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Distinct activity-gated pathways mediate attraction and aversion to CO₂ in *Drosophila*

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Supplementary Information

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Statistics and Reproducibility

Here we provide the exact number of trials / trajectories, individuals, and cohorts, for each experiment.

Figure 1	Number of trajectories	Mean trajectory length (sec)	Standard Deviation of trajectory lengths (sec)	Total number of individuals contributing to the experiment	Number of cohorts
Panel c (Clean air)	80930	1.83	2.56	108	9
Panel c (CO ₂)	101539	1.78	2.46	108	9
Panel d (Clean air)	79129	1.65	2.20	72	6
Panel d (Ethanol)	59970	1.80	2.46	72	6
Panel e (approach odor pad; Clean air; 15 scccm)	630			72	6
Panel e (approach odor pad; Clean air; 60 scccm)	1288			108	9
Panel e (approach odor pad; CO ₂ ; 15 scccm)	1021			72	6
Panel e (approach odor pad; CO ₂ ; 60 scccm)	1815			108	9
Panel e (approach odor pad; Clean air; 15 scccm)	229			36	3
Panel e (approach odor pad; Clean air; 60 scccm)	986			72	6
Panel e (approach odor pad; Ethanol; 15 scccm)	341			36	3
Panel e (approach odor pad; Ethanol; 60 scccm)	1720			72	6
Panel e (land; Clean air; 15 scccm)	94			72	6
Panel e (land; Clean air; 60 scccm)	184			108	9
Panel e (land; CO ₂ ; 15 scccm)	390			72	6
Panel e (land; CO ₂ ; 60 scccm)	896			108	9
Panel e (land; Clean air; 15 scccm)	44			36	3
Panel e (land; Clean air; 60 scccm)	153			72	6
Panel e (land; Ethanol; 15 scccm)	228			36	3
Panel e (land; Ethanol; 60 scccm)	1587			72	6
Panel e (approach dark spot; Clean air; 15 scccm)	630			72	6
Panel e (approach dark spot; Clean air; 60 scccm)	1288			108	9
Panel e (approach dark spot; CO ₂ ; 15 scccm)	1021			72	6
Panel e (approach dark spot; CO ₂ ; 60 scccm)	1815			108	9
Panel e (approach dark spot; Clean air; 15 scccm)	229			36	3
Panel e (approach dark spot; Clean air; 60 scccm)	986			72	6
Panel e (approach dark spot; Ethanol; 15 scccm)	341			36	3
Panel e (approach dark spot; Ethanol; 60 scccm)	1720			72	6

Figure 2	Number of trajectories	Total number of individuals contributing to the experiment	Number of cohorts
Panel e (H ₂ O)	183	60	3
Panel e (CO ₂)	125	80	4
Panel e (Ethanol)	171	18	3
Panel e (Ethanol-CO ₂)	121	12	2
Panel e (Vinegar)	193	40	4

Figure 3	Number of trials	Total number of individuals contributing to the experiment	Number of cohorts
Panel b	8	80	8
Panel c	10	100	10
Panel d (all sub panels; speed)	6	60	6
Panel d (all sub panels; afternoon)	42	60	6
Panel d (all sub panels; dusk)	30	60	6
Panel d (all sub panels; night)	48	60	6
Panel d (all sub panels; morning)	24	60	6
Panel e (high flow (HF))	30	60	6
Panel e (HF Arista clip)	30	60	6
Panel e (HF 32° C)	30	60	6
Panel e (EtOH 5%)	30	60	6
Panel f – data listed above			

Figure 4	Number of trials	Total number of individuals contributing to the experiment	Number of cohorts
Panel a	112	100	10
Panel b (IR64a; high activity)	46	100	10
Panel b (IR64a; low activity)	75	100	10
Panel b (Gr63a; high activity)	29	100	10
Panel b (Gr63a; low activity)	88	100	10
Panel b (IR64a, Gr63a; high activity)	68	100	10
Panel b (IR64a, Gr63a; low activity)	65	100	10
Panel c (HCS; high activity)	33	100	10
Panel c (HCS; low activity)	79	100	10
Panel c (IR8a, IR25a, Orco, Gr63a; high activity)	37	100	10
Panel c (IR8a, IR25a, Orco, Gr63a; low activity)	97	100	10
Panel c (Antennaless; high activity)	72	100	10
Panel c (Antennaless; low activity)	55	100	10
Panel c (Orco; high activity)	24	100	10
Panel c (Orco; low activity)	110	100	10
Panel c (IR8a; high activity)	37	100	10
Panel c (IR8a; low activity)	100	100	10
Panel c (IR25a; high activity)	45	100	10
Panel c (IR25a; low activity)	93	100	10
Panel c (IR25a + BAC rescue; high activity)	16	60	6
Panel c (IR25a + BAC rescue; low activity)	68	60	6
Panel c (IR8a, Orco, Gr63a; high activity)	47	100	10
Panel c (IR8a, Orco, Gr63a; low activity)	57	100	10
Panel c (IR40a; high activity)	34	60	6
Panel c (IR40a; low activity)	51	60	6

Figure 4 panel d	HCS	IR64a	Gr63a	IR64a, Gr63a	IR8a, IR25a, Orco, Gr63a	Antennaless	Orco	IR8a	IR25a	IR25a + BAC	IR8a, Orco, Gr63a	IR40a
Attraction N trials	33	46	29	68	37	72	24	37	45	16	47	34
Attraction Index	0.427	0.324	0.531	0.322	0.073	0.056	0.718	0.396	0.031	0.517	0.421	0.528
Attraction K-S test stat	NA	0.126	0.141	0.242	0.747	0.672	0.530	0.140	0.820	0.297	0.180	0.174
Attraction K-S p-value	NA	0.898	0.895	0.126	0	0	0	0.857	0	0.247	0.515	0.650
Aversion N trials	79	75	88	65	97	55	110	100	93	68	57	51
Aversion Index	-0.234	-0.234	-0.010	-0.003	-0.037	-0.034	-0.034	-0.172	-0.195	-0.015	-0.095	-0.191
Aversion K-S test stat	NA	.117	.520	.550	.336	.434	.303	.166	.154	.594	.301	.183
Aversion K-S p-value	NA	.64	0	0	0	0	0	.161	.242	0	.004	.225

Extended Data Figure 1	Number of trials	
Panel a	4	
Panel c (for each condition)	12	The mean and standard deviation of the total captured flies for each trial was 105 ± 59
Panel d (for each condition)	6	
Panel f (for each condition)	3	
Panel g (for each condition)	See panels d, f	

Extended Data Figure 2	Number of trajectories	Total number of individuals contributing to the experiment	Number of cohorts
Panel c (H ₂ O; 15 mL/min)	128	60	3
Panel c (H ₂ O; 60 mL/min)	183	60	3
Panel c (H ₂ O; 200 mL/min)	79	60	3
Panel c (CO ₂ ; 5 mL/min)	195	60	3
Panel c (CO ₂ ; 15 mL/min)	106	60	3
Panel c (CO ₂ ; 60 mL/min)	125	80	4
Panel c (CO ₂ ; 200 mL/min)	48	24	4
Panel c (Ethanol; 15 mL/min)	173	18	3
Panel c (Ethanol; 60 mL/min)	171	18	3
Panel c (Ethanol; 200 mL/min)	47	18	3
Panel c (Vinegar; 15 mL/min)	219	30	3
Panel c (Vinegar; 60 mL/min)	193	30	3
Panel c (Vinegar; 200 mL/min)	248	30	3
Panel d	See Panel c (CO ₂ ; 60 mL/min)		
Panel e	Red line: 30 trials; Magenta: see Panel d		
Panel f	See Panel c (Ethanol; 60 mL/min)		
Panel g	Red line: 30 trials; Black: see Panel f		

Extended Data Figure 4	Number of trials	Total number of individuals contributing to the experiment	Number of cohorts
Panel a (0% high speed)	131	29	29
Panel a (0% low speed)	153	29	29
Panel a (5% high speed)	200	29	29
Panel a (5% low speed)	210	29	29
Panel b, e, f (0%, high speed)	48	180	18
Panel b, e, f (0%, low speed)	170	180	18
Panel b, e, f (1.7%, high speed)	24	120	12
Panel b, e, f (1.7%, low speed)	64	120	12
Panel b, e, f (5%, high speed)	26	120	12
Panel b, e, f (5%, low speed)	70	120	12
Panel b, e, f (15%, high speed)	20	60	6
Panel b, e, f (15%, low speed)	56	60	6
Panel d	100 simulation runs		

Extended Data Figure 6	Number of trials	Total number of individuals contributing to the experiment	Number of cohorts
Panel a	90	100	10
Panel b (IR64a; high activity)	36	100	10
Panel b (IR64a; low activity)	66	100	10
Panel b (Gr63a; high activity)	25	100	10
Panel b (Gr63a; low activity)	81	100	10
Panel b (IR64a, Gr63a; high activity)	44	100	10
Panel b (IR64a, Gr63a; low activity)	44	100	10
Panel c (HCS; high activity)	29	100	10
Panel c (HCS; low activity)	61	100	10
Panel c (IR8a, IR25a, Orco, Gr63a; high activity)	21	100	10
Panel c (IR8a, IR25a, Orco, Gr63a; low activity)	66	100	10
Panel c (Antennaless; high activity)	55	100	10
Panel c (Antennaless; low activity)	41	100	10
Panel c (Orco; high activity)	17	100	10
Panel c (Orco; low activity)	71	100	10
Panel c (IR8a; high activity)	23	100	10
Panel c (IR8a; low activity)	63	100	10
Panel c (IR25a; high activity)	32	100	10
Panel c (IR25a; low activity)	60	100	10
Panel c (IR25a + BAC rescue; high activity)	10	70	7
Panel c (IR25a + BAC rescue; low activity)	47	70	7
Panel c (IR8a, Orco, Gr63a; high activity)	28	100	10
Panel c (IR8a, Orco, Gr63a; low activity)	42	100	10
Panel c (IR40a; high activity)	22	60	6
Panel c (IR40a; low activity)	38	60	6

Extended Data Figure 7	Number of trials	Total number of individuals contributing to the experiment	Number of cohorts
Panel a (IR25a[1]; high activity)	7	60	6
Panel a (IR25a[1]; low activity)	78	60	6
Panel a (IR25a[1] @ 32° C; high activity)	9	20	2
Panel a (IR25a[1] @ 32° C; low activity)	12	20	2
Panel a (IR25a[2]; high activity)	38	80	8
Panel a (IR25a[2]; low activity)	78	80	8
Panel a (IR25a[2] @ 32° C; high activity)	8	20	2
Panel a (IR25a[2] @ 32° C; low activity)	14	20	2
Panel a (IR25a[2] + BAC rescue; high activity)	16	60	6
Panel a (IR25a[2] + BAC rescue; low activity)	68	60	6
Panel a (IR25a[2] + BAC rescue @ 32° C; high activity)	17	40	4
Panel a (IR25a[2] + BAC rescue @ 32° C; low activity)	29	40	4
Panel b (IR25a[1]; high activity)	4	60	6
Panel b (IR25a[1]; low activity)	53	60	6
Panel b (IR25a[1] @ 32° C; high activity)	6	20	2
Panel b (IR25a[1] @ 32° C; low activity)	9	20	2
Panel b (IR25a[2]; high activity)	27	80	8
Panel b (IR25a[2]; low activity)	50	80	8
Panel b (IR25a[2] @ 32° C; high activity)	6	20	2
Panel b (IR25a[2] @ 32° C; low activity)	9	20	2
Panel b (IR25a[2] + BAC rescue; high activity)	10	60	6
Panel b (IR25a[2] + BAC rescue; low activity)	47	60	6
Panel b (IR25a[2] + BAC rescue @ 32° C; high activity)	13	40	4
Panel b (IR25a[2] + BAC rescue @ 32° C; low activity)	15	40	4
Panel c (IR40a; high activity)	34	60	6
Panel c (IR40a; low activity)	51	60	6
Panel c (IR40a @ 32° C; high activity)	15	30	3
Panel c (IR40a @ 32° C; low activity)	20	30	3
Panel d (IR40a; high activity)	22	60	6
Panel d (IR40a; low activity)	38	60	6
Panel d (IR40a @ 32° C; high activity)	10	30	3
Panel d (IR40a @ 32° C; low activity)	11	30	3

Extended Data Figure 8	Number of trials	Total number of individuals contributing to the experiment	Number of cohorts
Panel a	70	50	5
Panel b (HCS)	59	50	5
Panel b (IR8a, IR25a, Orco, Gr63a)	51	50	5
Panel b (Antennaless)	53	50	5
Panel b (Orco)	27	50	5
Panel b (IR8a)	38	50	5
Panel b (IR25a)	58	50	5
Panel b (IR25a + BAC rescue)	19	50	5
Panel b (IR8a, Orco, Gr63a)	64	50	5
Panel c (HCS)	40	50	5
Panel c (IR8a, IR25a, Orco, Gr63a)	32	50	5
Panel c (Antennaless)	37	50	5
Panel c (Orco)	20	50	5
Panel c (IR8a)	27	50	5
Panel c (IR25a)	39	50	5
Panel c (IR25a + BAC rescue)	14	50	5
Panel c (IR8a, Orco, Gr63a)	40	50	5
Panel d	57	40	4
Panel e (HCS)	49	40	4
Panel e (IR8a, IR25a, Orco, Gr63a)	36	40	4
Panel e (Orco)	41	40	4
Panel e (IR8a)	37	40	4
Panel e (IR25a)	36	40	4
Panel e (IR8a, Orco, Gr63a)	20	40	4
Panel f (HCS)	34	40	4
Panel f (IR8a, IR25a, Orco, Gr63a)	25	40	4
Panel f (Orco)	30	40	4
Panel f (IR8a)	26	40	4
Panel f (IR25a)	22	40	4
Panel f (IR8a, Orco, Gr63a)	15	40	4

Effects of CO₂ concentration

The overall effect of concentration on the flies' behavior is complex, and it deserves some explanation. The time course of preference indices shown in Extended Data Fig. 4b suggests that flies find a stimulus of 5% briefly attractive, whereas lower concentrations appear to have an overall neutral effect, followed by aversion after the end of the CO₂ stimulus. This is, however, likely an artifact of the convolution of the stochastic nature of free behavior and the analysis we chose.

To show that flies are indeed attracted to the low (1.7%) concentration of CO₂, we used a different analysis. In the new analysis, we counted the number of times that flies approach the CO₂ source over the course of the 10-minute trial and found that compared to a 0% CO₂ control stimulus, flies are significantly more likely to approach 1.7%, 5%, and 15% CO₂ (Extended Data Fig. 4e). Next, we looked at the time course of this approach behavior (Extended Data Fig. 4f), which suggests that in the presence of a 5% or 15% CO₂ stimulus, most of the flies respond to the CO₂ within the first 5 minutes, and subsequently ignore (or avoid) the CO₂. In the 1.7% concentration case, however, the approaches are more evenly distributed between the first and second 5-minute epochs of the 10-minute stimulus.

To explain the complex dynamics of the approach behavior under the different CO₂ concentrations, we made a very simple agent-based model. In our model we assume that the probability that a fly detects CO₂ is proportional to the concentration (because high concentrations of CO₂ will diffuse more effectively across

the arena). Next, we assume that flies will spend less time near very high concentrations of CO₂ (which we observed in the wind tunnel experiments, see Extended Data Fig. 2). Specifically, we assume that both 1.7% and 5% concentrations of CO₂ cause flies to spend 2 minutes searching near CO₂, whereas 15% results in 0.5 minutes of exploration (these values are roughly based on Extended Data Fig. 2). Finally, based on the long-term aversion seen throughout our experiments, we assume that after a fly has explored a CO₂ source, it will find the source aversive for (at least) 30 minutes (even after the stimulus has disappeared). Below we outline our model in pseudo-code; we have provided a jupyter notebook of our simulation on our github repository here: https://github.com/florisvb/drosophila_co2_attraction/blob/master/napproaches_notebook/model.ipynb

Although this model is extraordinarily simple, it is able to capture the qualitative dynamics of the real flies' behavior. The key insight the model offers is that even if 1.7% and 5% trigger similar types of behavior (i.e. 2 minutes of CO₂ exploration), the combination of a concentration dependent likelihood of detecting the odor, and the long-term aversion, results in a lower apparent attraction.

Supplementary Videos

V1 – *Drosophila* find CO₂ aversive during periods of low activity. Flies' responses to the onset of CO₂ (red) during an afternoon trial in our walking arena, corresponding to data shown in Fig. 3d (5% CO₂, 24 hrs starved). Playback is approximately 10x real time. This video is a representative example of the 42 trials.

V2 – *Drosophila* find CO₂ attractive during periods of high activity. Flies' responses to the onset of CO₂ (red) during a dusk trial in our walking arena, corresponding to data shown in Fig. 3d (5% CO₂, 24 hrs starved). Same group of flies as video 1. Playback is approximately 10x real time. This video is a representative example of the 30 trials.

V3 – *Drosophila's* response to CO₂ during high activity is qualitatively similar to their response towards ethanol. Flies' responses to the onset of ethanol (red) during a dusk trial in our walking arena, corresponding to data shown in Fig. 3d (5% Ethanol, 24 hrs starved). Playback is approximately 10x real time. This video is a representative example of the 30 trials.