Cell Reports, Volume *31*

Supplemental Information

Heterogeneous Habenular Neuronal Ensembles during Selection of Defensive Behaviors

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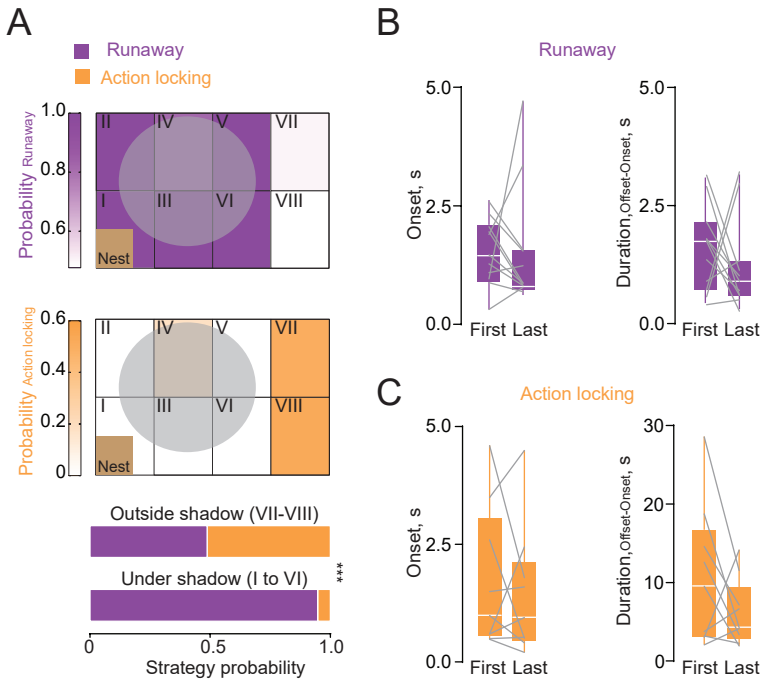

Figure S1. Shadow projection modulate runaway and action locking responses that are stable in time, Related to Figure 1.
(A) Top: Arena representation divided in zones (from I to VIII) reporting the max loom shadow projection and the probability (heat-plots) for runaway (R) and action locking (AL) responses plotted in each zone (n_{mice}=11; $n_{runaway}$= 56; Zone = trials; I = 2, II = 5, III = 5, IV = 9, V = 8, VI = 7, VII = 10, VIII = 10. $n_{action\ locking}$= 23; I = 0, II = 0, III = 0, IV = 2, V = 0, VI = 0, VII = 11, VIII = 10). Bottom. Bar graph reporting the probability of R and AL respect to the max looming shadow projection (R vs AL, Outside shadow, zone VII and VIII: 20 vs 21; Under shadow, zone I to VI: 36 vs 2; $X^2_1 = 20.18$; ***p<0.0001, Chi Square test).
(B) Reaction onset (n_{mice}= 11, first vs last trial; 1.52 ± 0.22 s vs 1.56 ± 0.43 s; t_{10}=0.094, p=0.93, paired t-test) and duration (first vs last trial; 1.58 ± 0.27 s vs 1.2 ± 0.29 s; t_{10}= 0.794, p=0.44, paired t-test) for the first and last Runaway response for each mouse.
(C) Same as (B) but for Action-locking trials (n_{mice}=9, first vs last trial; Onset: 1.71 ± 0.50 s vs 1.44 ± 0.46 s; t_8= 0.561, p=0.59, paired t-test. Duration: 10.72 ± 2.98 s vs 6.2 ± 1.4 s; t_8=1.50, p=0.17, paired t-test).

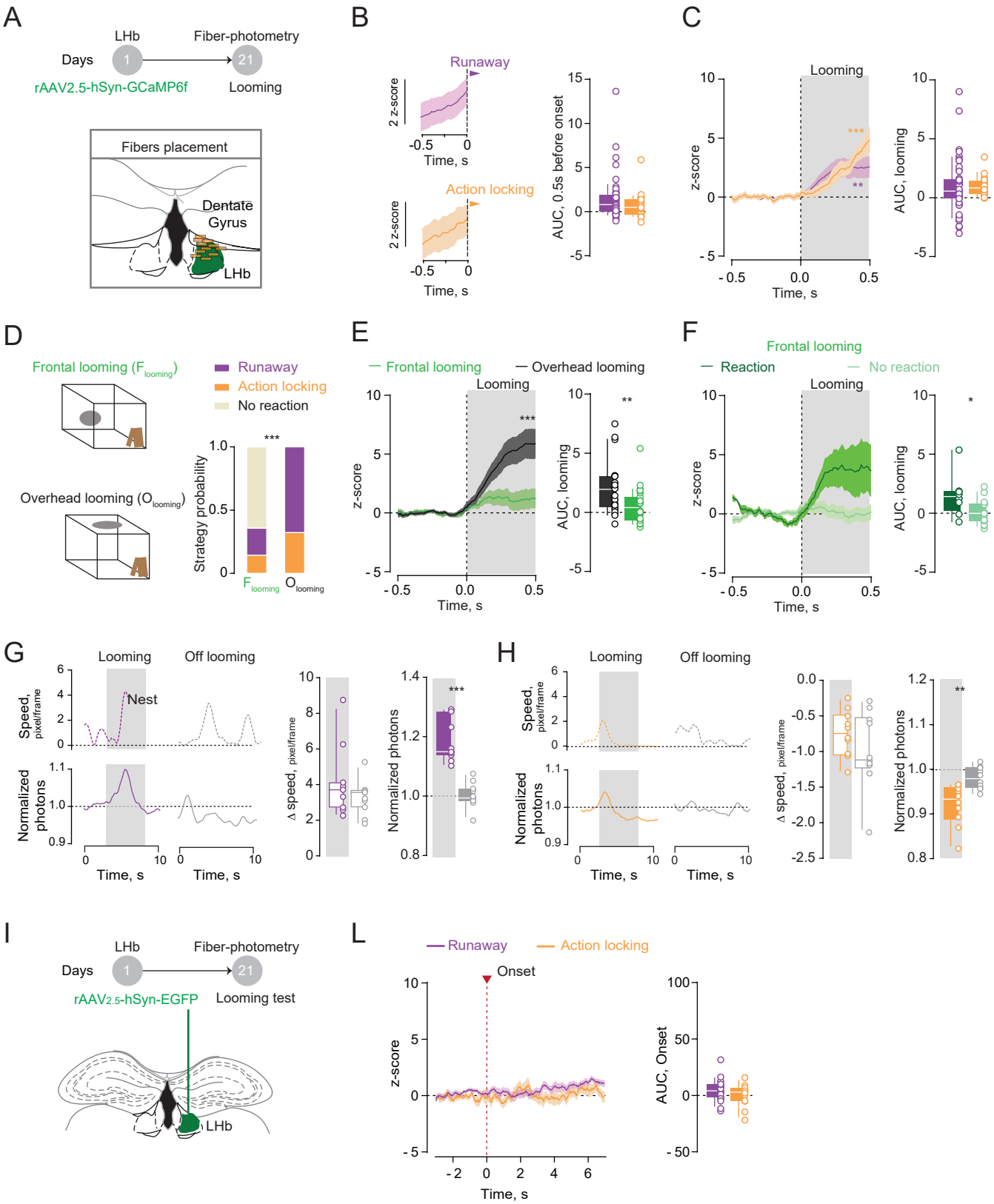
A
LHb
Fiber-photometry
Days
1
21
rAAV2.5-hSyn-GCaMP6f
Looming
Fibers placement
Dentate Gyrus
LHb
B
Runaway
2 z-score
-0.5
0
Time, s
Action locking
AUC, 0.5s before onset
C
Looming
z-score
***
**
AUC, looming
D
Frontal looming (F looming)
Overhead looming (O looming)
Runaway
Action locking
No reaction
Strategy probability
E
Frontal looming
Overhead looming
F
Reaction
No reaction
G
Looming
Off looming
Nest
Speed, pixel/frame
Normalized photons
Δ speed, pixel/frame
H
I
rAAV2.5-hSyn-EGFP
Looming test
L
Onset
AUC, Onset

Figure S2. Looming-driven Ca2+ dynamics within the LHb are threat dependent but independent of locomotion, Related to Figure 2.

(A) Schematic of fiber placement in the LHb (brown rectangles represent fiber tip placement)

(B) Averaged traces and boxplots for Runaway (R, 56 trials) and Action locking (AL, 23 trials) reporting the AUC 0.5 s before the behavioral onset (R vs AL, 1.45 ± 0.31 vs 0.75 ± 0.30, t_{77}= 1.348, p= 0.182 Unpaired t-test)

(C) z-scored traces and area under curve (AUC) showing the LHb activity time-locked with the looming onset for Runaway (51 trials, F_{2550}= 5.79; **p=0.008, RM One way ANOVA) and Action locking trials (18 trials, F_{867}= 10.12; ***p=0.0003, RM One way ANOVA). Boxplots reported the AUC for the same set of data (R vs AL, 0.94 ± 0.30 vs 0.97± 0.20; t_{67}= 0.066, p=0.94 Unpaired t-test).

(D) Left. Representative drawings illustrating the arena setting for the frontal looming (top, $F_{looming}$) and the classical overhead looming (bottom, $O_{looming}$). Right. Bar graph reporting the probability of Runaway (R), Action locking (AL) or no reaction (NR) in response to the frontal or overhead looming (n_{mice}= 5; $F_{looming}$: $n_{runaway}$= 6, $n_{action\ locking}$= 4, $n_{no\ reaction}$= 18. $O_{looming}$: $n_{runaway}$= 19, $n_{action\ locking}$= 9, $n_{no\ reaction}$= 0. X^2_2= 26.68; ***p<0.0001, Chi Square test).

(E) z-scored traces and AUC showing the LHb activity time-locked with the looming for the $F_{looming}$ (26 trials, F_{1250}= 0.671; p=0.502, RM One way ANOVA) and the $O_{looming}$ (25 trials, F_{2250}= 10.15; ***p=0.0005, RM One way ANOVA). Boxplots reported the AUC for the same set of data ($F_{looming}$ vs $O_{looming}$, 0.53 ± 0.27 vs 1.97± 0.42; t_{49}= 2.87, **p=0.0061 Unpaired t-test).

(F) Same as (E) but only for the $F_{looming}$ trials divided in trials triggering a reaction (R or AL) and trials were the mouse do not react (Reaction vs No reaction, 8 vs 18 trials; 1.49 ± 0.64 vs 0.10 ± 0.23; t_{24}= 2.54, *p=0.017, Unpaired t-test)

Note that for this analysis trials displaying a behavioral onset < 0.5 sec were discarded to avoid behavior-dependent signal contamination.

(G) Representative traces and boxplots reporting increase in speed (Looming on vs Looming off; 11 vs 11; 4.1 ± 0.56 vs 3.3 ± 0.28 pixel/frame; t_{20}=1.28, p=0.21 Unpaired t-test) and the relative LHb activity (Looming on vs Looming off; 11 vs 11, 1.19±0.02 vs 1.00 ± 0.012 normalized photon; t_{20} = 7.35, ***p<0.0001 Unpaired t-test) in presence or absence of the looming stimulus.

(H) Same as (G) but for decrease in speed (Looming on vs Looming off; 10 vs 10; -0.7558 ± 0.10 vs -0.9987 ± 0.176 pixel/frame; t_{18}=1.190, p=0.249 Unpaired t-test) and relative LHb photon change (Looming on vs Looming off; 10 vs 10, 0.9204±0.014 vs 0.9807±0.007 normalized photons; t_{18}=3.61, **p=0.002 Unpaired t-test).

For this comparison, we selected the first runaway and action locking response for each mouse. Then we looked for a single episode outside looming presentation with a comparable change in speed for the same mouse. Note that one mouse did not display any action locking response throughout the recording session.

(I) Schematic of the experiment and representative brain coronal section showing eGFP injections in the LHb (n_{mice}= 5).

(L) Left: z-score time-course graph showing averaged traces of Runaway (R, n_{trials}= 21, F_{1400}=2, p=0.074 RM One way ANOVA) and Action-locking (AL, n_{trials}= 12, F_{770}=3.347, p=0.269 RM One way ANOVA) trials time-locked with the behavioral onset. Right: area under curve (AUC) for the same data set (R vs AL, 4.87 ± 2.12 vs 0.191 ± 2.96 t31=1.303, p=0.202, Unpaired t-test).

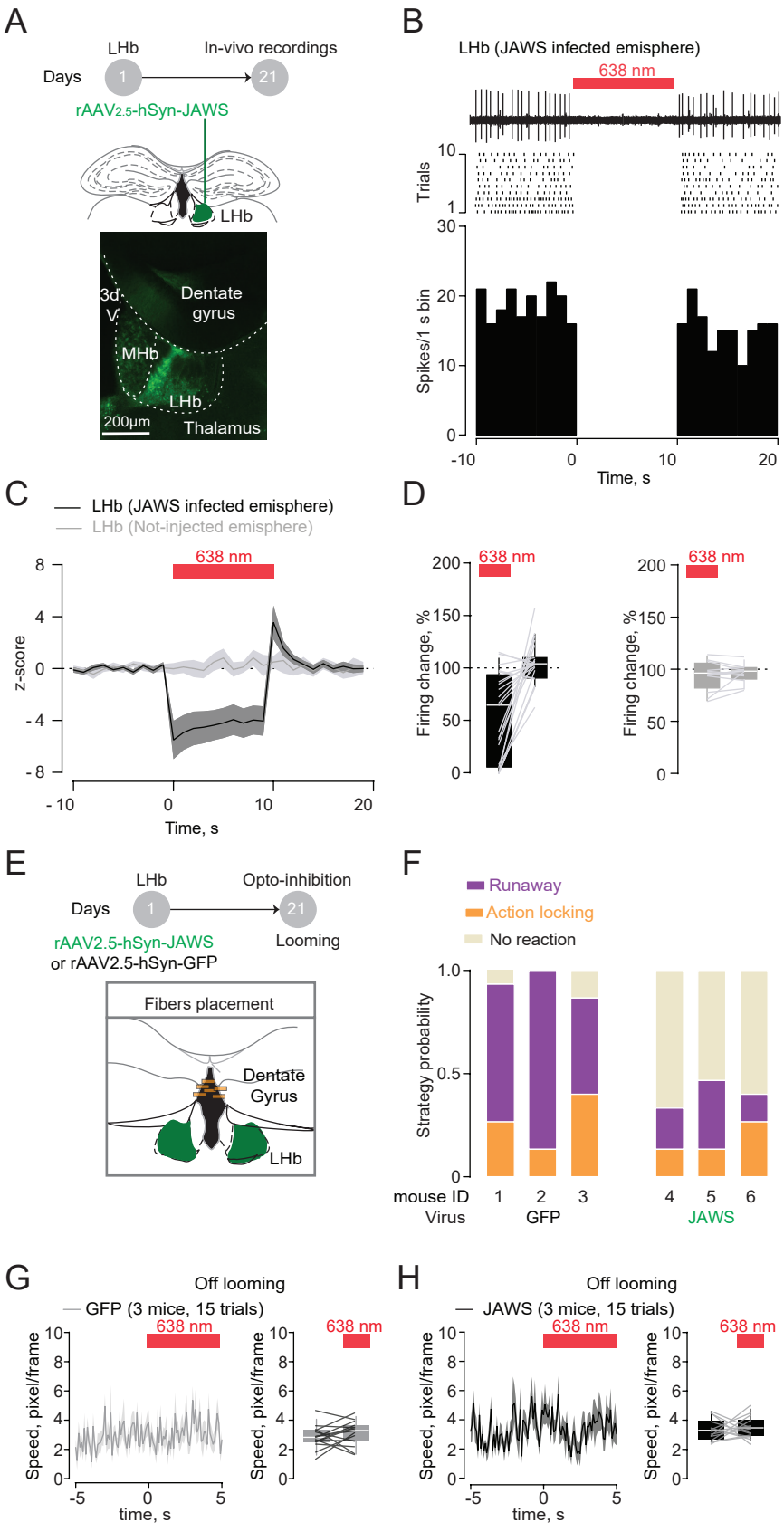
A
LHb
In-vivo recordings
Days
1
21
rAAV2.5-hSyn-JAWS
LHb
3d V.
Dentate gyrus
MHb
LHb
200µm
Thalamus
B
LHb (JAWS infected emisphere)
638 nm
Trials
Spikes/1 s bin
Time, s
C
LHb (JAWS infected emisphere)
LHb (Not-injected emisphere)
638 nm
z-score
Time, s
D
638 nm
Firing change, %
638 nm
Firing change, %
E
LHb
Opto-inhibition
Days
1
21
rAAV2.5-hSyn-JAWS
or rAAV2.5-hSyn-GFP
Looming
Fibers placement
Dentate Gyrus
LHb
F
Runaway
Action locking
No reaction
Strategy probability
mouse ID
Virus
GFP
JAWS
G
Off looming
GFP (3 mice, 15 trials)
638 nm
Speed, pixel/frame
time, s
638 nm
Speed, pixel/frame
H
Off looming
JAWS (3 mice, 15 trials)
638 nm
Speed, pixel/frame
time, s
638 nm
Speed, pixel/frame

Figure S3. LHb silencing during looming-driven defensive responses, Related to Figure 2.
(A) Schematic of the experiment and representative brain coronal section showing JAWS transduction unilaterally in the LHb.
(B) Sample trace, raster plots and peri-stimulus time histogram of a representative LHb neuron in response to 10 trials of 10s continuous red light (638 nm, 8mW).
(C) Averaged z-scored traces (n_{mice}= 3) showing the response to the light activation in the JAWS-infected (23 cells) and not-infected (12 cells) LHb hemisphere (F_{29}= 5.61, ***p<0.0001, Two Way ANOVA RM).
(D) Bar graph and lines reporting the single cell % of change in Firing rate upon the light activation compared to baseline firing (baseline vs light on vs after. Infected hemisphere: 4.51 ± 0.88 Hz vs 3.2 ± 0.93 Hz vs 4.72 ± 0.94 Hz; Bas vs light on: q_{22}= 2.98, *p=0.013; Not-infected hemisphere: 6.12 ± 1.84 Hz vs 6.16 ± 1.89 Hz vs 6.09 ± 1.83 Hz; Bas vs light on: q_{11}= 0.23, p= 0.95; Dunnett's multiple comparisons test).
(E) Schematic of fiber placement in the LHb (brown rectangles represent fiber tip placement).
(F) Bar graph reporting the probability of Runaway (R), Action locking (AL) or no reaction (NR) in response to the looming paired with the light for GFP- (n_{mice}= 3) or JAWS- (n_{mice}= 3) injected mice (GFP_{mouse1}: $n_{runaway}$= 10, $n_{action\ locking}$= 4, $n_{no\ reaction}$= 1. GFP_{mouse2}: $n_{runaway}$= 13, $n_{action\ locking}$= 2, $n_{no\ reaction}$= 0. GFP_{mouse3}: $n_{runaway}$= 7, $n_{action\ locking}$= 6, $n_{no\ reaction}$= 2. X^2_4= 5.8; p= 0.214. $JAWS_{mouse1}$: $n_{runaway}$= 3, $n_{action\ locking}$= 2, $n_{no\ reaction}$= 10. $JAWS_{mouse2}$: $n_{runaway}$= 5, $n_{action\ locking}$= 2, $n_{no\ reaction}$= 8. $JAWS_{mouse3}$: $n_{runaway}$= 2, $n_{action\ locking}$= 4, $n_{no\ reaction}$= 9; X^2_4= 2.622; p=0.623, Chi Square test).
(G) Time-course graph and boxplots depicting the effect of 5s 638nm light activation on the speed of GFP LHb-injected mice (n_{mice} = 3; n_{trials}= 15) during spontaneous locomotion (off looming periods; Baseline (-5s to 0s) vs Light on (0s to 5s): 2.82 ± 0.2 vs 3.09 ± 0.21 pixel/frame; t_{14}= 1.05, p= 0.31, paired t-test).
(H) Same as (G) but for JAWS LHb-injected mice (n_{mice}= 3, n_{trials}= 15; Baseline (-5s to 0s) vs Light on (0s to 5s): 3.29 ± 0.19 vs 3.44 ± 0.19 pixel/frame; t_{14}= 0.45, p= 0.65, paired t-test).

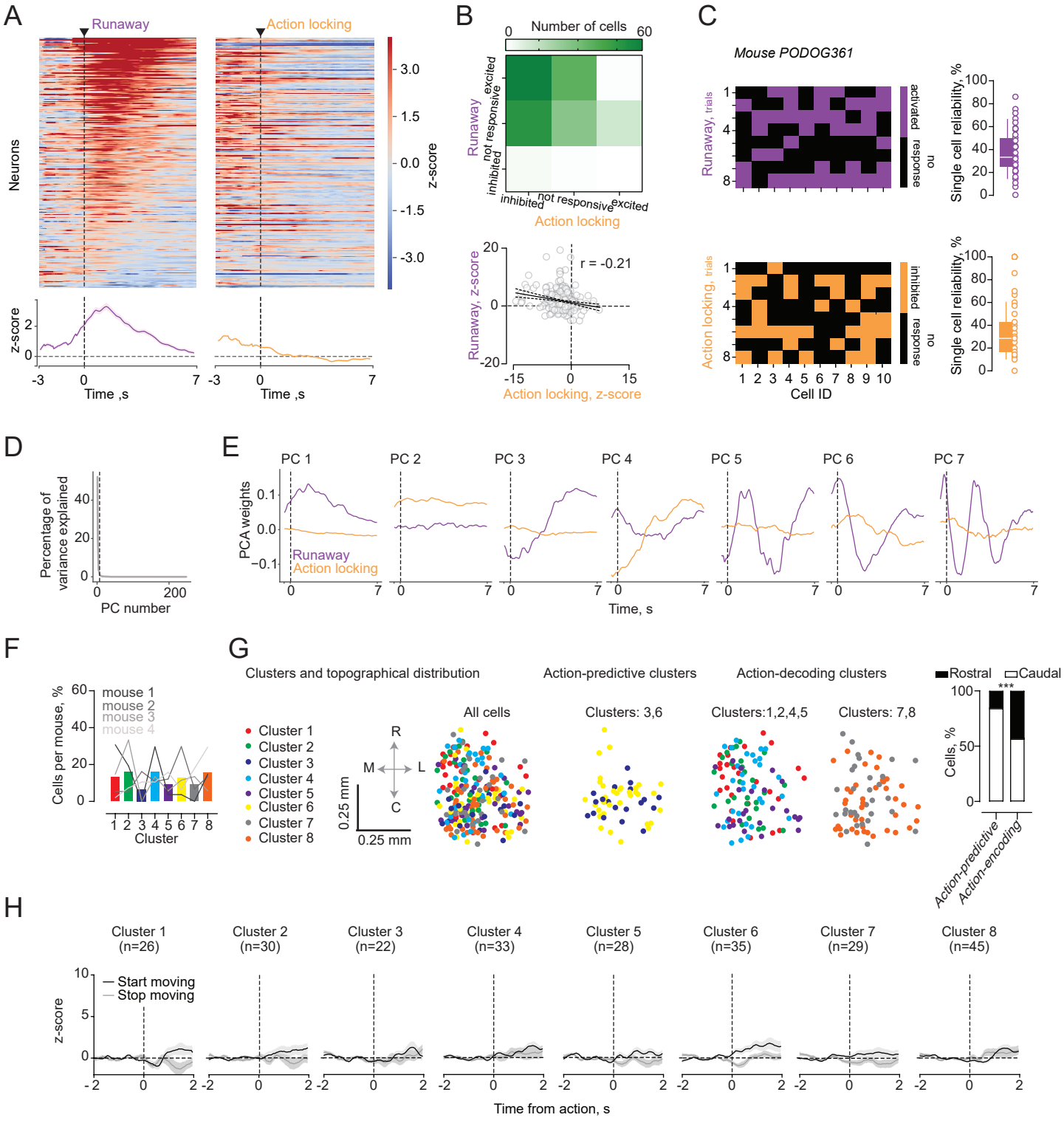
A
Runaway
Action locking
Neurons
z-score
3.0
1.5
0.0
-1.5
-3.0
Time ,s
B
Number of cells
0
60
Runaway
excited
not responsive
inhibited
Action locking
r = -0.21
Runaway, z-score
Action locking, z-score
C
Mouse PODOG361
Runaway, trials
activated
no response
Single cell reliability, %
Action locking, trials
inhibited
Cell ID
D
Percentage of variance explained
PC number
E
PC 1
PC 2
PC 3
PC 4
PC 5
PC 6
PC 7
PCA weights
Runaway
Action locking
Time, s
F
Cells per mouse, %
mouse 1
mouse 2
mouse 3
mouse 4
Cluster
G
Clusters and topographical distribution
Action-predictive clusters
Action-decoding clusters
Rostral
Caudal
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
R
M
L
C
0.25 mm
All cells
Clusters: 3,6
Clusters:1,2,4,5
Clusters: 7,8
Cells, %
***
Action-predictive
Action-encoding
H
Cluster 1 (n=26)
Cluster 2 (n=30)
Cluster 3 (n=22)
Cluster 4 (n=33)
Cluster 5 (n=28)
Cluster 6 (n=35)
Cluster 7 (n=29)
Cluster 8 (n=45)
Start moving
Stop moving
z-score
Time from action, s

Figure S4. Single-cell LHb dynamics, cluster detection, topography and their relationship with spontaneous locomotion, Related to Figure 3 and 4.

(A) Mean Ca2+ responses for runaway (left) and action-locking (right) trials time-locked with the behavioral onset, including all cells recorded in 4 mice (n=248). Cells are sorted for response magnitude in runaway trials.
On the bottom, runaway- and action-locking-locked averaged signals. Data are reported as z-score.

(B) On the top, heat-map showing the cell distribution in the different categories according to their response to runaway and action-locking (Runaway/Action-locking: excited/inhibited=73, excited/non responsive=47, excited/excited=4, non responsive/inhibited=62, non responsive/non responsive=35, non responsive/excited=16, inhibited/inhibited=6, inhibited/non responsive=4, inhibited/excited=3). On the bottom, correlation analysis of single cell average Ca2+ responses (z-score) to runaway vs action locking displaying variability (Runaway vs Action-locking; ncells=248, r=-0.208; R^2 =0.043; ***p=<0.0001, Pearson correlation coefficient).

(C) Top: Raster plots showing active (purple squares) and non-active cells (black squares), imaged over different runaway trials in a single mouse. On the right, the boxplot reports single cell reliability (%) for runaway responses (n_{cells}=248, Runaway, 38.01 ± 1.3 %). Bottom, same mouse as top. Raster plots showing cells inhibited (orange squares) or not (black squares), imaged over different action-locking trials. On the right, the boxplot show reliability for single cells in percentage for action-locking responses (n_{cells}= 248, Action-locking, 32.71± 1.37%).

(D) Plot of the percentage variance explained per principal component, showing the number of principal components retained (dashed line).

(E) Individual retained principal components, showing response vectors to both runaway and action-locking trials.

(F) The graph reports the percentage of cells in each cluster (Cluster 1 to 8, number of cells per cluster: 26, 30, 22, 33, 28, 35, 29, 45) and single-mouse contribution per cluster (Cluster 1 to 8, number of cells per cluster. Mouse 1: 16, 10, 0, 7, 3, 6, 7, 3. Mouse 2: 4, 4, 12, 8, 9, 22, 5, 10. Mouse 3: 4, 9, 0, 8, 1, 1, 0, 4. Mouse 4: 2, 7, 10, 10, 15, 6, 17, 28).

(G) Topographical distribution of the clusters in LHb (action-predictive vs action-decoding clusters; rostral vs caudal cell distribution; Action-predictive: 57 cells, 9 rostral vs 48 caudal. Action-decoding: 191 cells, 83 rostral vs 108 caudal. X^2_1= 14.4; z=3.79; ***p=0.0001, Chi-Square test).

(H) Average traces across all neurons within cluster aligned to the start (black traces) or stop (gray traces) moving onset.