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Supplemental information

Distinct subpopulations of ventral pallidal cholinergic projection neurons encode valence of olfactory stimuli

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Fig S1

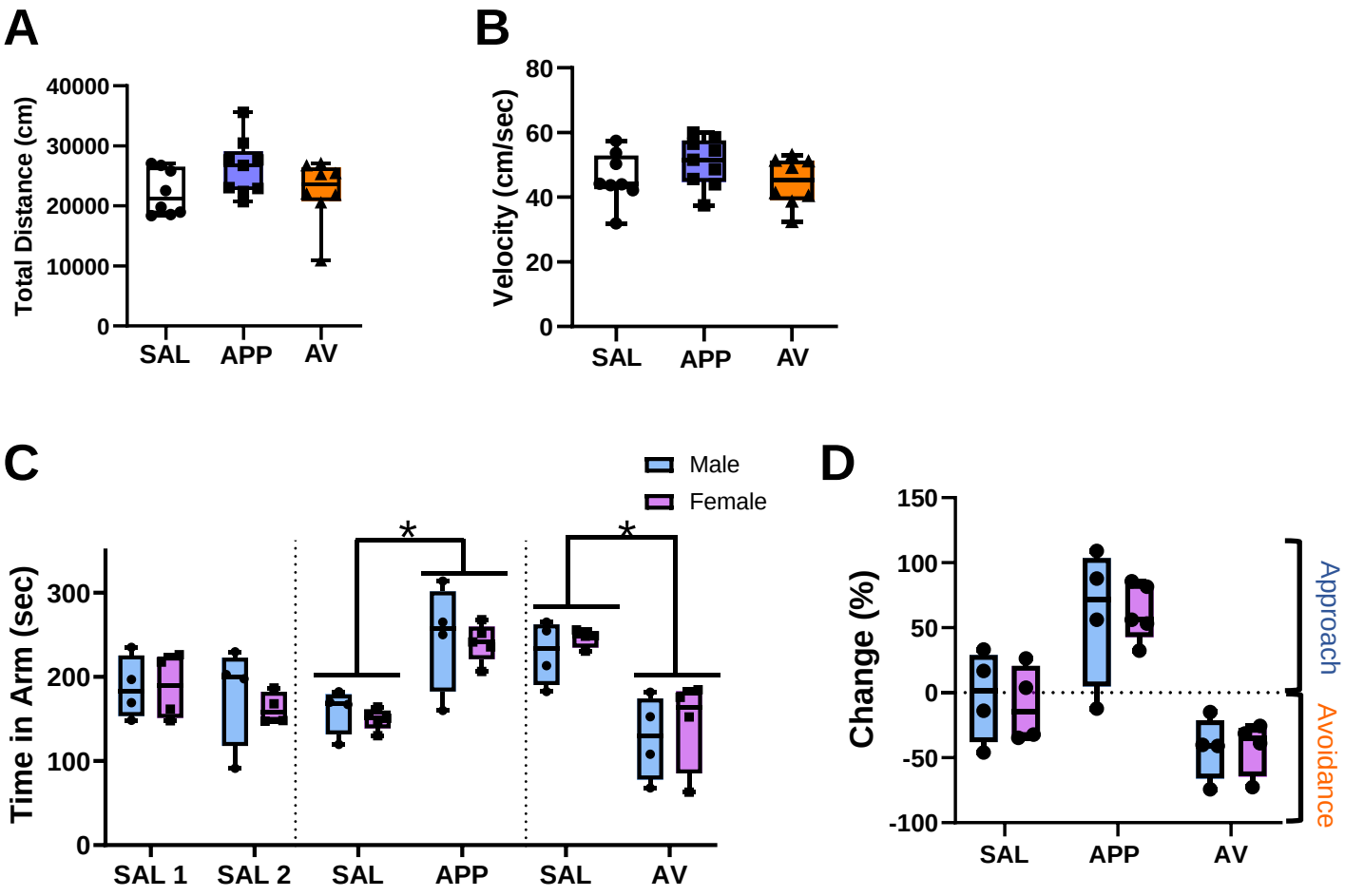


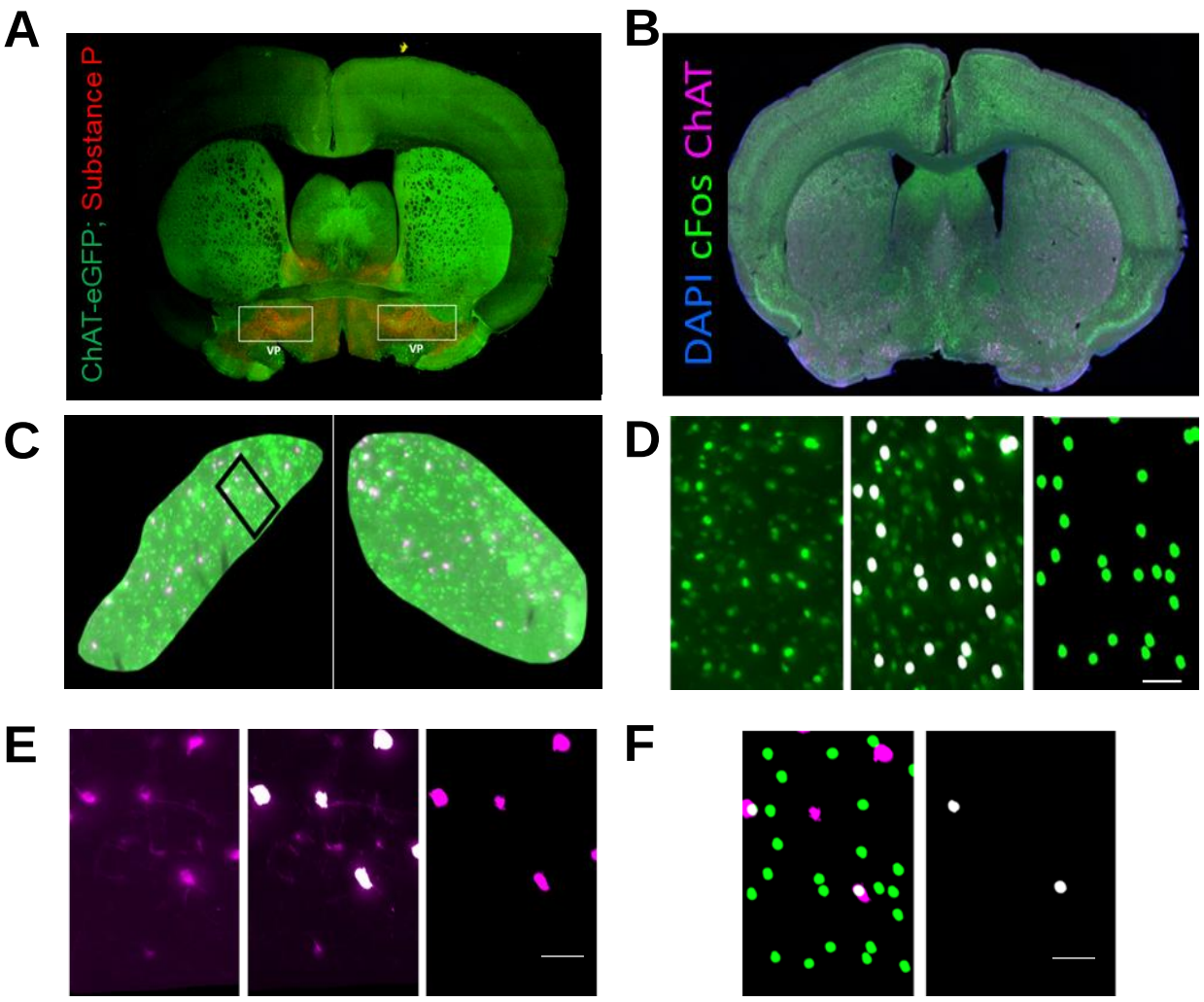
Figure S1 (supplementary to Fig 1): Odor induced approach and avoidance behaviors are not due to odor induced changes in locomotor activity and there is no sex difference in approach and avoidance behaviors.

Neither the appetitive odor (APP) nor the aversive odor (AV) significantly altered **(A)** total distance traveled or **(B)** velocity during the odor preference test vs saline (SAL).

No sex differences were observed in **(C)** time spent in each arm during odor preference test. Left: Male and female mice show equal time spent in both arms of the Y-Maze during a preference test with saline in both arms. Middle: Both male and female mice demonstrate more time spent in the arm with the APP odor vs SAL (i.e., approach). Right: Both male and female mice demonstrate more time spent in the arm with SAL vs the AV odor (i.e., avoidance).

No sex differences were observed in **(D)** percentage of time spent in each arm vs. SAL. Both male and female mice exhibit approach behavior in response to APP odor exposure and avoidance behavior in response to AV odor exposure.

Fig S2



<u>Figure S2 (supplementary to Fig 1):</u> Workflow of the analysis pipeline for the detection of activated VP cholinergic neurons.

A. Immunohistochemistry (IHC) for Substance-P was conducted in a Chat-tau-eGFP mouse to assist in delineating VP borders.

B. Following the odor preference test, tissue was collected and processed for ChAT (to mark cholinergic neurons) and cFos (to label activated neurons). A whole slice image was acquired using the Olympus VS200 slide scanner (20x objective, minimal exposure times for 405/488/594/647 nm channels). Files were converted to Imaris files and uploaded to Imaris software for quantification.

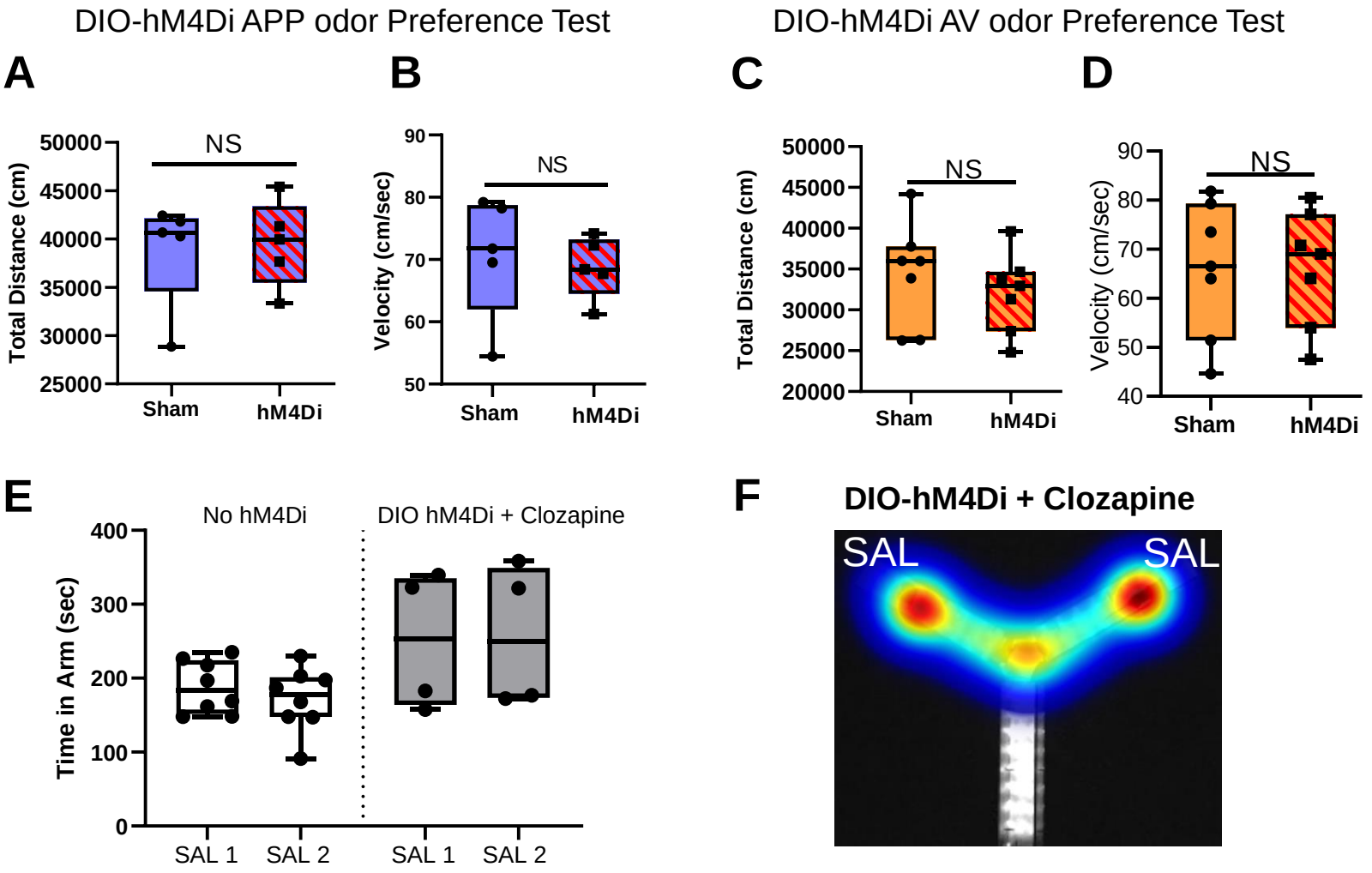
C. The whole slice image is cropped and masked to contain only signal within the VP. Cell counts for all experiments were obtained from the entire VP. Black rectangle indicates area for high magnification images show in S2D-S2F.

D. A signal-based intensity and diameter threshold is set using the spots function in Imaris to detect cFos signal. This threshold is then used to create a new masked channel with only the cFos signal (Left: raw cFos signal, Middle: threshold for cFos signal, Right: masked cFos signal).

E. The surfaces function in Imaris was used to set a signal-based intensity for ChAT detection. This threshold is then used to create a new masked channel containing only ChAT signal (Left: raw ChAT signal, Middle: threshold for ChAT signal, Right: masked ChAT signal)

F. Imaris is used to detect the colocalized pixels of the two masked channels.

Fig S3



<u>Figure S3 (supplementary to Fig 2):</u> Chemogenetic inhibition of VP cholinergic neurons induce changes in odor preference but not changes in locomotor activity.

To assess the effects of the inhibition of VP cholinergic neurons on approach and/or avoidance behaviors, Chat-Cre mice were injected with AAV.hSyn.DIO-hM4Di and AAV.Syn.eGFP (hM4Di experimental group) or AAV.Syn.eGFP only (Sham) in the VP. Following recovery from surgery, all mice were injected IP with 0.1 mg/kg clozapine 15-minutes prior to an odor preference test in a Y-Maze. In the odor preference test, mice were allowed access to two arms of the Y-Maze (appetitive (APP) odor vs. saline **(A & B)** or aversive (AV) odor vs. saline **(C & D)**). Time spent in each arm, as well as locomotor activity (distance traveled and velocity) were assessed.

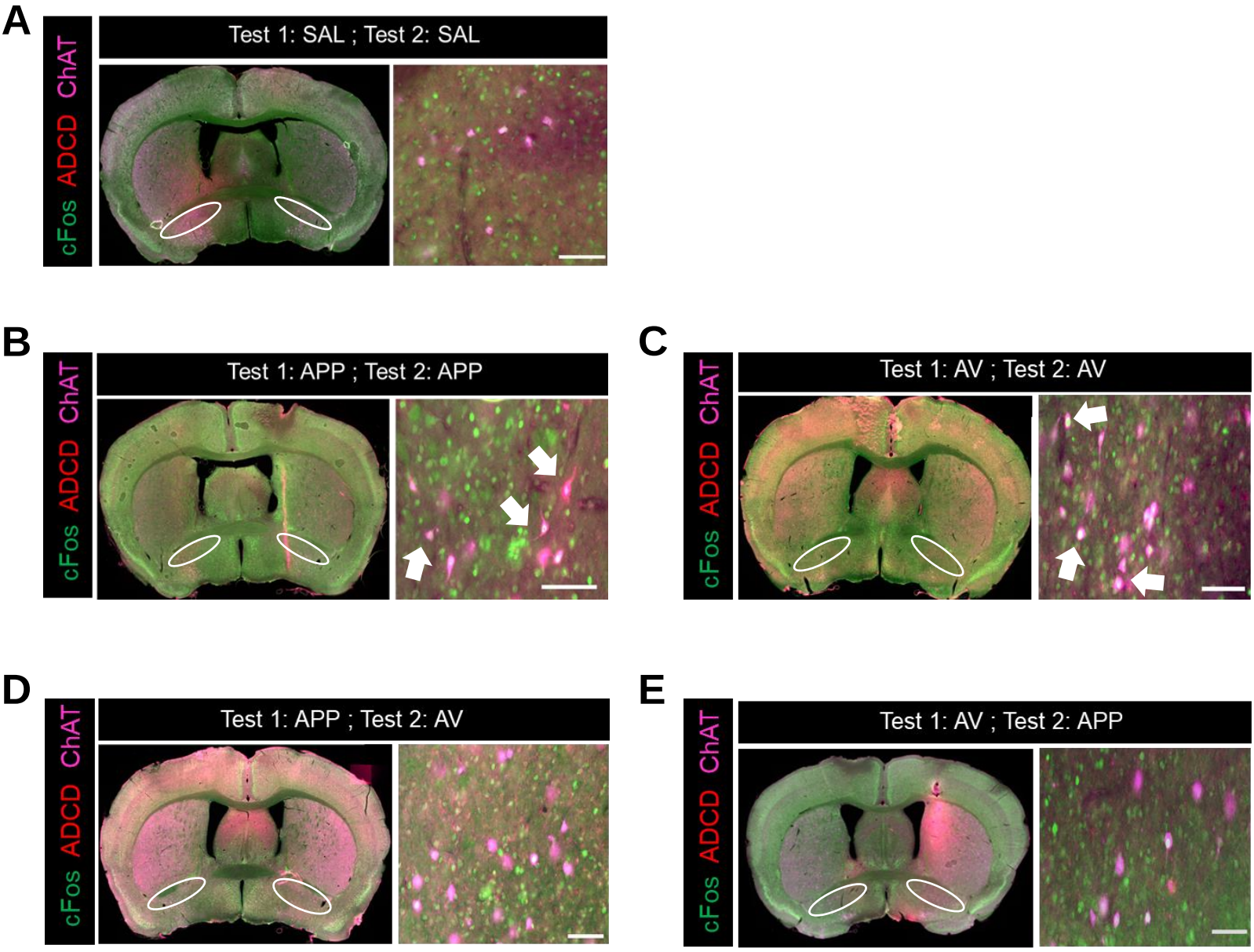
(A & B): DIO-hM4Di APP odor preference test. There is no difference between mice in the sham group and mice that express DIO-hM4Di in **(A)** distance traveled or **(B)** velocity during the APP odor preference test.

(C & D): DIO-hM4Di AV odor preference test. There is no difference between mice in the sham group and mice that express DIO-hM4Di in **(C)** distance traveled or **(D)** velocity during the AV odor preference test.

(E) Chat-Cre mice injected with DIO-hM4di in the VP and administered 0.1 mg/kg clozapine show no change in behavior in a saline/saline preference test. Left: From Fig 1A & 1B, mice without DIO-hM4Di in the VP show equal time spent in each saline arm. Right: Mice injected with DIO-hM4Di and clozapine demonstrate equal time spent in each saline arm.

(F): Representative heatmap from a Chat-Cre mouse injected with DIO-hM4Di in the VP and administered clozapine demonstrating equal time spent in each saline arm.

Fig S4



<u>Figure S4 (supplementary to Fig 3 & 4):</u> Representative raw fluorescent images from ADCD/cFos labeling experiments.

Representative, non-thresholded images from ADCD/cFos labeling experiments showing cFos activated cells (green), ADCD positive VP cholinergic neurons (red), and ChAT labeled cells (magenta). White circles demarcate VP boundaries and an approximation of the area selected as an ROI for analysis. Left = whole slice images; Right = zoomed in image of an area within the VP. Arrows indicate re-activated (i.e., ADCD+/cFos+ VP cholinergic neurons) observed only when exposed to the same odor on Test 1 and Test 2. Scale bar = 50 µm.

A. Test 1 = saline (SAL); Test 2 = SAL; from Fig 3B.

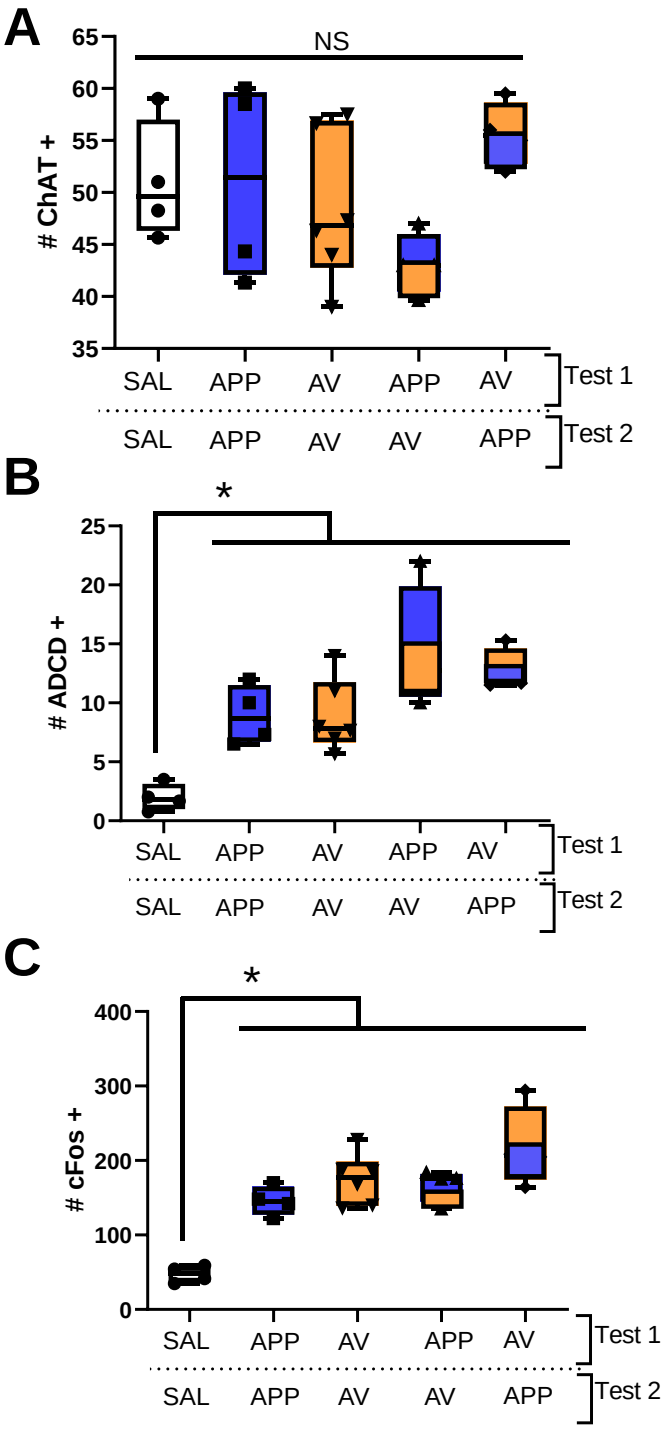
B. Test 1 = appetitive odor (APP); Test 2 = APP; from Fig 3C.

C. Test 1 = aversive odor (AV); Test 2 = AV; from Fig 3D.

D. Test 1 = APP; Test 2 = AV; from Fig 4A.

E. Test 1 = AV; Test 2 = APP; from Fig 4B.

Fig S5



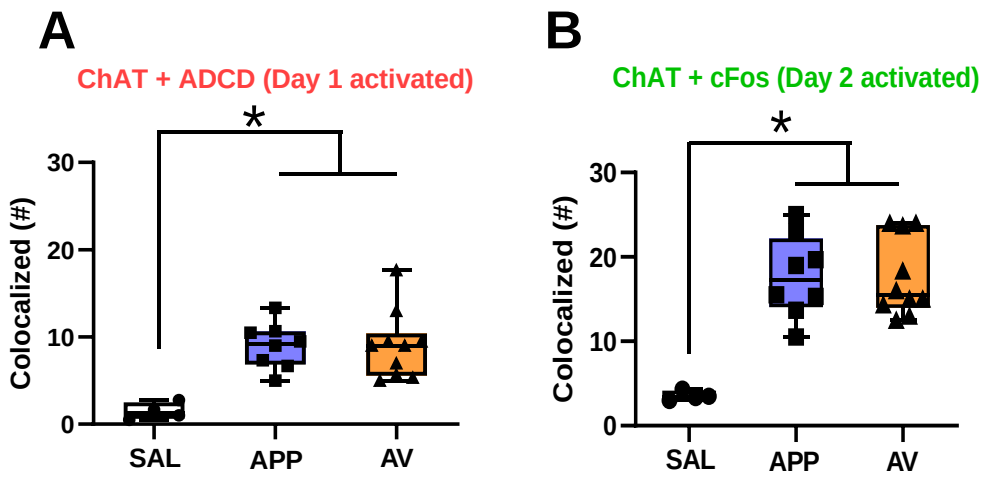
<u>Figure S5 (supplementary to Fig 3 & 4):</u> Total cell counts from all ADCD/cFos labeling experiments. White bars: Test 1 = Saline (SAL) & Test 2 = SAL (from Fig 3B). Blue bars: Test 1 = appetitive odor (APP) & Test 2 = APP (from Fig 3C). Orange bars: Test 1 = aversive odor (AV) & Test 2 = AV (from Fig 3D). Blue/orange bars: Test 1 = APP & Test 2 = AV (from Fig 4B). Orange/blue bars: Test 1 = AV & Test 2 = APP (from fig 4C).

A. Total cholinergic neurons measured using ChAT immunohistochemistry. There was no significant difference between experiments in the total number of ChAT neurons.

B. Total number ADCD labeled cells. Exposure to either odor significantly increased the number of ADCD+ neurons.

C. Total number of activated cells measured using cFos immunohistochemistry. Exposure to either the APP or AV odor on Test 2 significantly increased the number of cFos+ cells. (* $p < 0.05$).

Fig S6

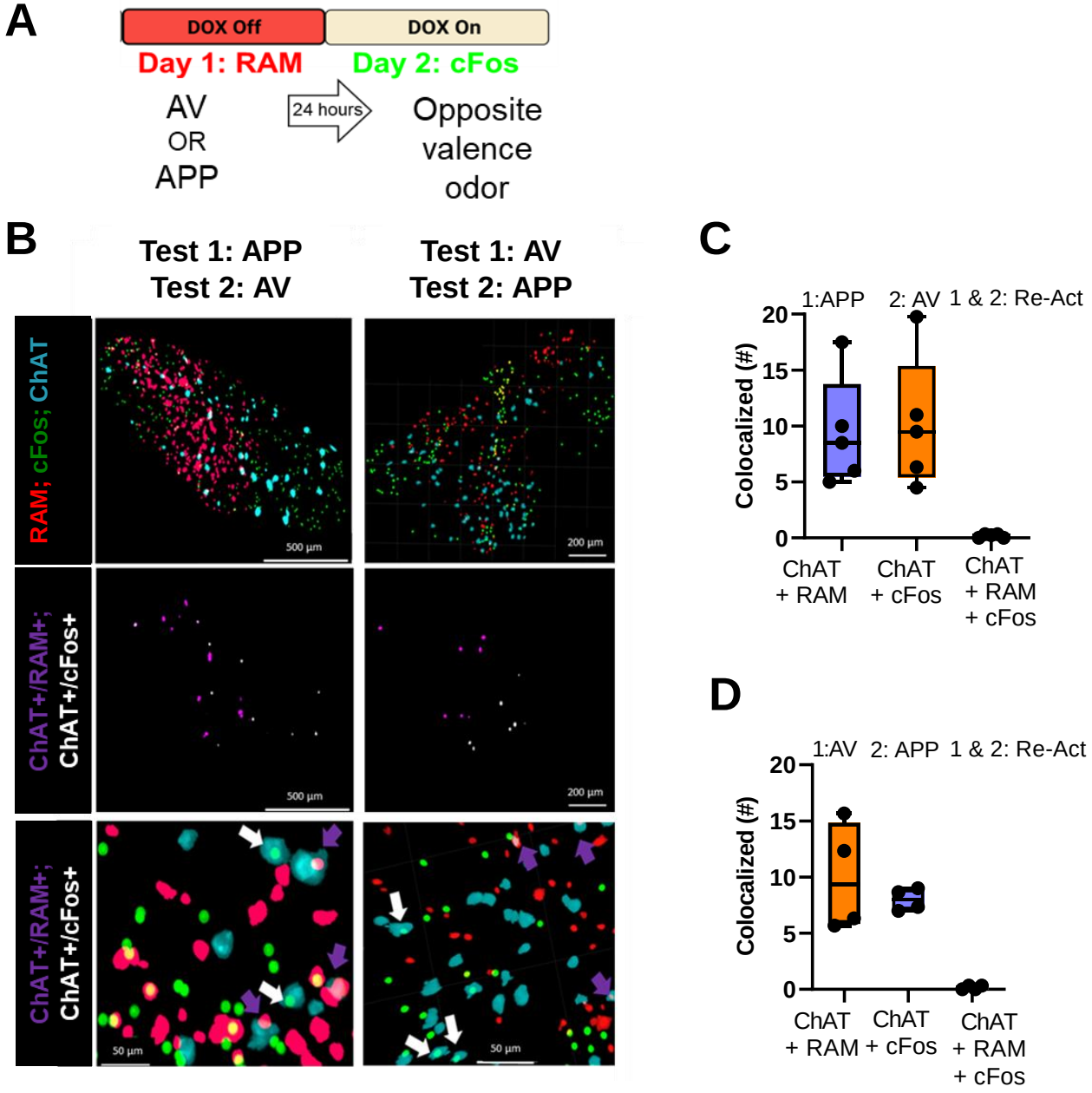


<u>Figure S6 (supplementary to Fig 3 & 4):</u> The order of odor presentation does not affect the total number of VP cholinergic neurons activated. Regardless of order of odor presentation (i.e., (**A**) Day 1 vs. (**B**) Day 2) and method in which activated VP cholinergic neurons are assayed (i.e., (**A**) ChAT and ADCD vs. (**B**) ChAT and cFos IHC), both odors (APP or AV) significantly increase the number of activated VP Cholinergic neurons vs. SAL. * $p < 0.05$.

A. The number of ADCD+ VP cholinergic neurons (i.e., activated on Test 1), is significantly higher following APP or AV odor exposure.

B. The number of ChAT/cFos+ VP cholinergic neurons (i.e., activated on Test 2) is significantly increased following APP or AV odor exposure.

Fig S7

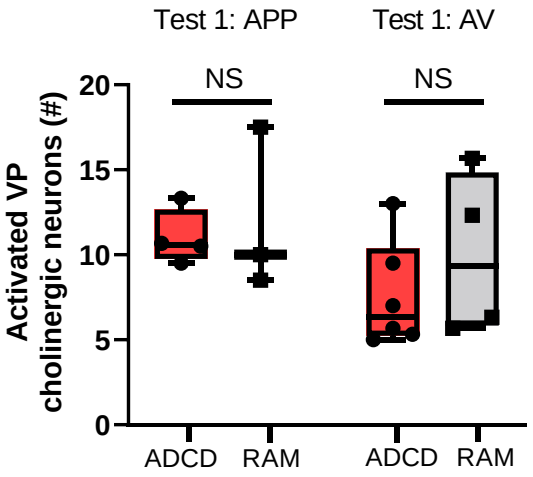


<u>Figure S7 (supplementary to Fig 3 & 4):</u> Experiments using the robust activity marker (RAM) confirm ADCD and cFos labeling experiments.

A. A distinct activity-dependent viral vector was used to verify results from ADCD and cFos labeling experiments. The robust activity marker (RAM) viral vector utilizes a synthetic activity-dependent promoter and a Tet-Off system to label activated neurons. RAM was injected in the VP of wild-type C57/BL6J mice. The RAM construct was used in conjunction with ChAT and cFos IHC to label activated VP cholinergic neurons in two distinct contexts. The behavioral paradigm used was identical to the protocol used for ADCD and cFos labeling experiments. Representative images from RAM experiments showing RAM positive (activated neurons on Day 1), cFos positive (activated cells on Day 2), ChAT (cholinergic marker), and the colocalization of RAM + ChAT and cFos + ChAT.

B. & C. Confirming results from the ADCD and cFos labeling experiments (in Fig 4), mice exposed to a different odor on Day 2 exhibited no overlap between ChAT+/RAM+ and ChAT+/cFos+ neurons.

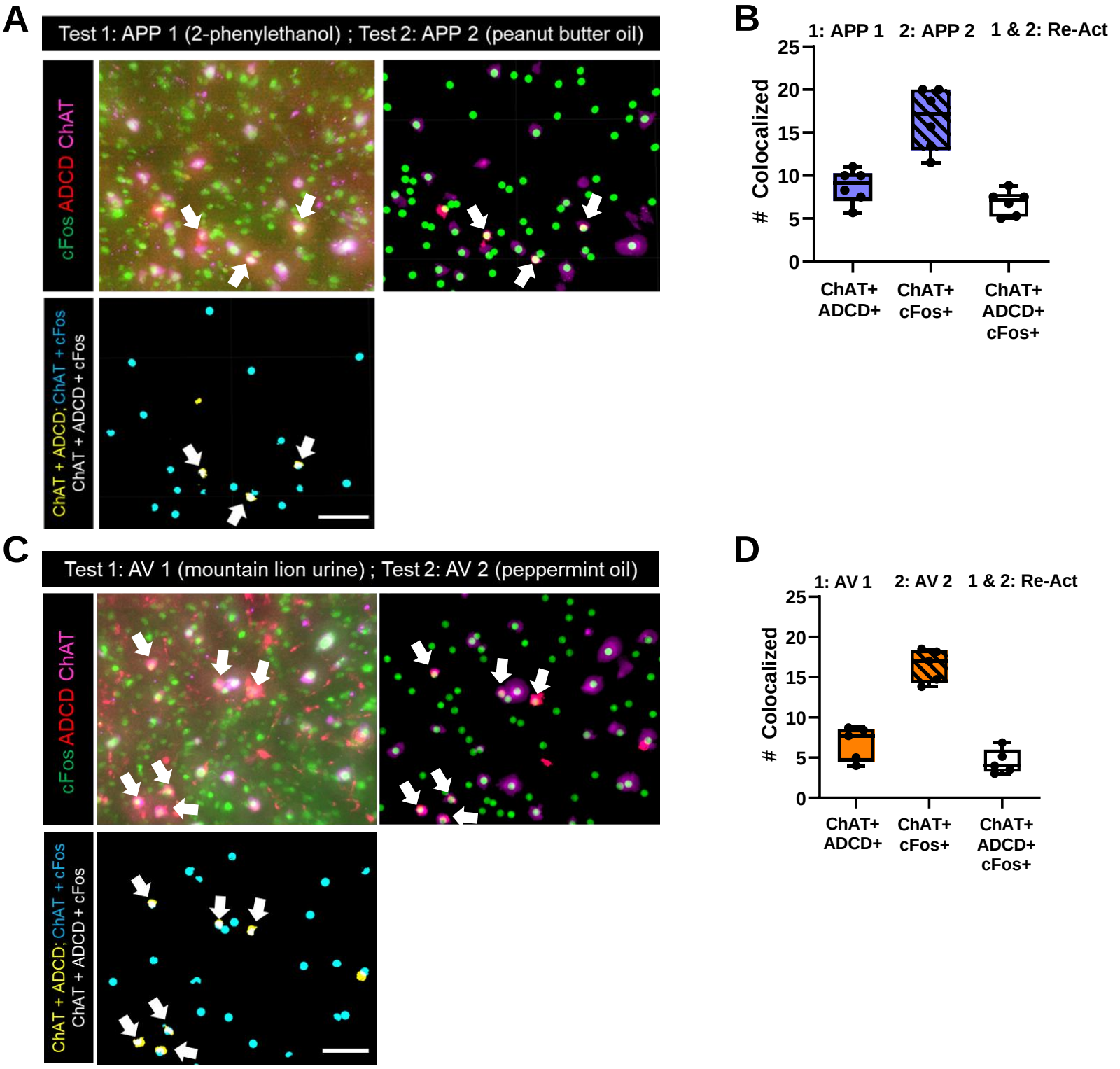
Fig S8



<u>Figure S8 (supplementary to Fig 3 & 4):</u> ADCD and RAM are comparable in labeling activated VP cholinergic neurons.

For ADCD experiments, Chat-Cre x Fos-tTA/GFP mice were injected with ADCD in the VP. For Test 1, ADCD was used to assess the number of activated VP cholinergic neurons. On Test 2, the colocalization of ChAT and cFos-GFP (examined using IHC) was used to assess the number of activated cholinergic neurons (see Fig 3 and Fig 4 for details). For RAM experiments, c57/BL6J mice were injected with RAM in the VP. For Test 1, the number of RAM+ neurons co-labeled with ChAT IHC was used to assess the number of activated VP cholinergic neurons. For Test 2, the colocalization of ChAT and cFos (examined using IHC) was used to assess the number of activated VP cholinergic neurons (see Fig S8 for details). Regardless of order of odor presentation (Left = appetitive odor (APP), Right = aversive odor (AV)), ADCD and RAM labeled similar number of activated VP cholinergic neurons.

Fig S9



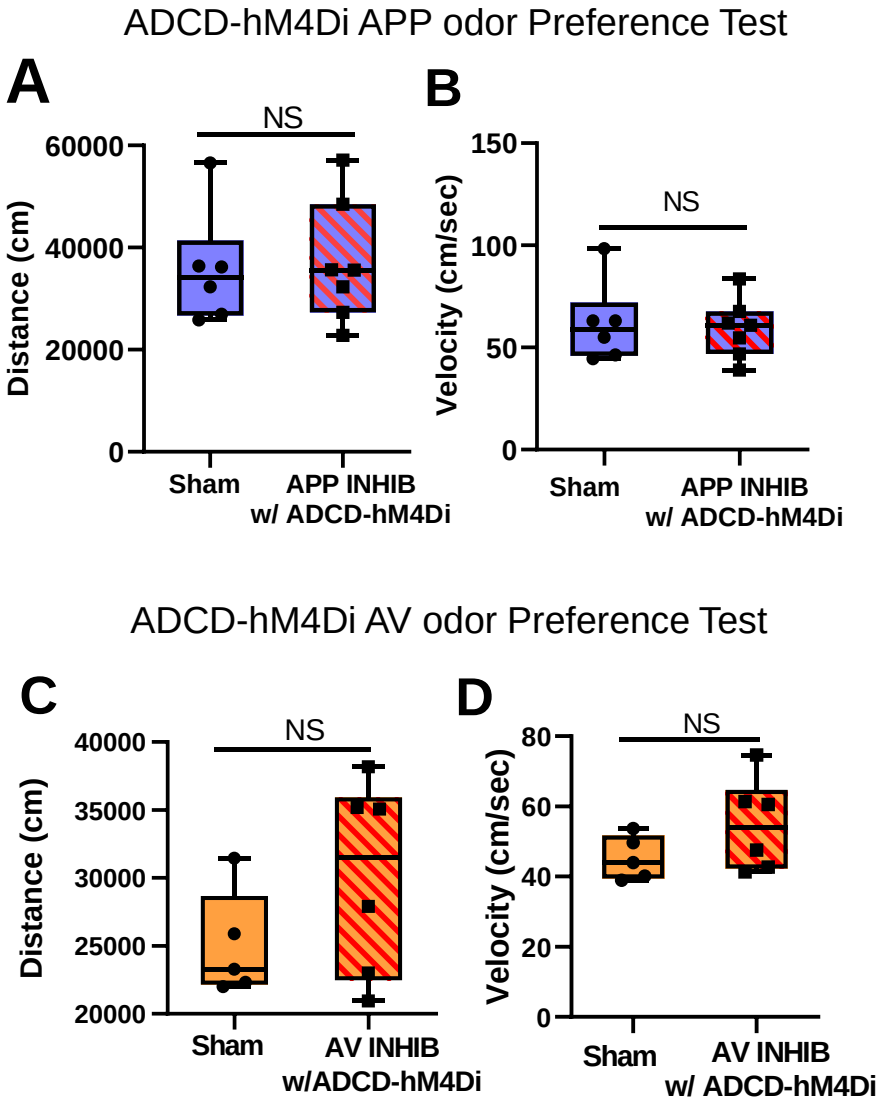
<u>Figure S9 (supplementary to Fig 3 & 4):</u> Distinct appetitive and distinct aversive odors reactivate VP cholinergic neurons.

To test if the reactivation of VP cholinergic neurons was valence-specific or due to the unique properties of each odor, Chat-Cre x Fos-tTA/GFP mice were injected with ADCD in the VP and exposed to distinct appetitive (APP, **A & B**) or distinct aversive (AV, **C & D**) odors (see Fig 3 and Fig 4 legends for details on ADCD/cFos labeling experiments).

(A) Representative images and **(B)** quantification from mice (n = 6 mice, 4 male and 2 female) exposed to two distinct APP odors: 2-phenylethanol and peanut butter oil. ~80% of VP cholinergic neurons activated by APP 1 (2-phenylethanol) are re-activated when exposed to APP 2 (peanut butter oil). Scale bar = 50 µm.

(C) Representative images and **(B)** quantification from mice (n = 5 mice, 1 male and 4 female) exposed to two distinct AV odors: mountain lion urine and peppermint oil. ~70% of VP cholinergic neurons activated by AV 1 (mountain lion urine) are re-activated when exposed to AV 2 (peppermint oil). Scale bar = 50 µm.

Fig S10



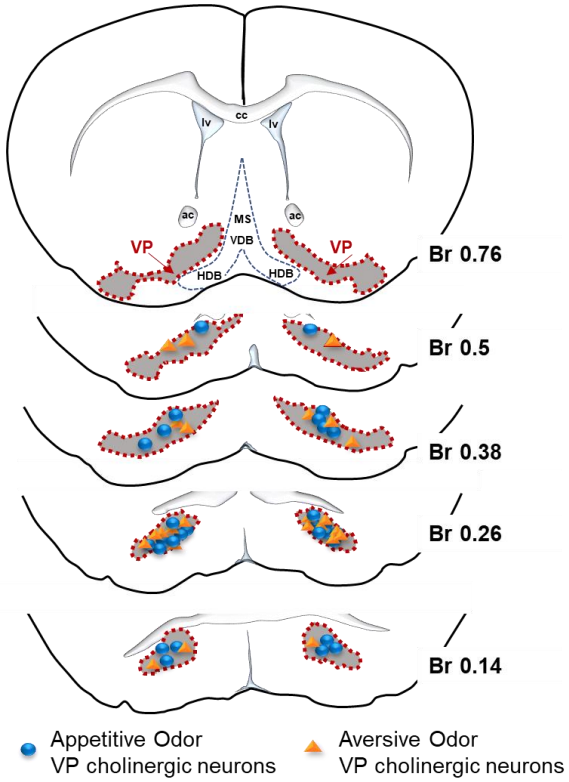
<u>Figure S10 (supplementary to Fig 5):</u> ADCD-hM4di induced changes in odor preference are not due to changes in locomotor activity.

To assess the effects of the inhibition of previously activated VP cholinergic neurons on approach and/or avoidance behaviors in response to odor exposure, Chat-Cre x Fos-tTA/GFP mice were injected with ADCD and AAV.Syn.eGFP (experimental group), or AAV.Syn.eGFP only (Sham) in the VP (see Fig 5 legend and methods for details). Following recovery from surgery, mice were habituated in the Y-Maze (2 x 10 min) and taken off a DOX diet. Approximately 24-hours later, mice were exposed to an odor (either appetitive (APP) or aversive (AV)) in one arm of the Y-Maze. Following odor exposure, mice were placed on a DOX diet to prevent further expression of ADCD. Approximately 24-hours later, all mice were injected IP with 0.1 mg/kg clozapine 15-minutes prior to an odor preference test in a Y-Maze. In the odor preference test, mice were allowed access to two arms of the Y-Maze (previously exposed odor, either APP or AV, vs. saline). Time spent in each arm, as well as locomotor activity (distance traveled and velocity) were assessed.

Top = ADCD-hM4Di appetitive (APP) odor preference test. There is no difference between mice in the sham group and mice that express ADCD-hM4Di in **(A)** distance traveled or **(B)** velocity during the APP odor preference test.

Bottom = ADCD-hM4Di aversive (AV) odor preference test. There is no difference between mice in the sham group and mice that express ADCD-hM4Di in **(C)** distance traveled or **(D)** velocity during the AV odor preference test.

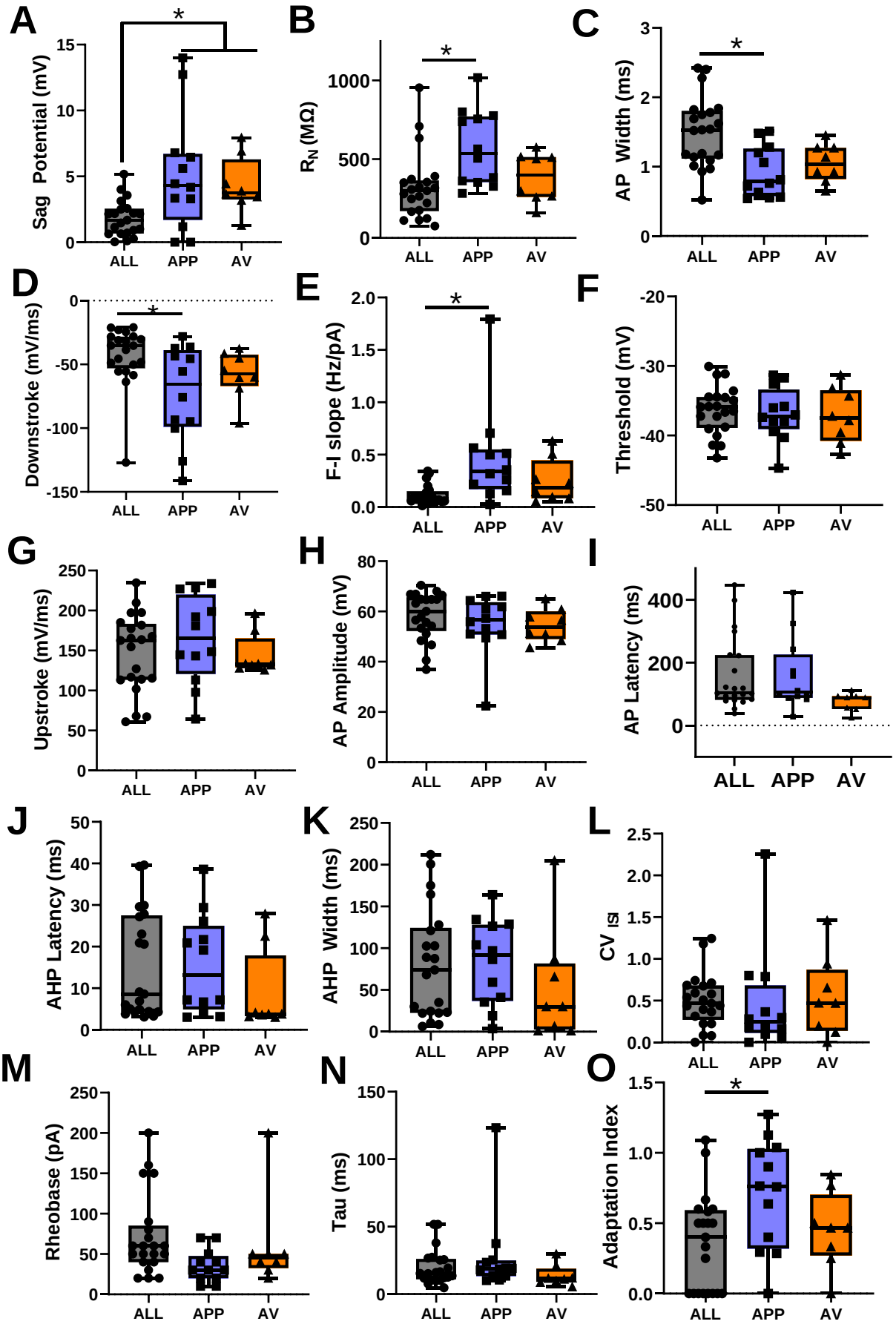
Fig S11



<u>Figure S11 (supplementary to Fig 6 & 7):</u> Appetitive and aversive odor activated VP cholinergic neurons are intermingled within the VP.

Viral injection sites from mice studied in Figures 3 & 4 were used to relocalize the approximate positions of VP cholinergic neurons activated by the appetitive odor vs. those activated by the aversive odor. Appetitive and aversive odor activated VP cholinergic are intermingled across the anterior-posterior axis of the VP assayed (Bregma +0.76 – Bregma +0.14).

Fig S12



<u>Figure S12 (supplementary to Fig 6):</u> Comparison of the electrophysiological properties of appetitive (APP) vs. AV (AV) VP cholinergic neurons demonstrates that they are largely similar to one another and to the overall population of VP cholinergic neurons (see Fig 6 for differences).

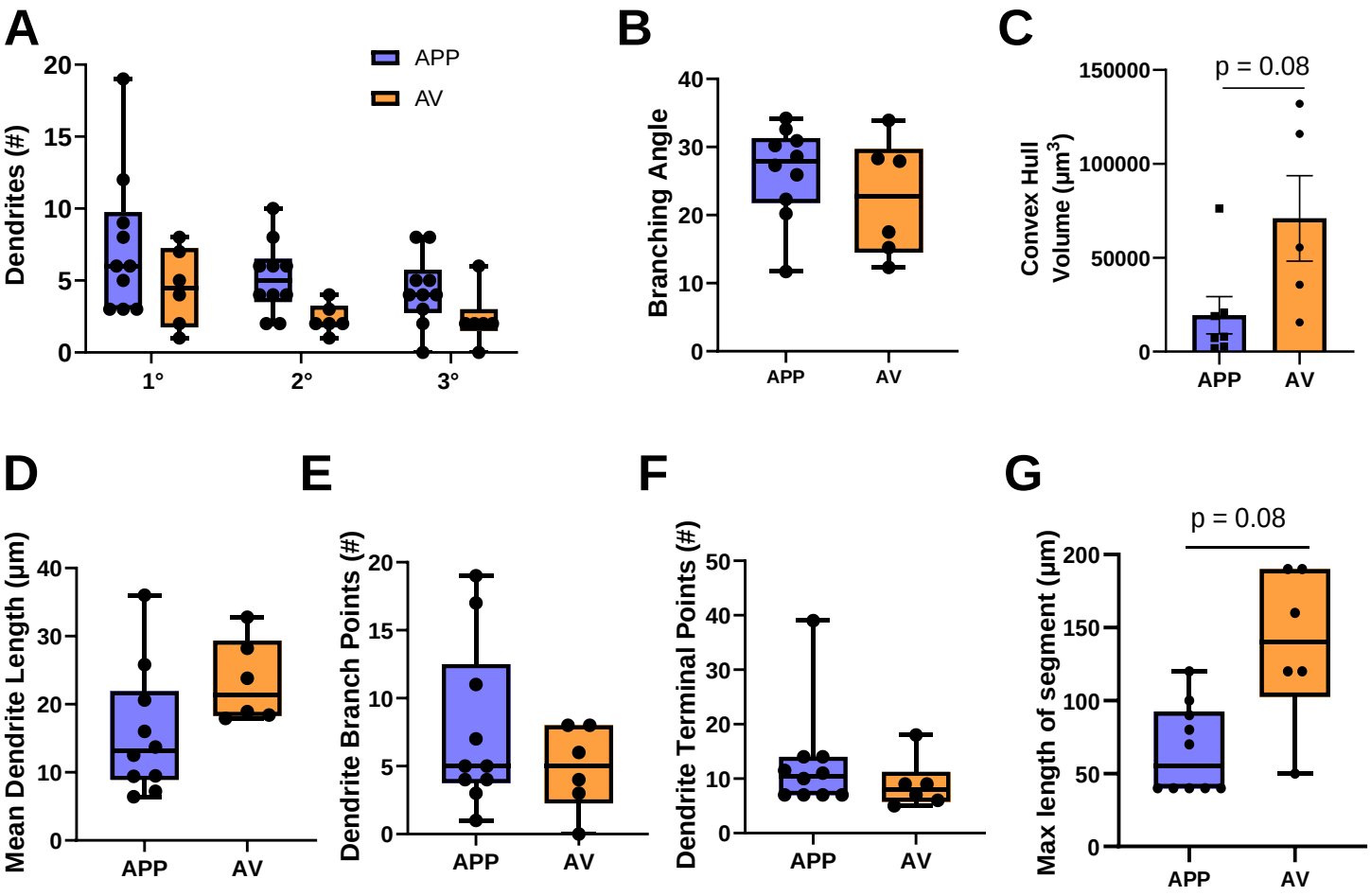
The majority of both passive and active membrane properties are the same whether the recordings are from APP or AV activated VP cholinergic neurons.

Both APP and AV activated VP neurons significantly differ from ALL VP cholinergic neurons in **(A)** sag potential.

APP odor activated VP cholinergic neurons significantly differ from ALL VP cholinergic neurons in **(B)** input resistance, **C)** action potential width, **(D)** action potential downstroke and **(E)** frequency-current slope.

Similarities between APP, AV and ALL VP cholinergic neurons are observed in **(F)** threshold, **(G)** action potential upstroke, **(H)** actional potential amplitude, **(I)** action potential latency, **(J)** afterhyperpolarization latency, **(K)** afterhyperpolarization width, **(L)** coefficient variation, **(M)** rheobase, **(N)** tau, and **(O)** adaptation index.

Fig S13

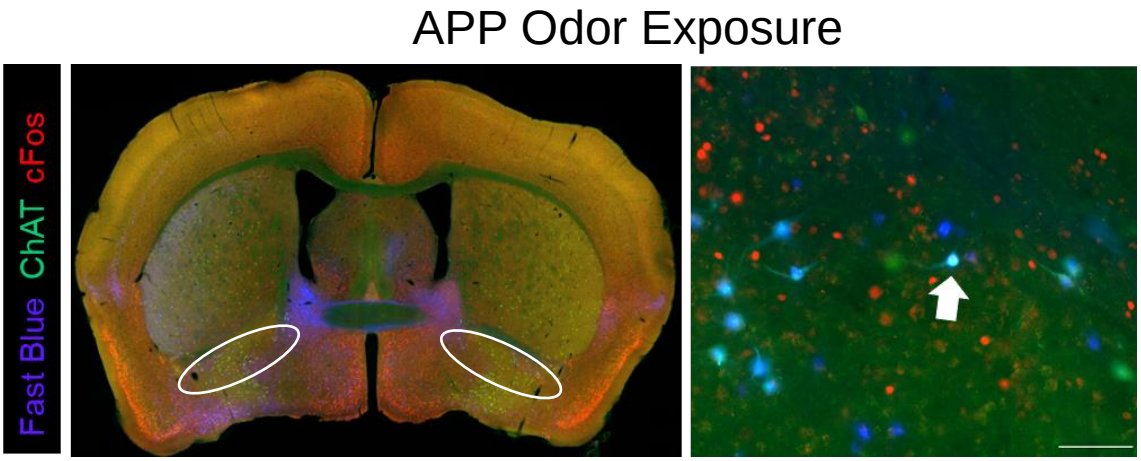


<u>Fig S13 (supplementary to Fig 6):</u> Additional morphological properties between appetitive odor activated (APP) and aversive odor activated (AV) VP cholinergic neurons.

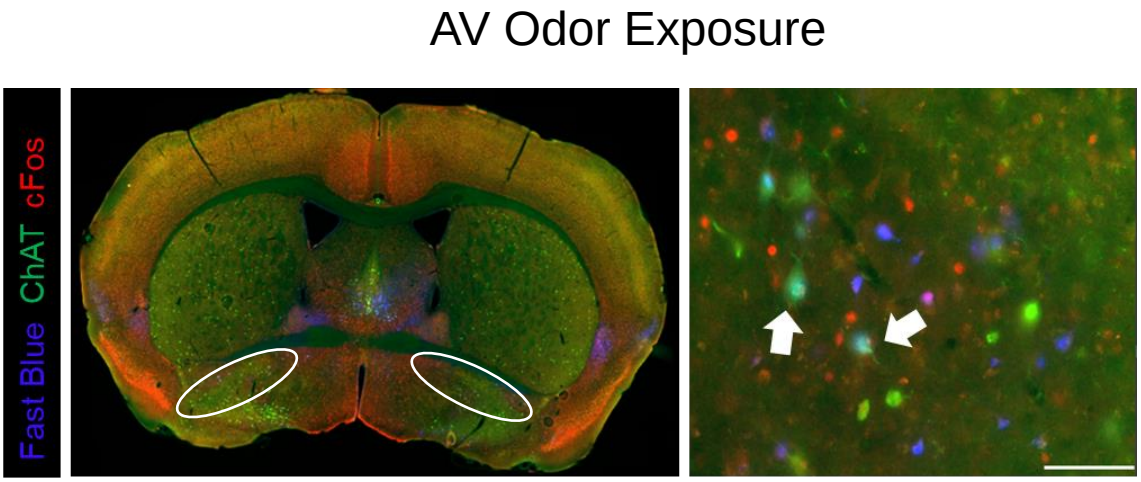
A. Assay of the number of 1° , 2° , and 3° dendrites revealed no statistically significant differences between APP vs. AV VP cholinergic neurons Despite differences in proximal complexity (see Fig 6), there were no significant differences between groups in **(B)** branching angle, **(C)** volume of the convex hull, **(D)**mean dendrite length, **(E)** number of dendrite branch points, **(F)** number of dendrite terminal points or **(G)** max length of segment.

Fig S14

A



B



C

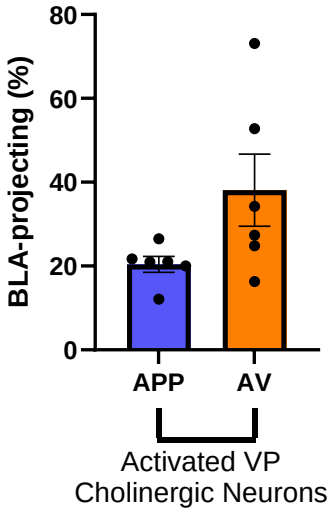


Figure S14 (supplementary to Fig 7): Percentage of appetitive (APP) and aversive (AV) odor activated VP cholinergic neurons that project to the BLA

A. Left = Representative whole slice image, right = zoomed in image within the VP from a mouse with Fast Blue injection in the BLA and exposed to the APP odor. White circles represent VP boundaries and the approximate area selected for analysis. Blue = BLA-projecting cells within the VP, ChAT = cholinergic marker, cFos = marker of neuronal activation. Scale bar = 100 µm. Arrows indicate odor activated BLA-projecting VP cholinergic neurons.

B. Left = Representative whole slice image, right = zoomed in image within the VP from a mouse with Fast Blue injection in the BLA and exposed to the AV odor.

C. Percentage of APP and AV odor activated VP cholinergic neurons that are BLA-projecting.