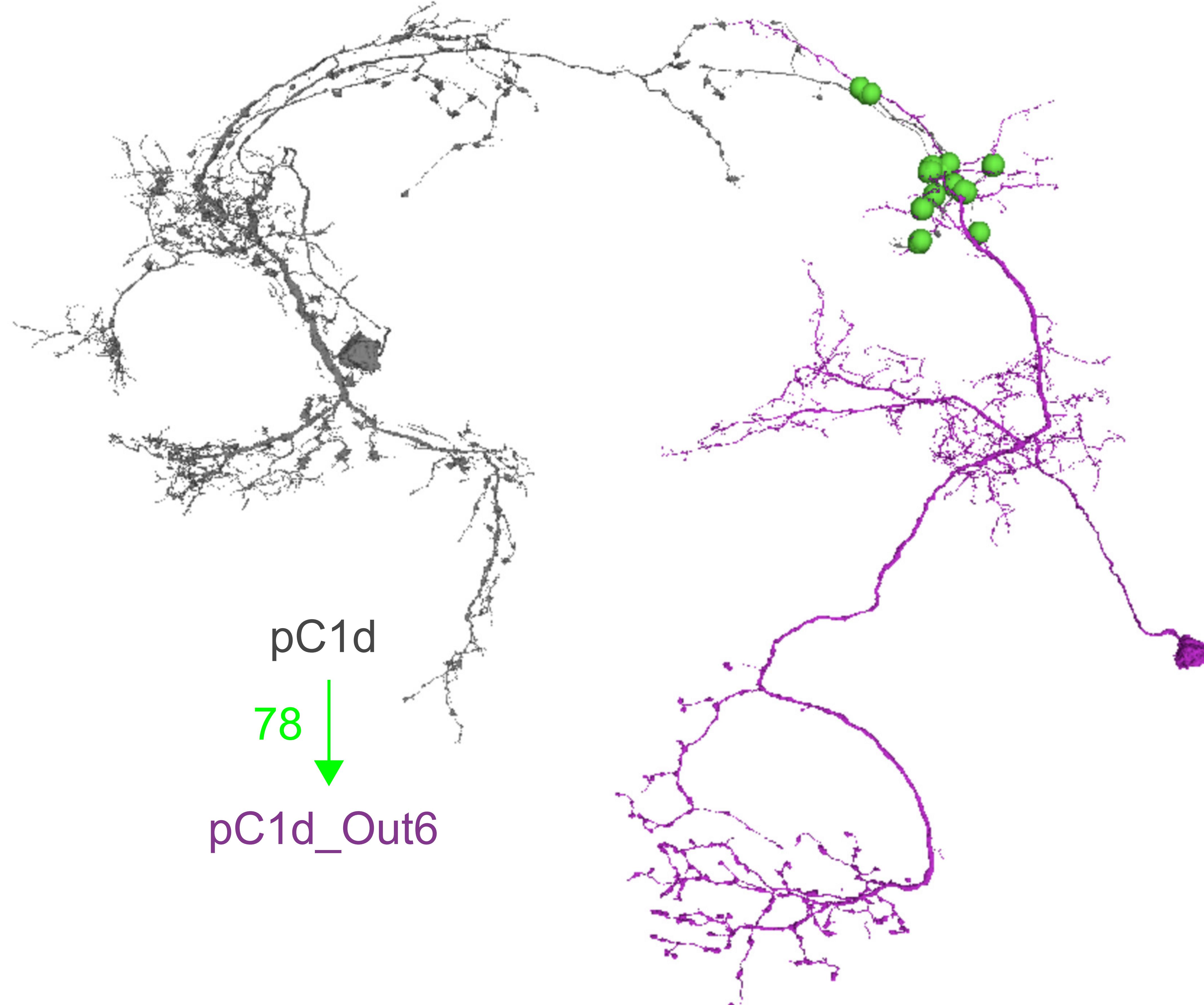


A pC1d top inputs

pC1d top outputs

**B** pC1d input example

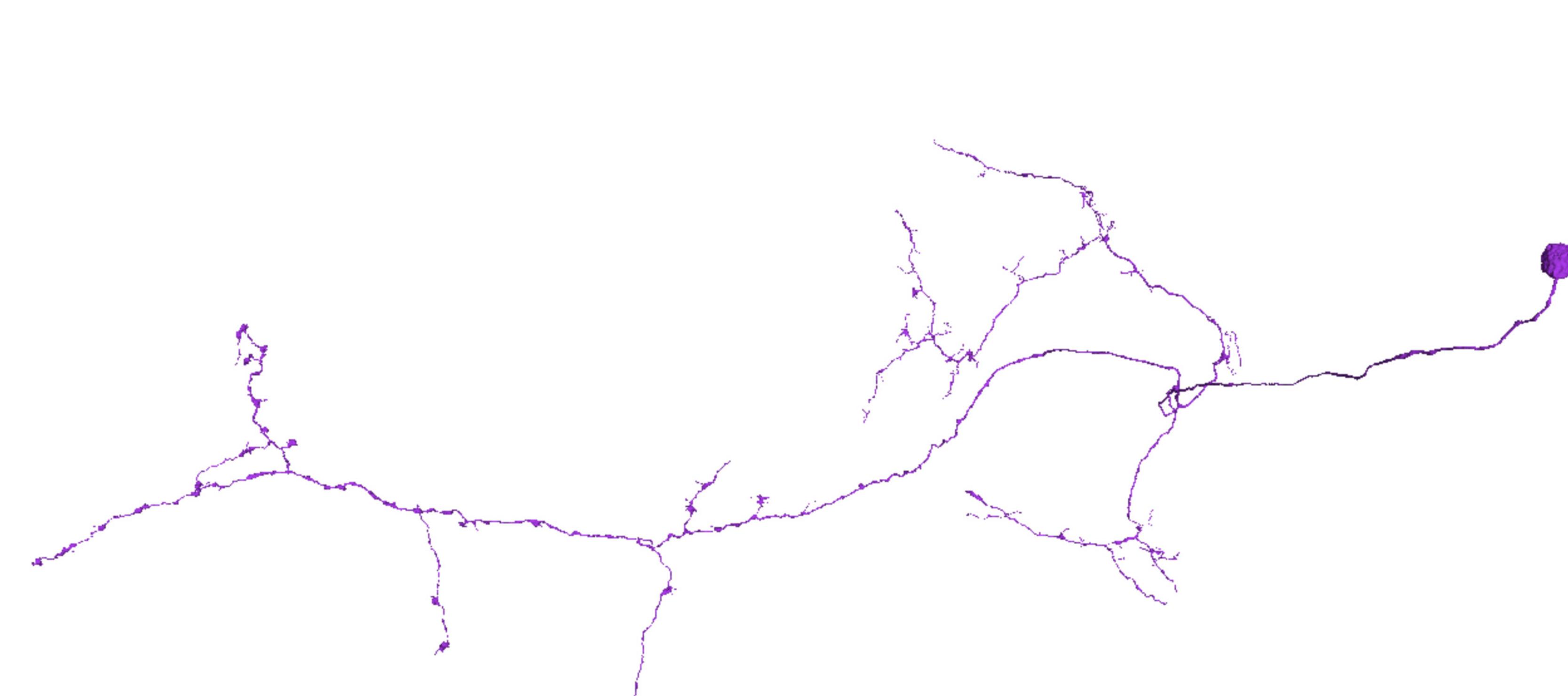
pC1d output example

**C**

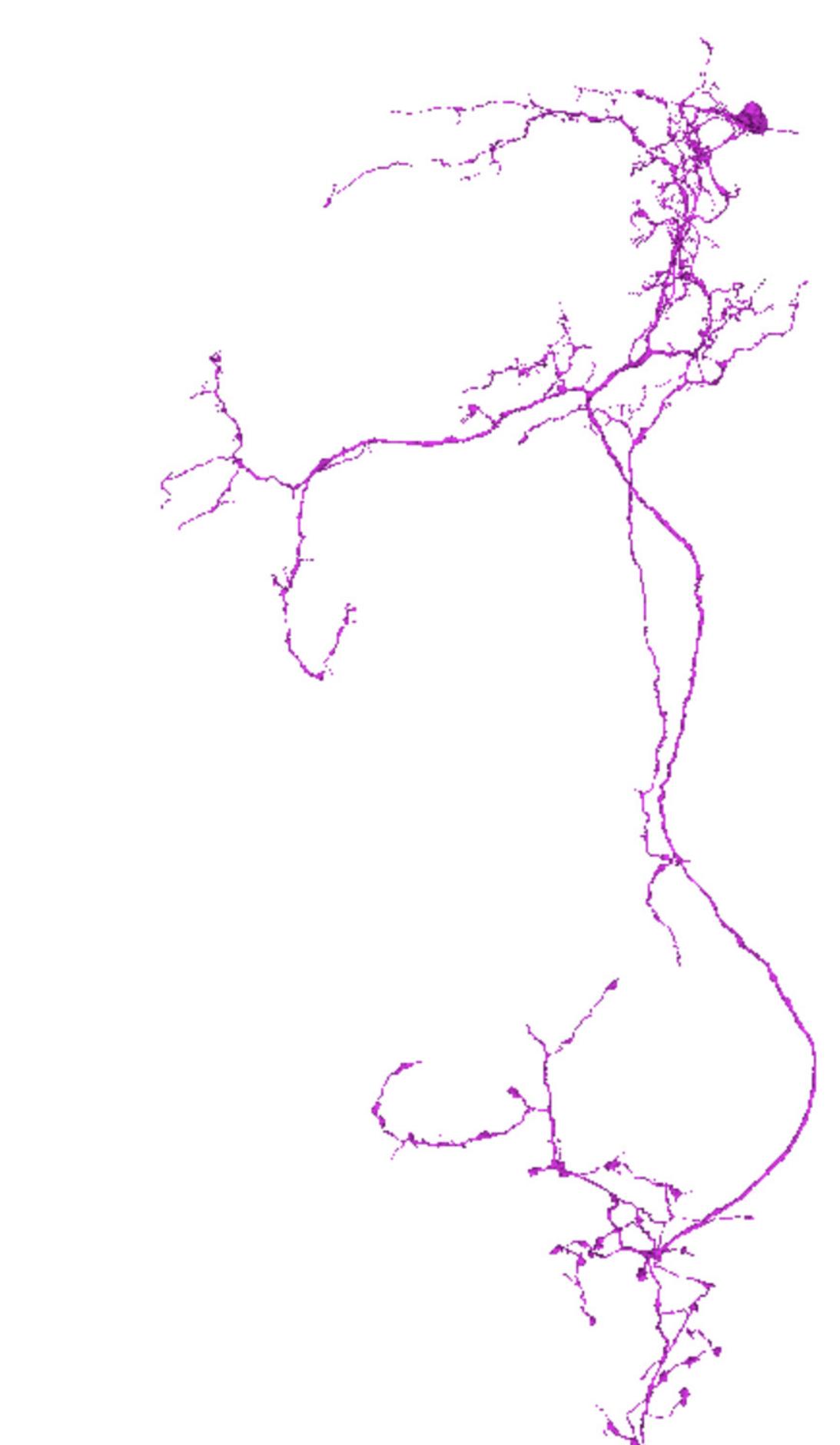
FlyWire



AOTU064

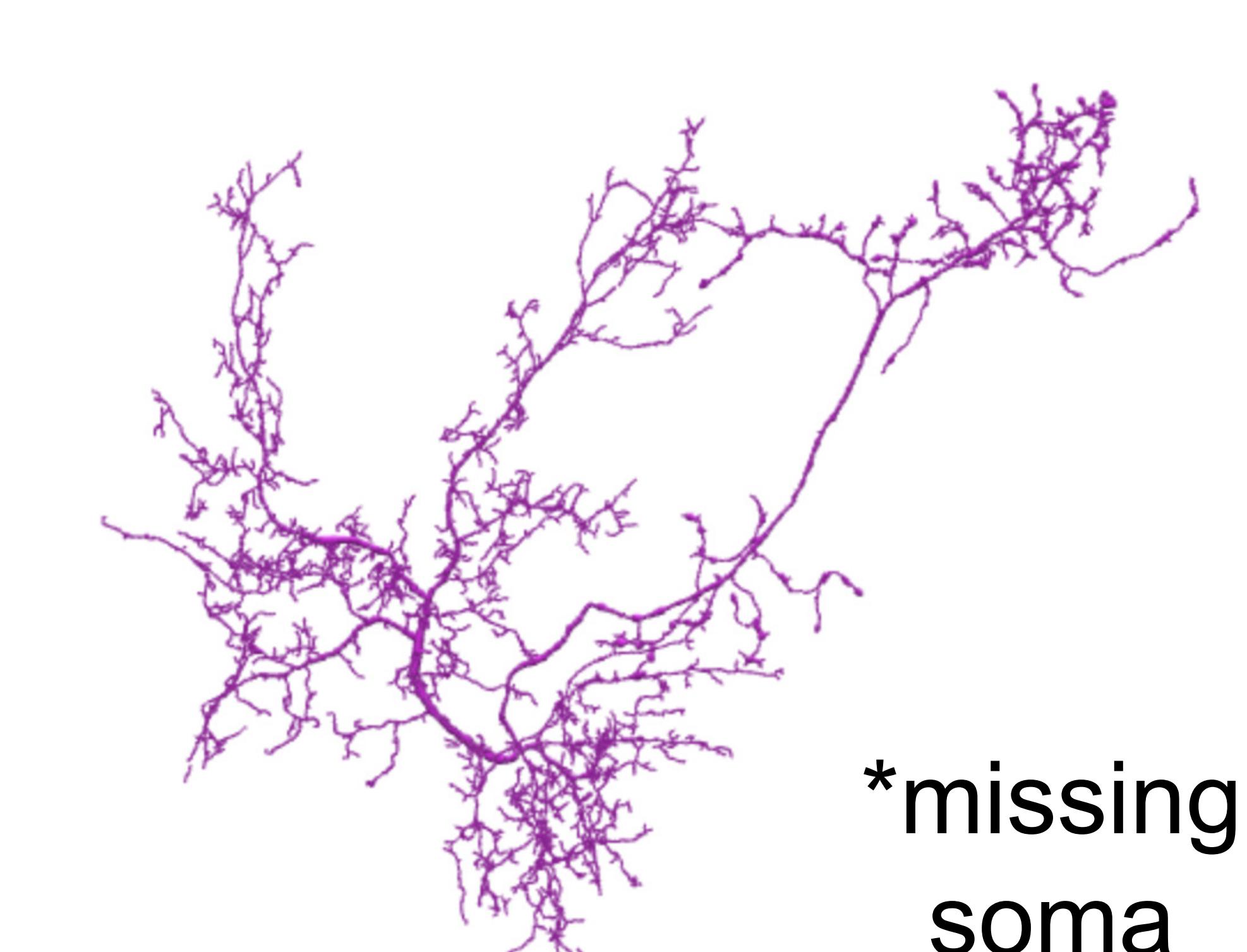


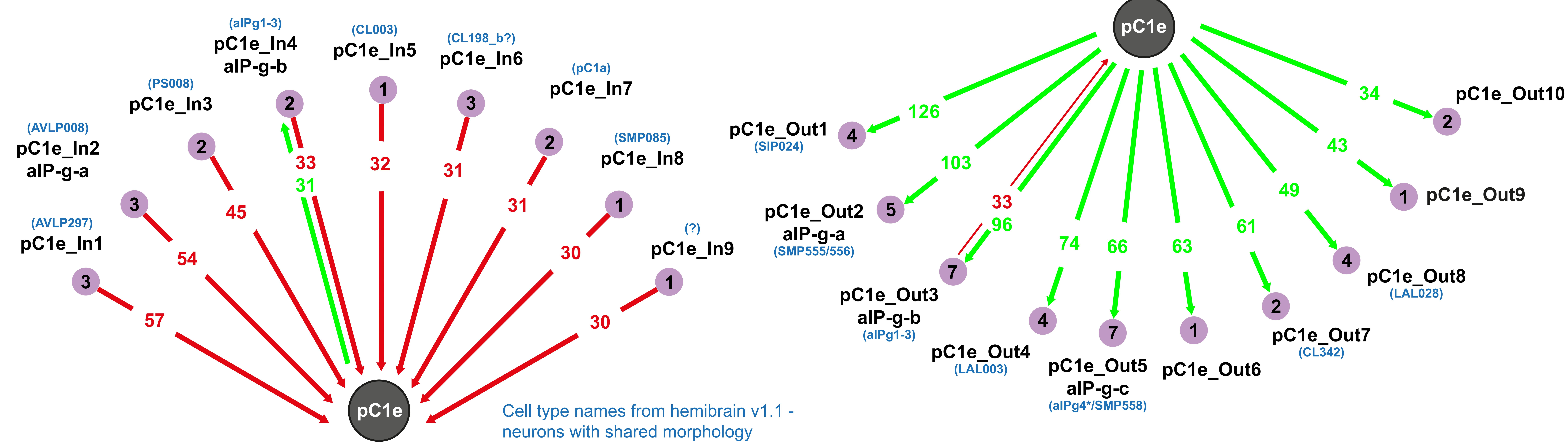
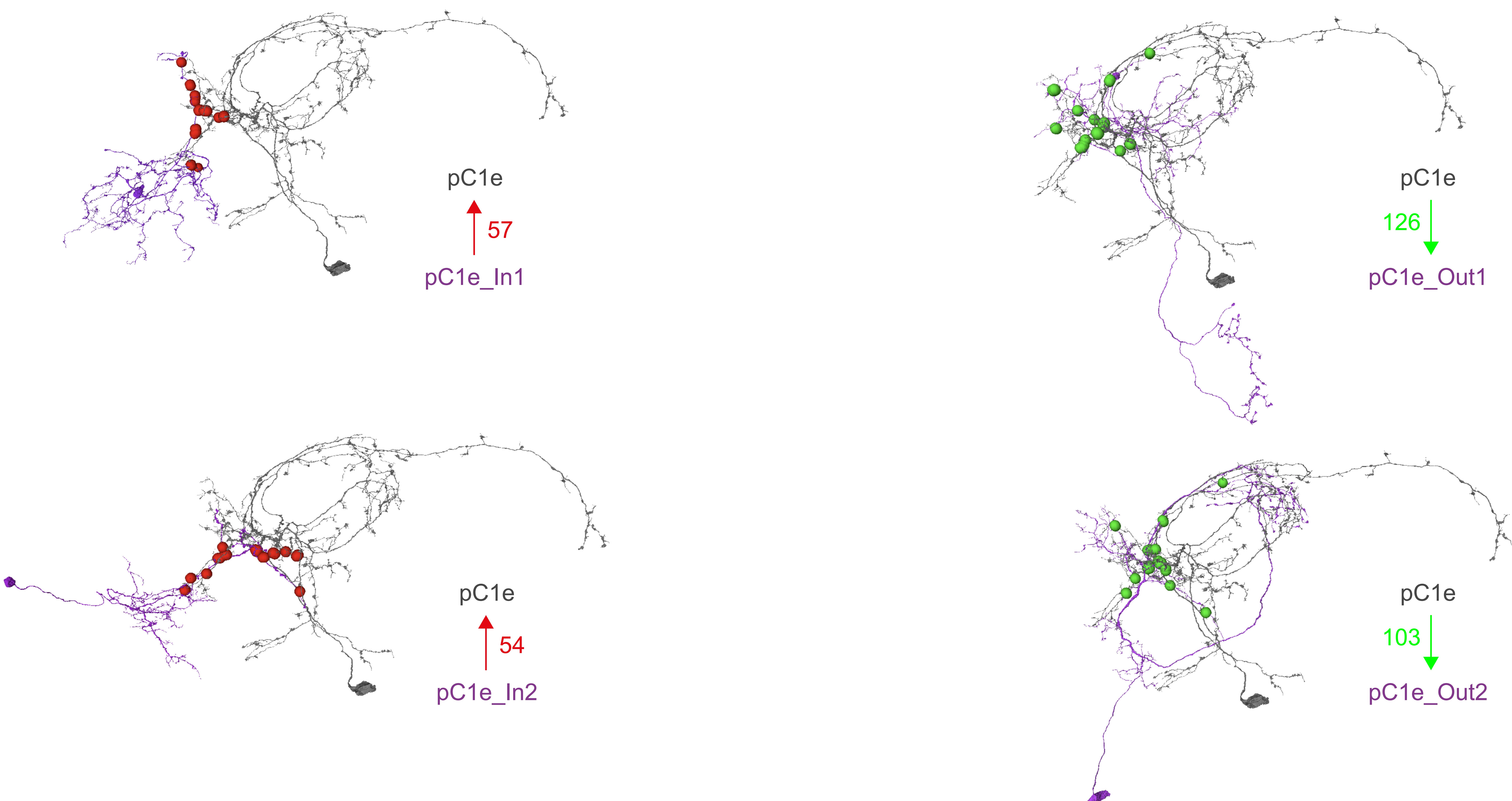
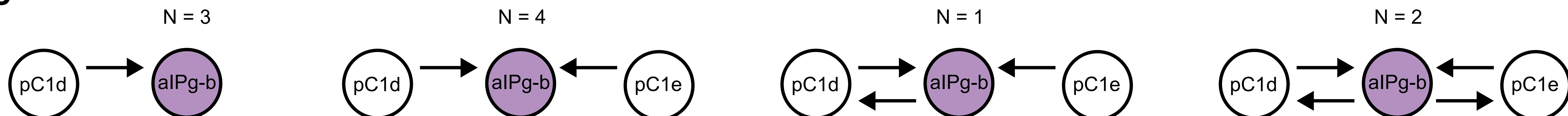
CL278

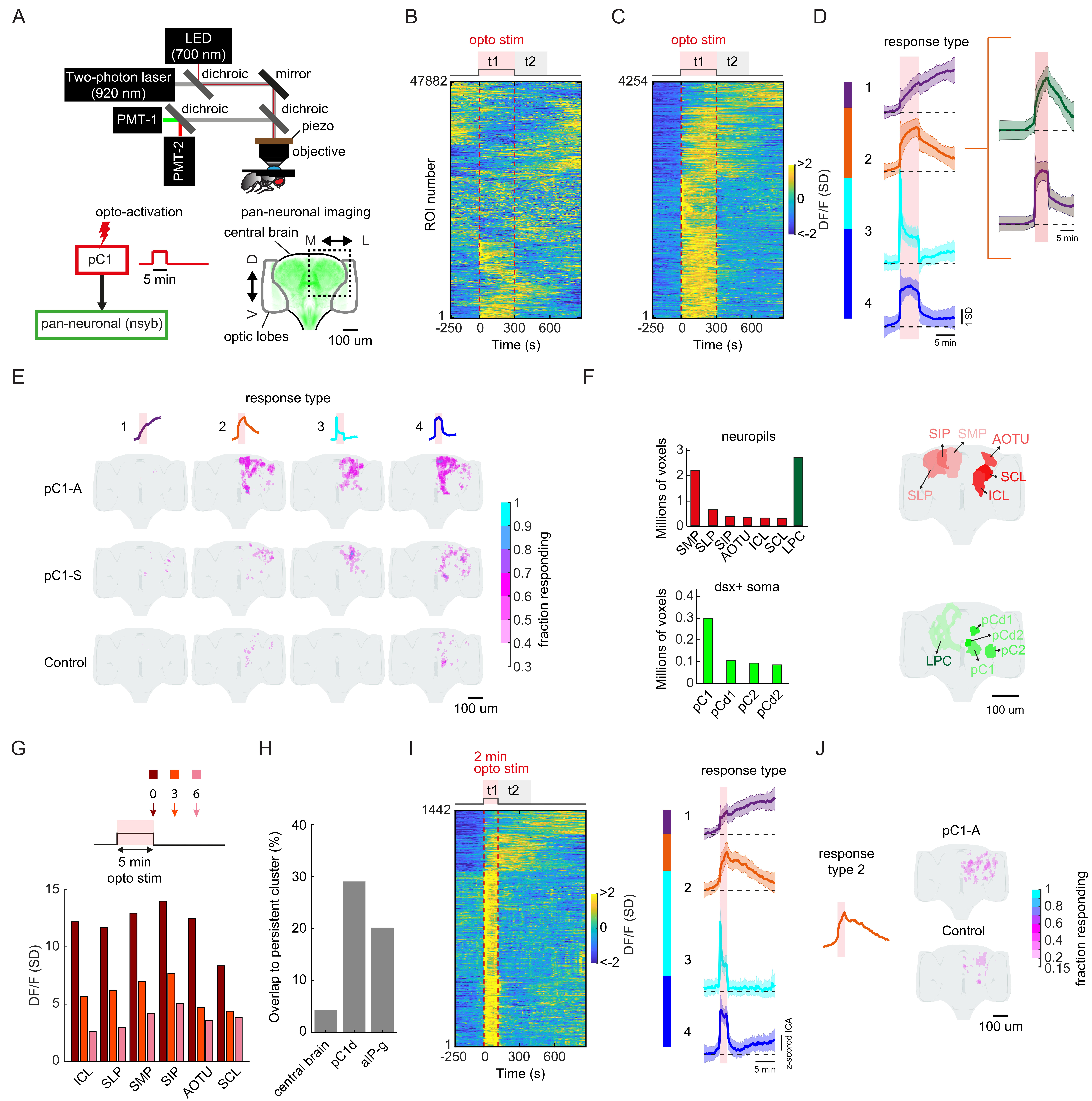


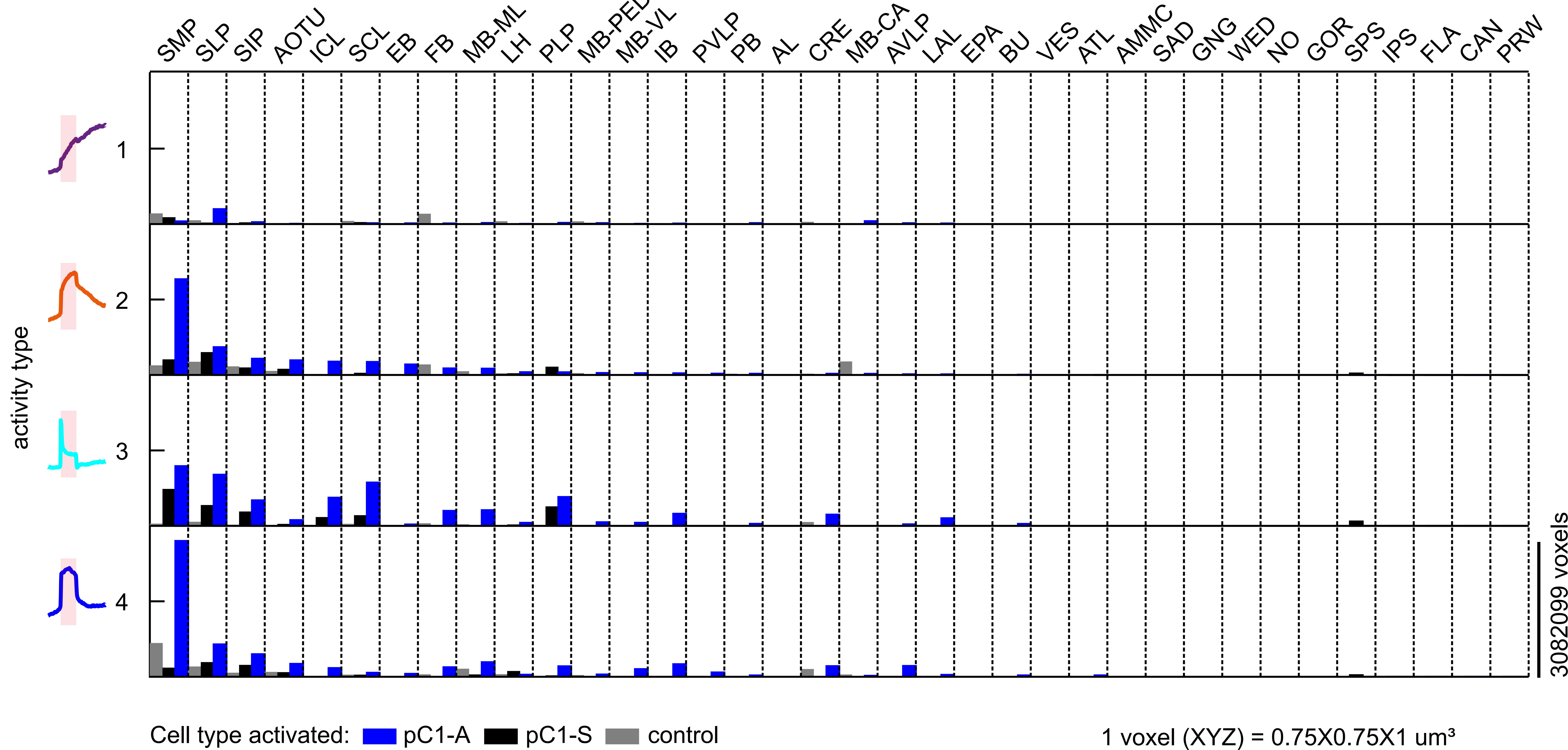
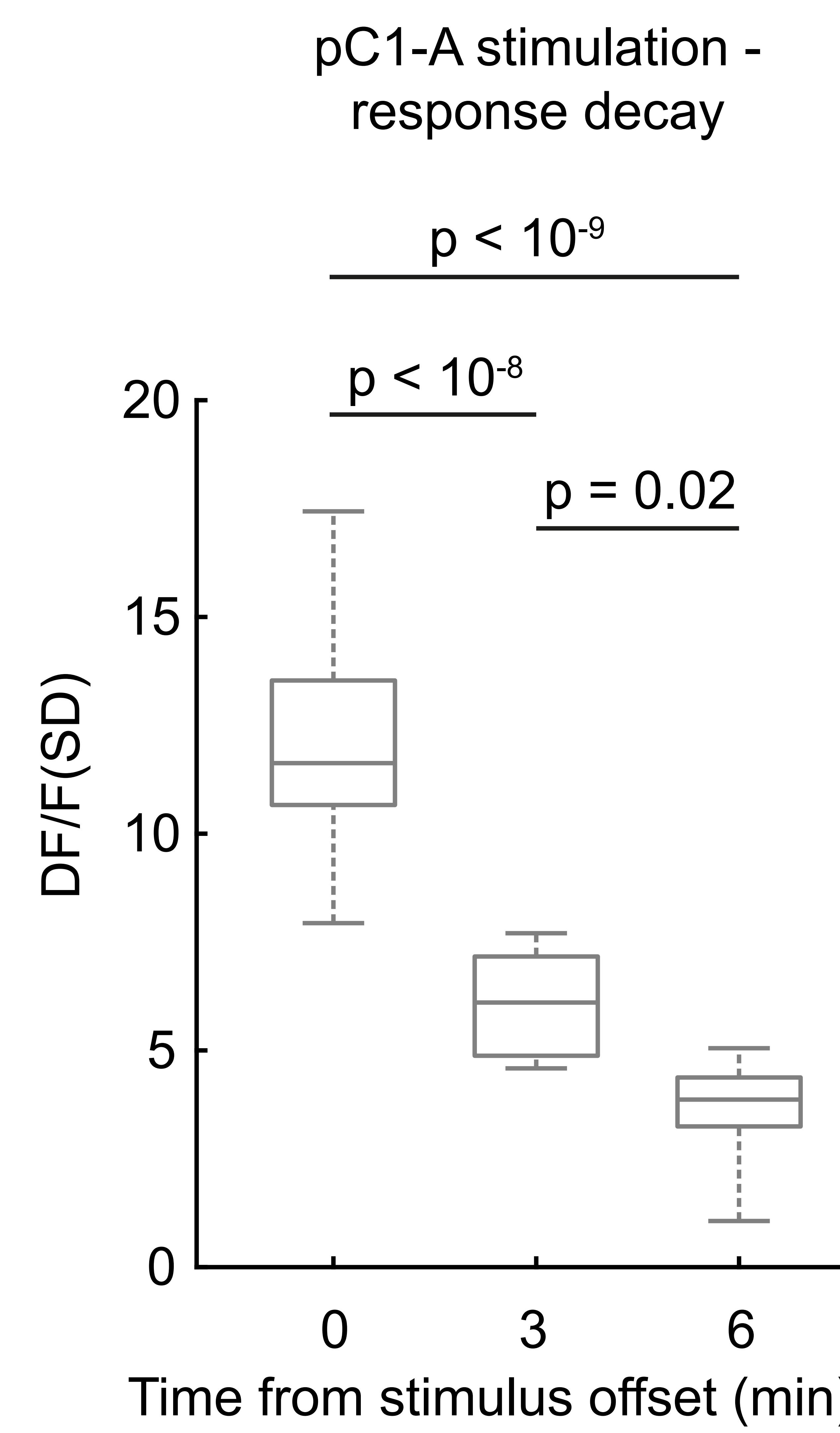
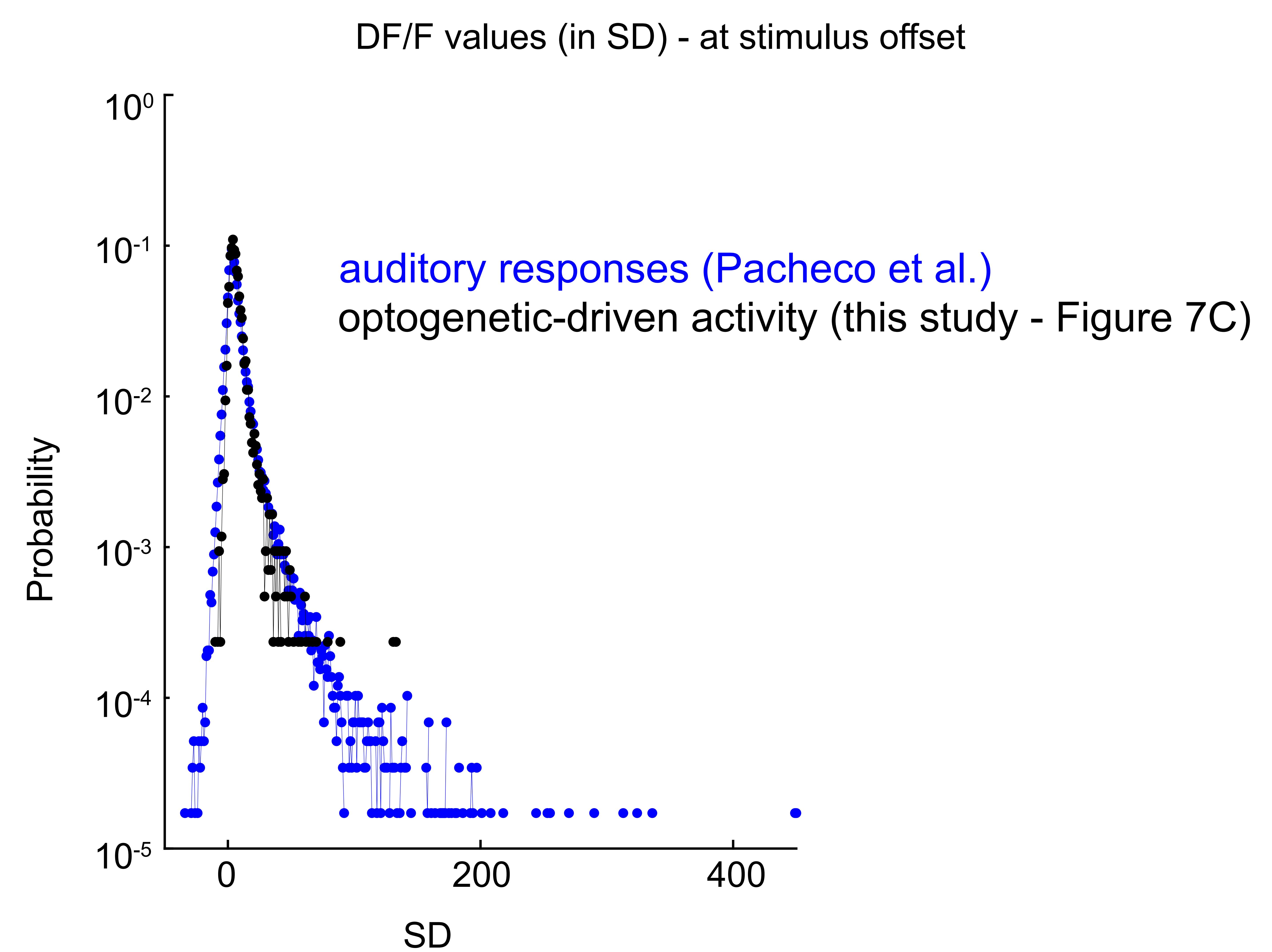
SIP024

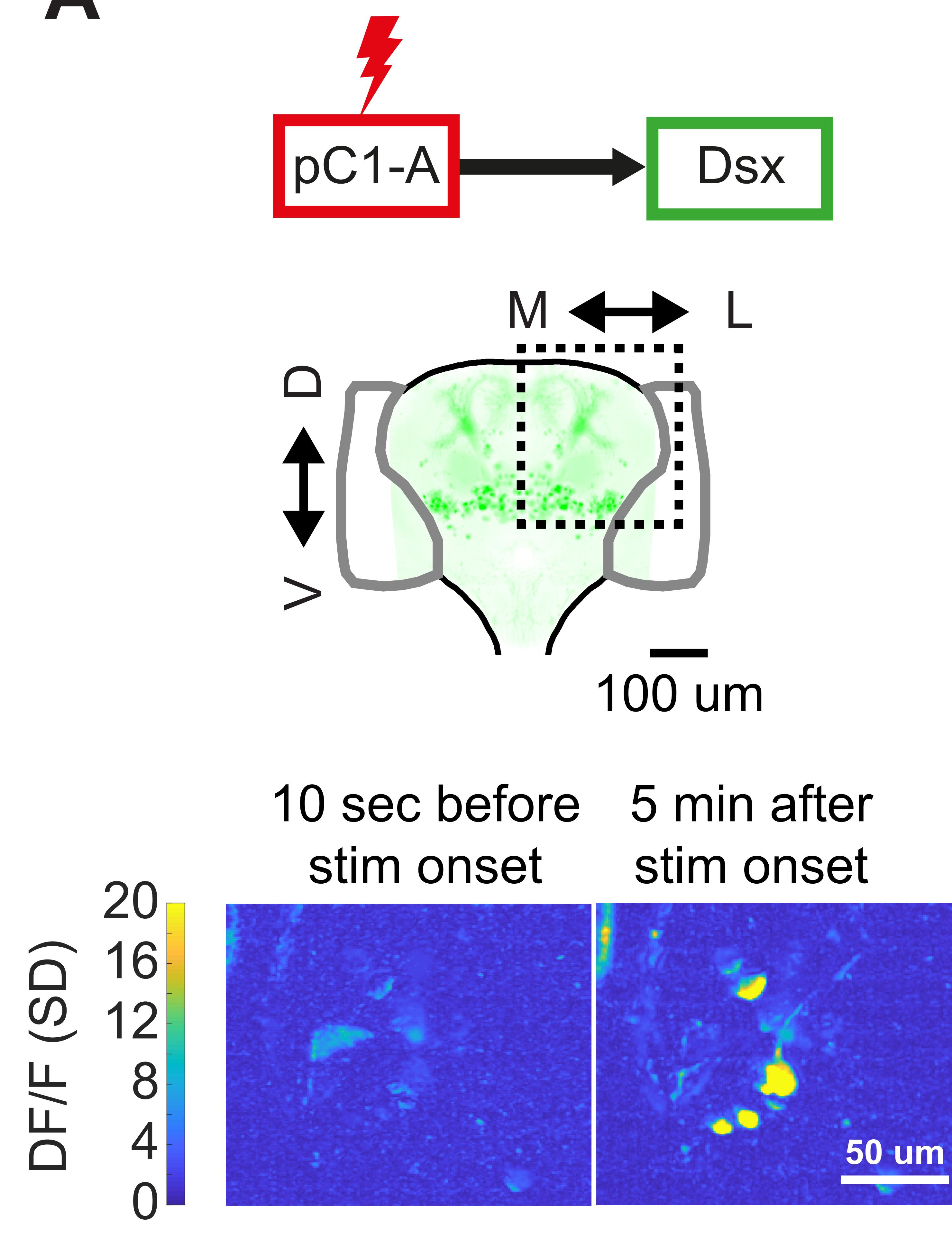
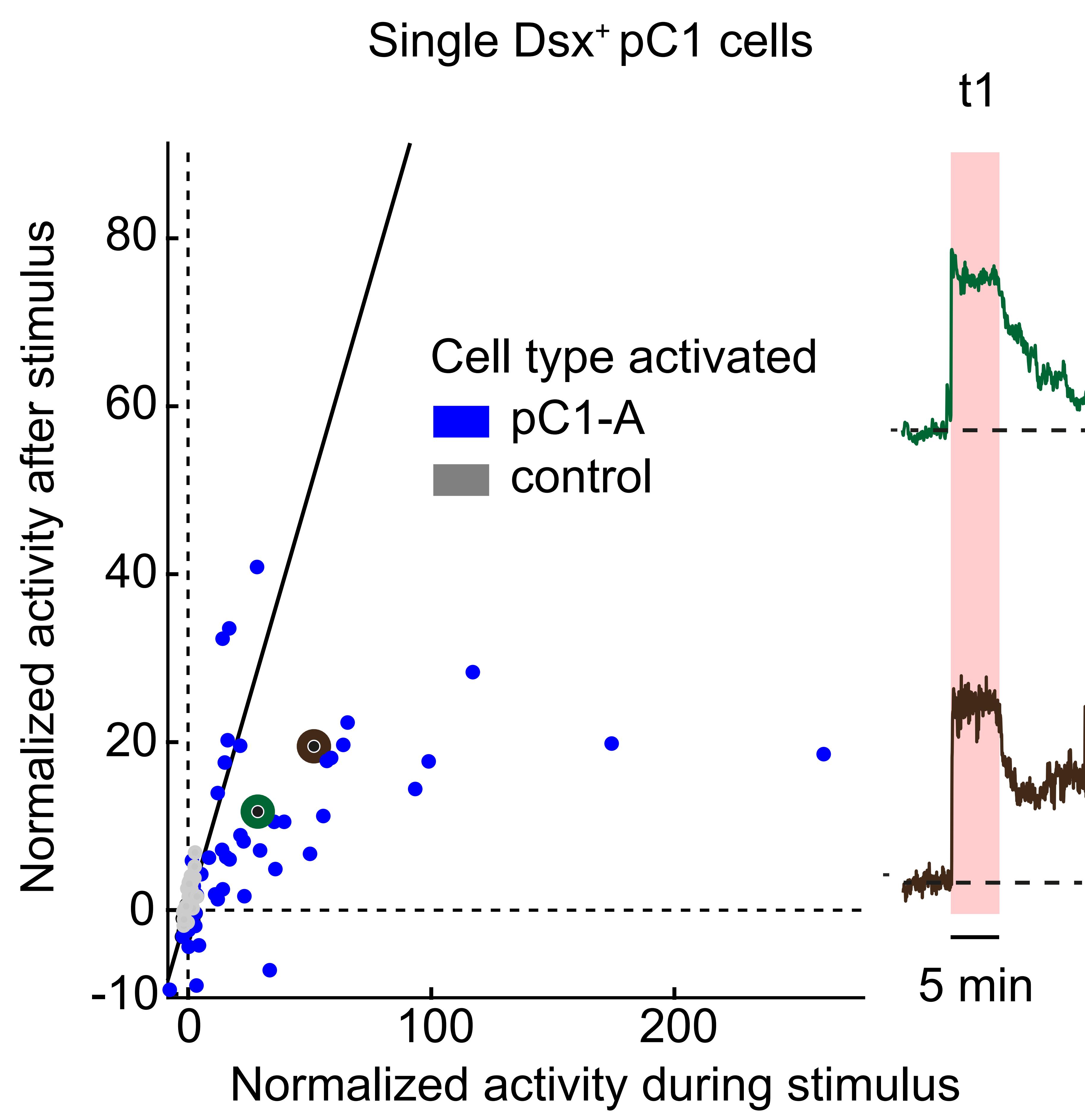
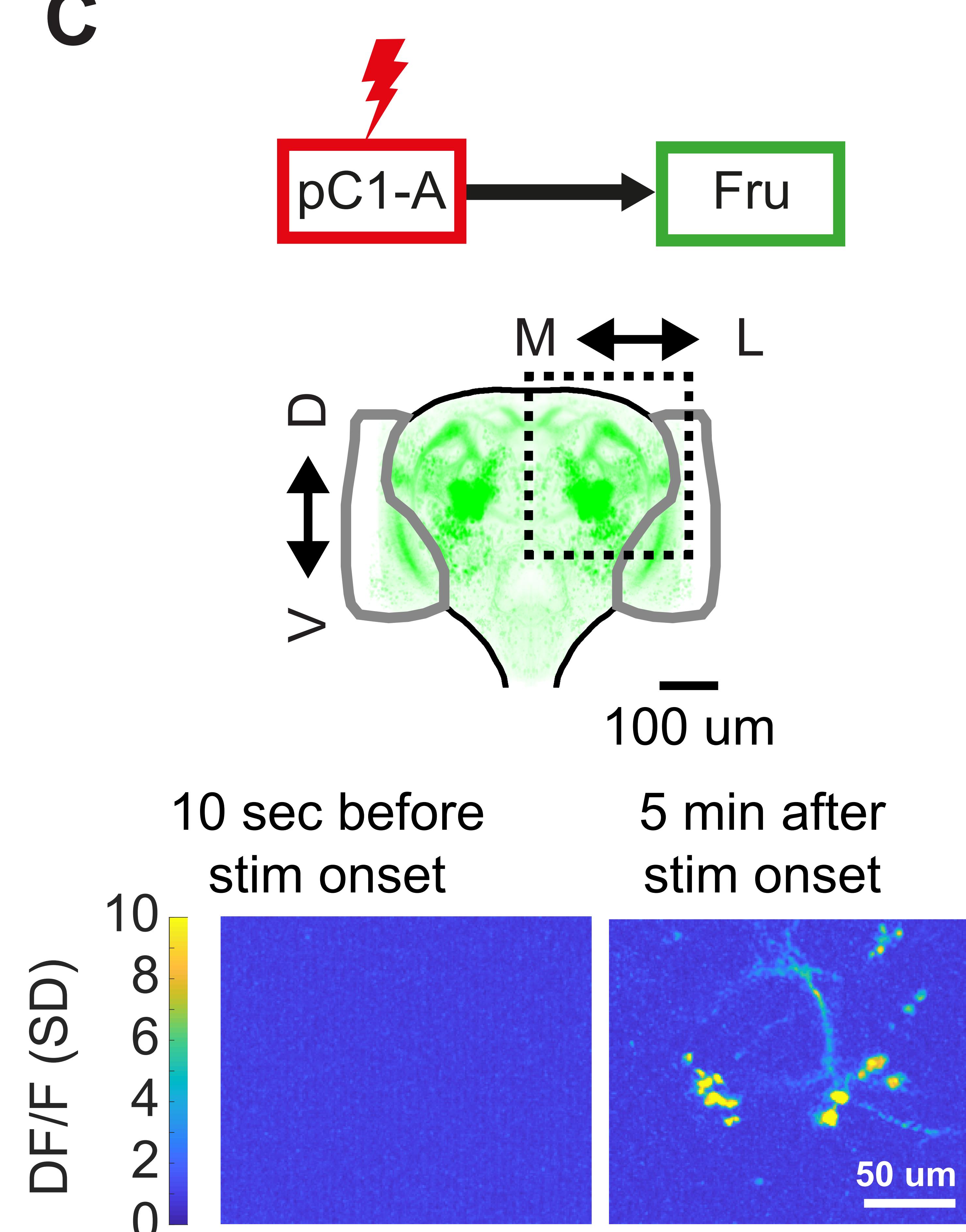
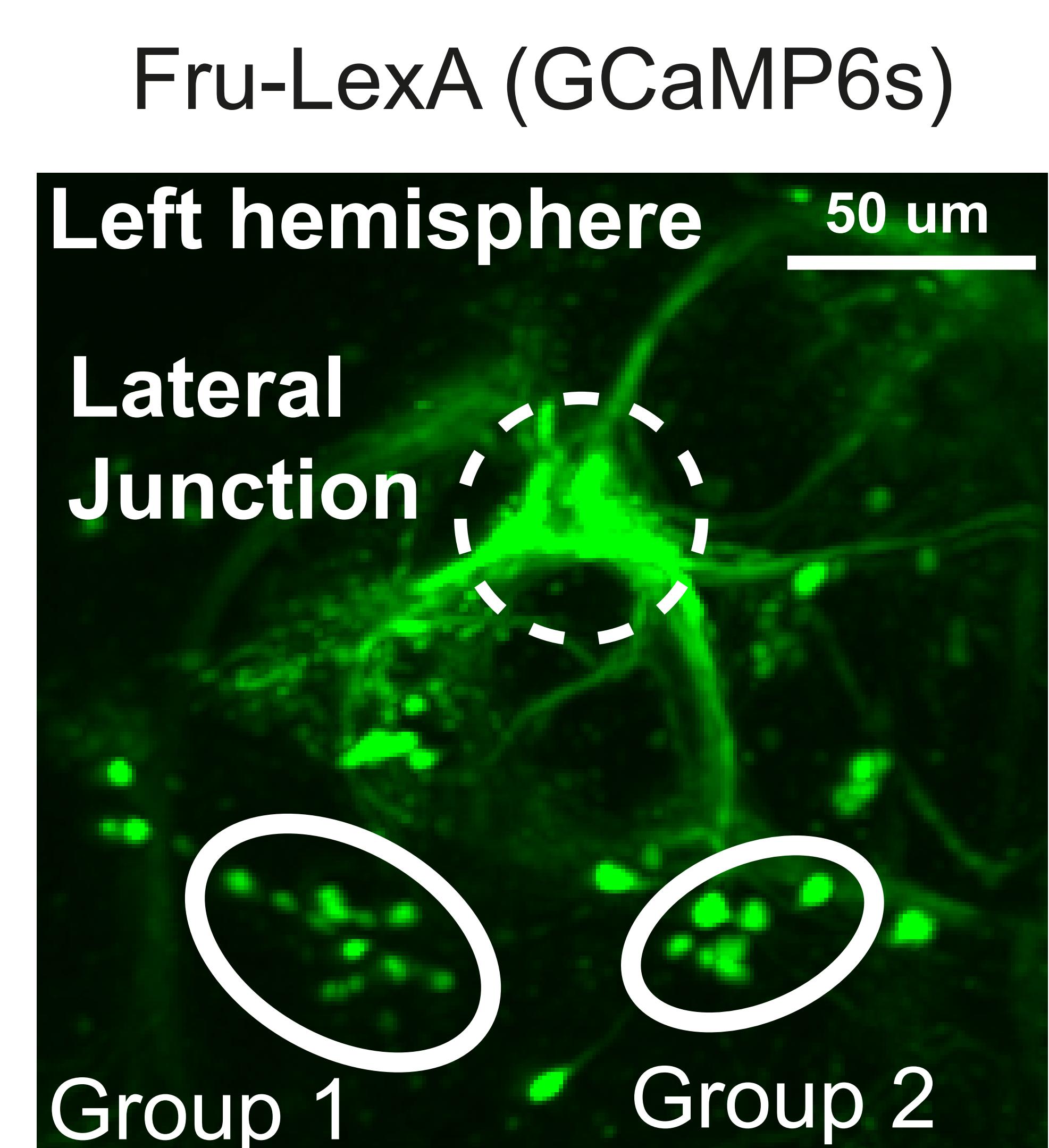
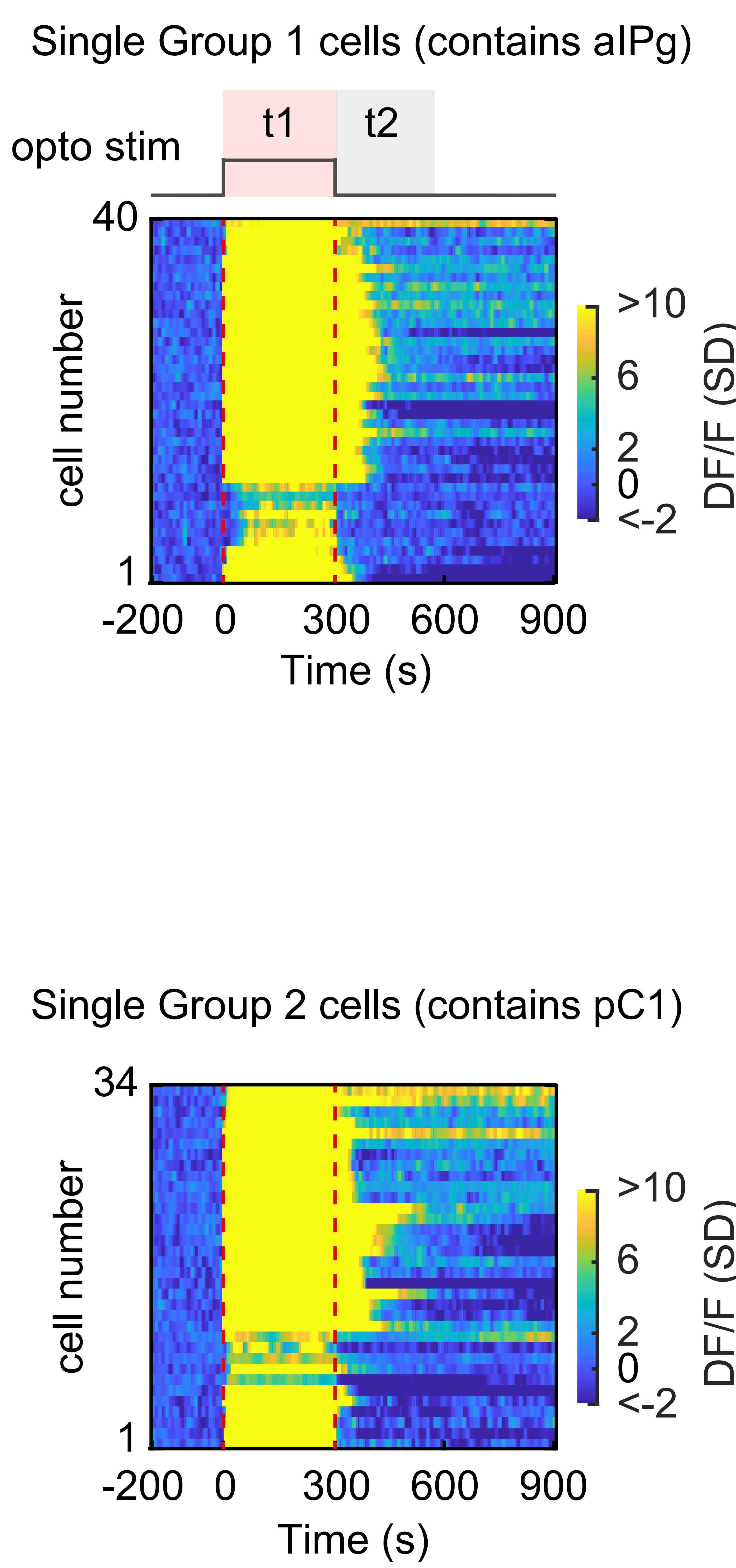
hemibrain v1.1

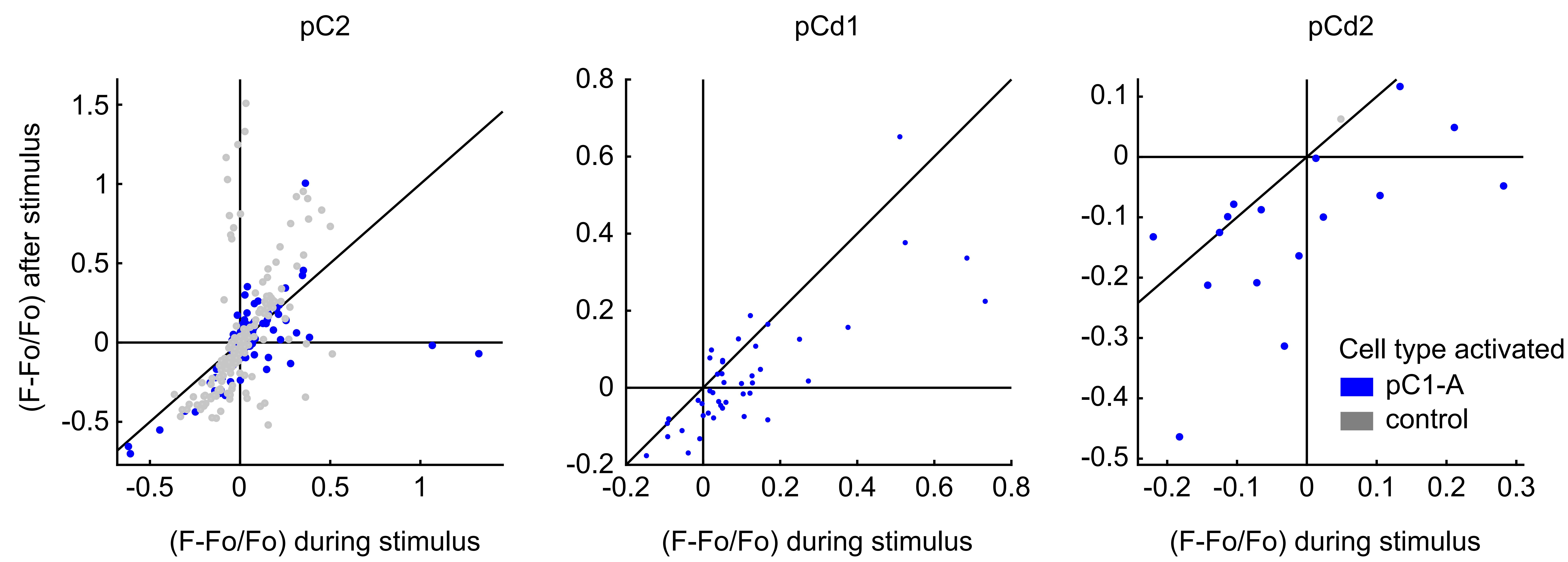
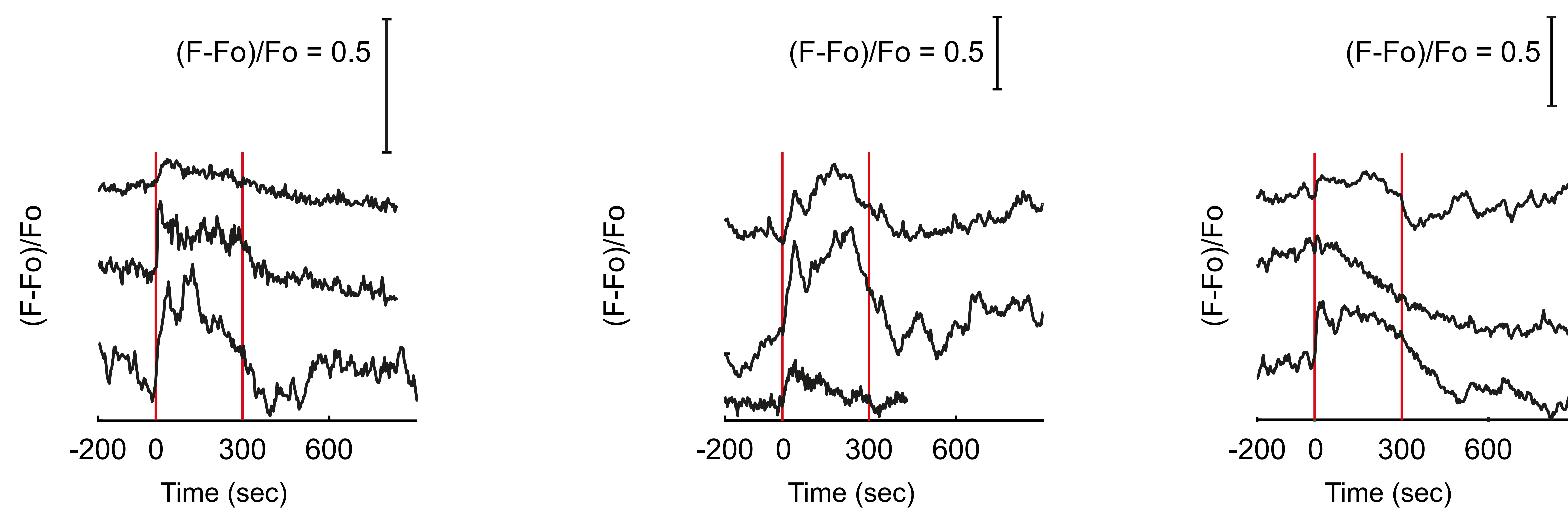


A**pC1e top inputs****pC1e top outputs****B****C**

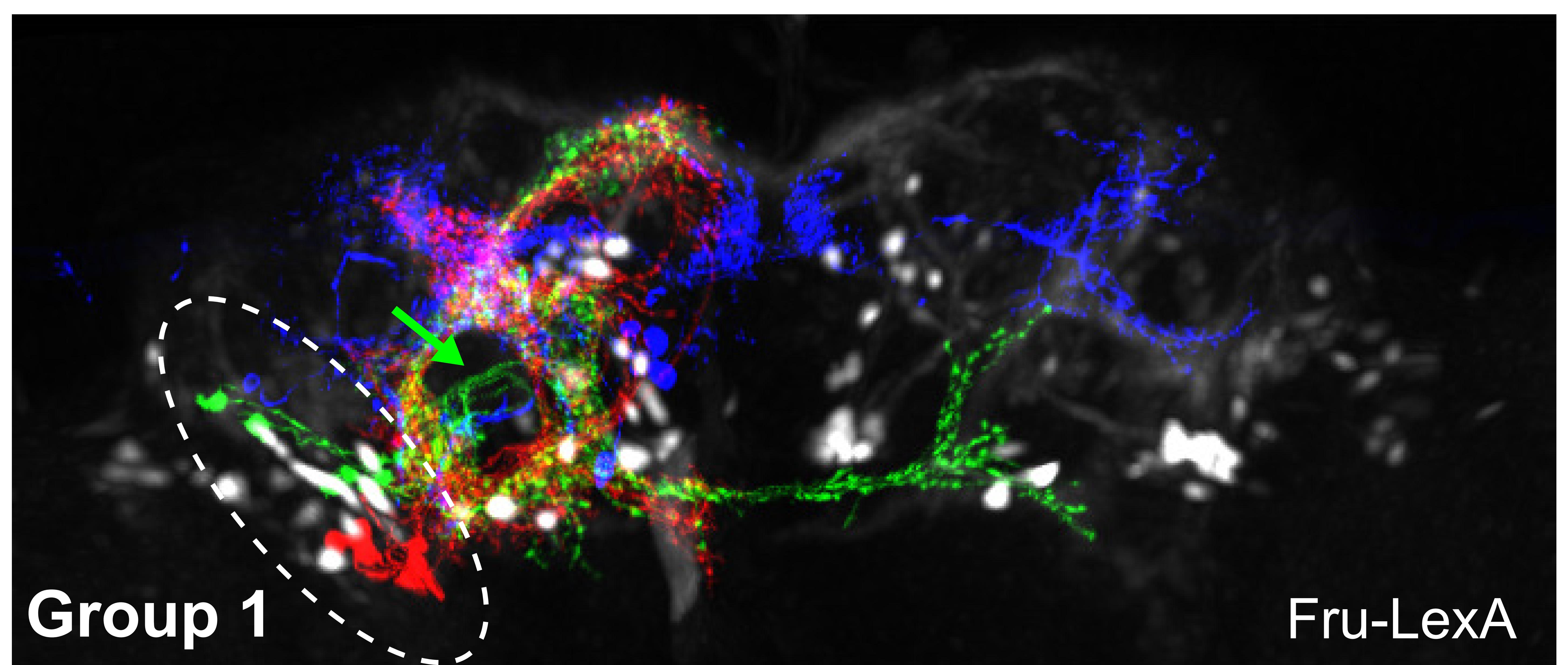


A**B****C**

A**B****C****D**

A**B**

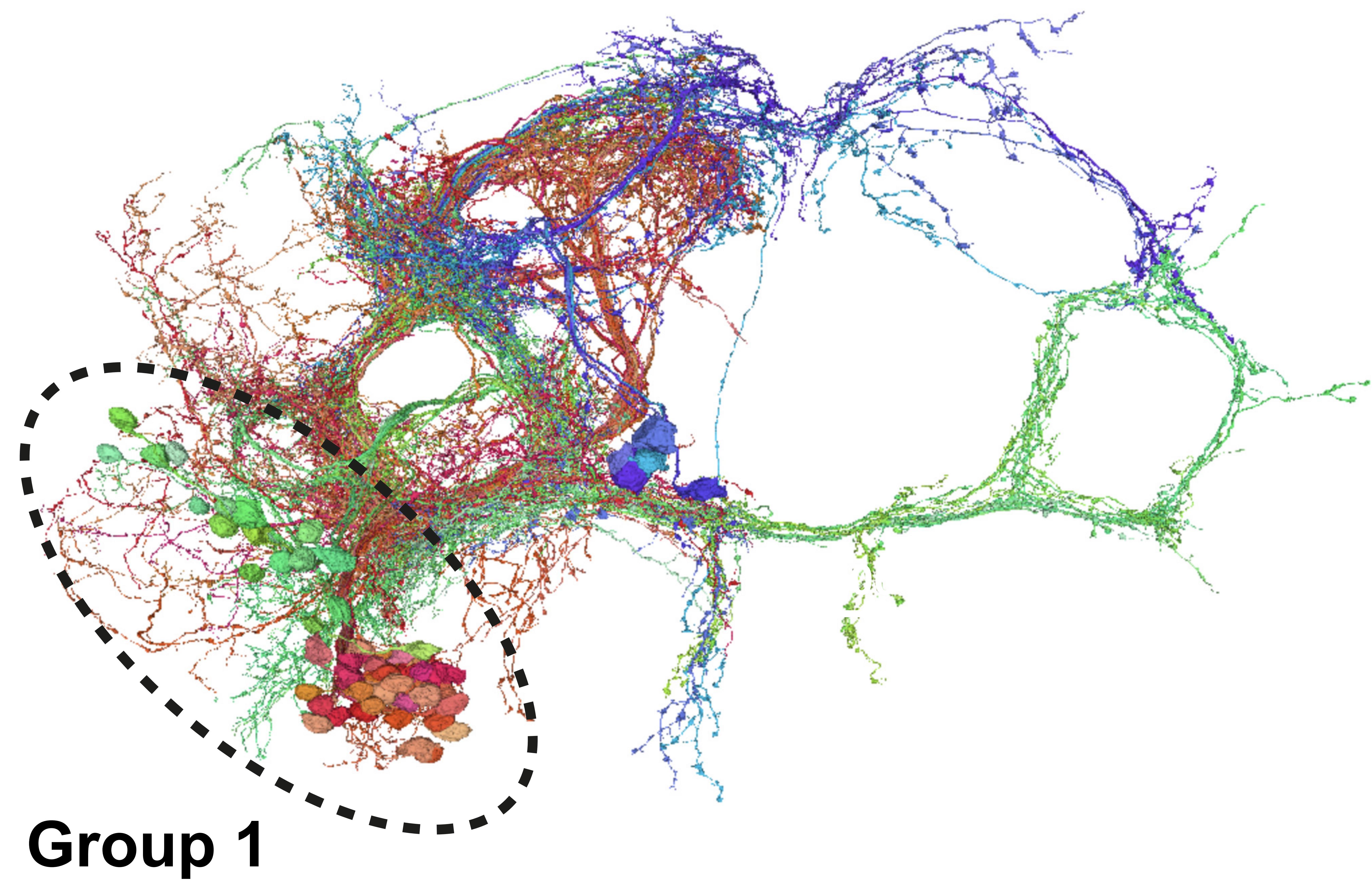
A Fru-LexA (20X) and FlyCircuit co-registered



- FlyCircuit pIP-e Fru+ cells (contains pC2-like cells)
- FlyCircuit aIP-g Fru+ cells (contains aIPg cells)
- FlyCircuit pMP-e Fru+ cells (contains pC1 cells)

B

FlyWire



- pC2/pIP5
- aIPg
- pC1