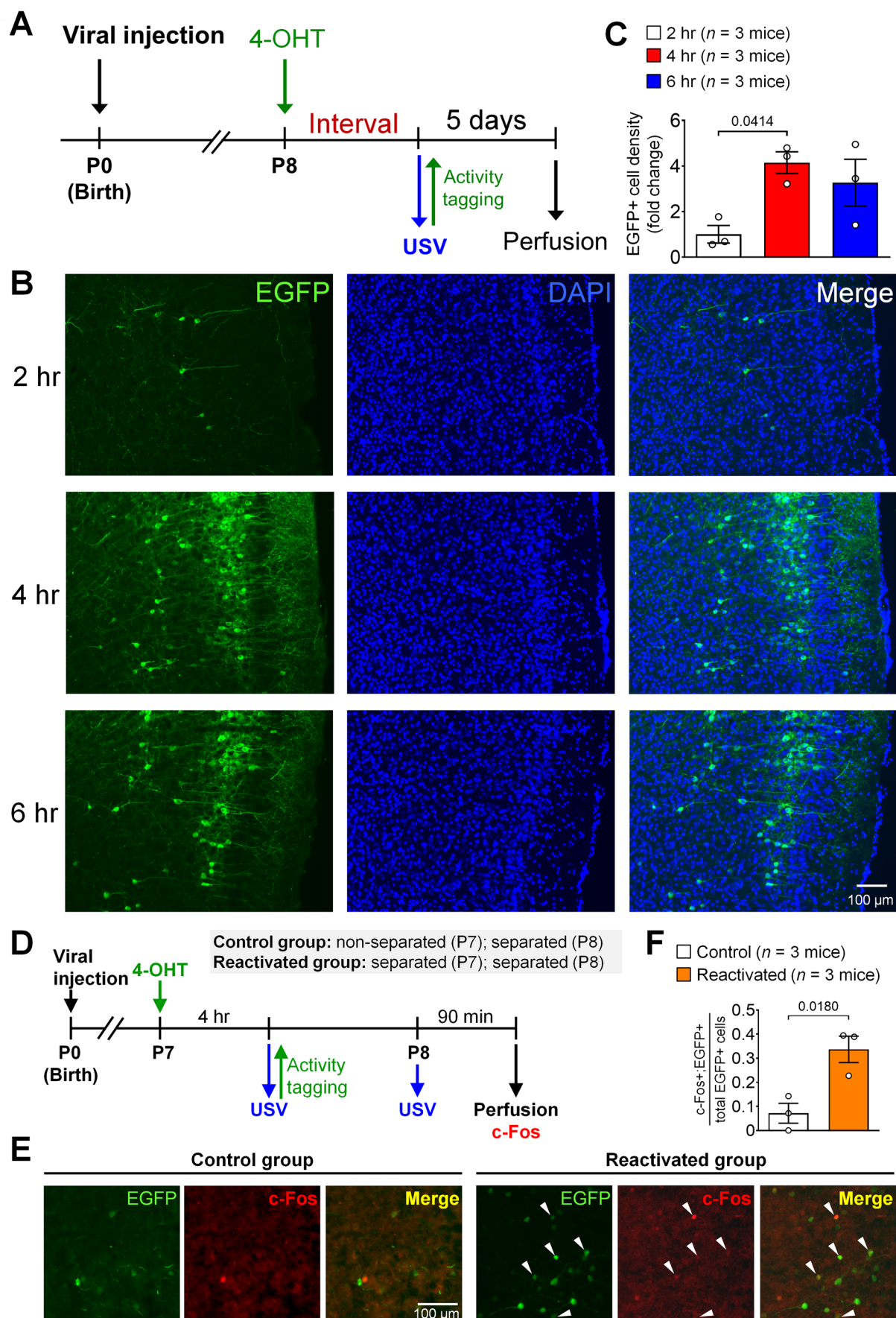


Expanded View Figures

Figure EV1. Optimal interval of 4-OHT injection and reactivated neurons in the vmPFC for USV experiment.

(A) To determine the optimal interval for detecting activated cells during USV emission, we injected 4-OHT into P8 TRAP2 mice whose striatum had been injected with AAVrg-*hSyn-DIO-EGFP* at P0. The mice were separated from the dam 2, 4, or 6 h after 4-OHT injection, and then perfused 5 days later. (B) We found fewer EGFP-positive (+) neurons in the prelimbic cortex (PL) in the 2-h interval group compared with the 4-h and 6-h interval groups. (C) Quantification of EGFP+ neurons. (D) At P7, all mice received an injection of 4-OHT. Four hours after injection, the mice were separated from their mother to trigger isolation stress-induced USV. Littermates that remained with the dam served as non-separated controls. At P8, both groups underwent a 5-min maternal separation and were perfused 90 min later for the detection of c-Fos expression. Cellular reactivation was quantified by measuring the proportion of c-Fos+ cells within the EGFP+ population. (E) Reactivated cells (arrowheads) are identified by co-expression of EGFP (green) and c-Fos (red). (F) Quantification of reactivated neurons. The proportion of c-Fos+ cells within the EGFP+ population was significantly higher in the reactivated group compared with the control group. One-way ANOVA is used in (C). Independent *t*-test is used in (F).



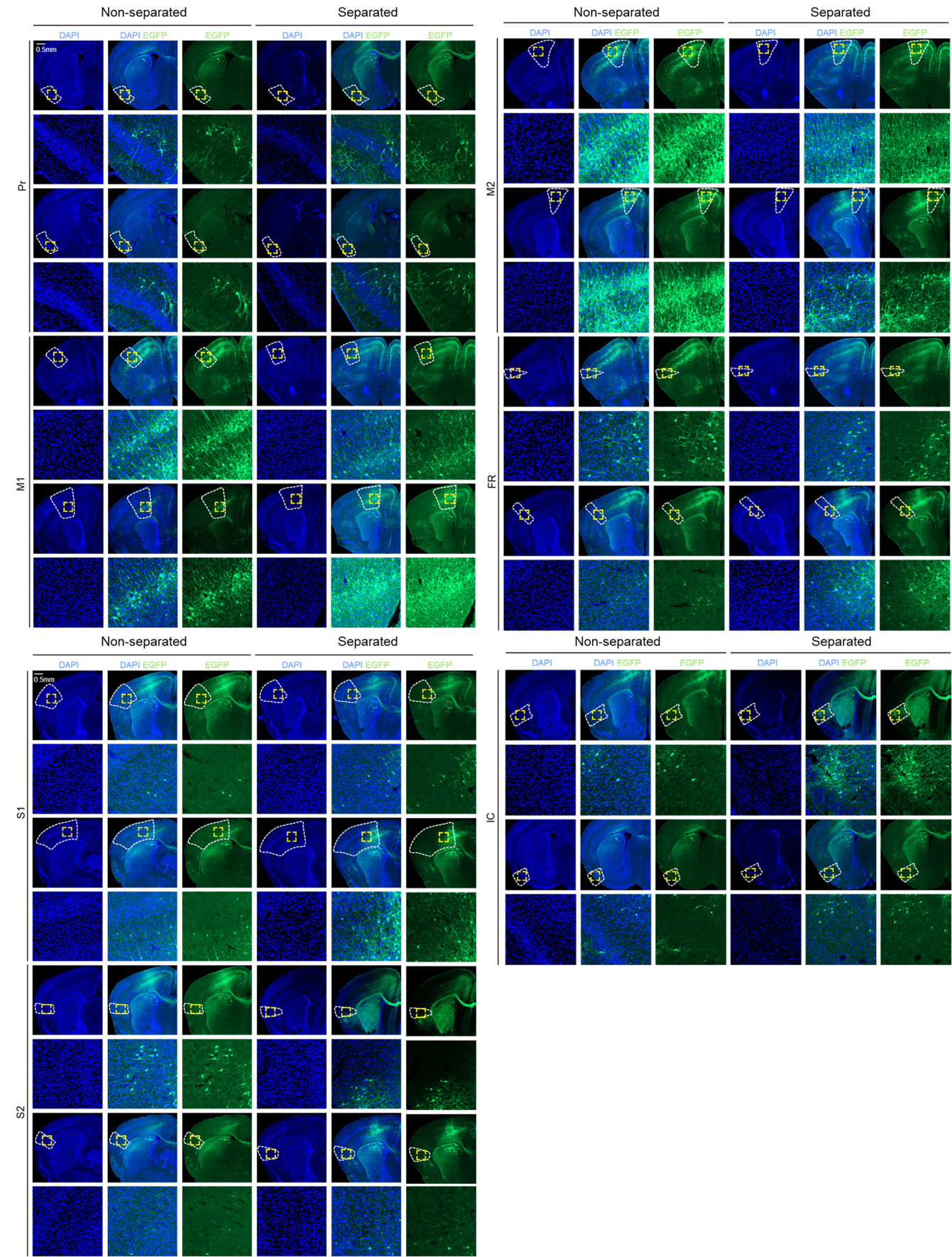


**Figure EV2. Localization of vocalization-related corticostriatal ensembles in neonatal mice.**

EGFP signals, identified by GFP immunostaining, indicate the activated neurons during USV emission. EGFP-positive neurons were quantified among 13 cortical regions, including prelimbic, infralimbic, medial orbital, cingulate, lateral/ventral orbital, dorsal peduncular (shown in Fig. 1), piriform (Pr), primary motor (M1), secondary motor (M2), frontal area (FR), primary somatosensory (S1), secondary somatosensory (S2), and insular (IC) cortices. This figure provides additional representative images. In a subset of panels, images are derived from the same sections as those shown in Fig. 1C but correspond to distinct, non-overlapping regions of interest.

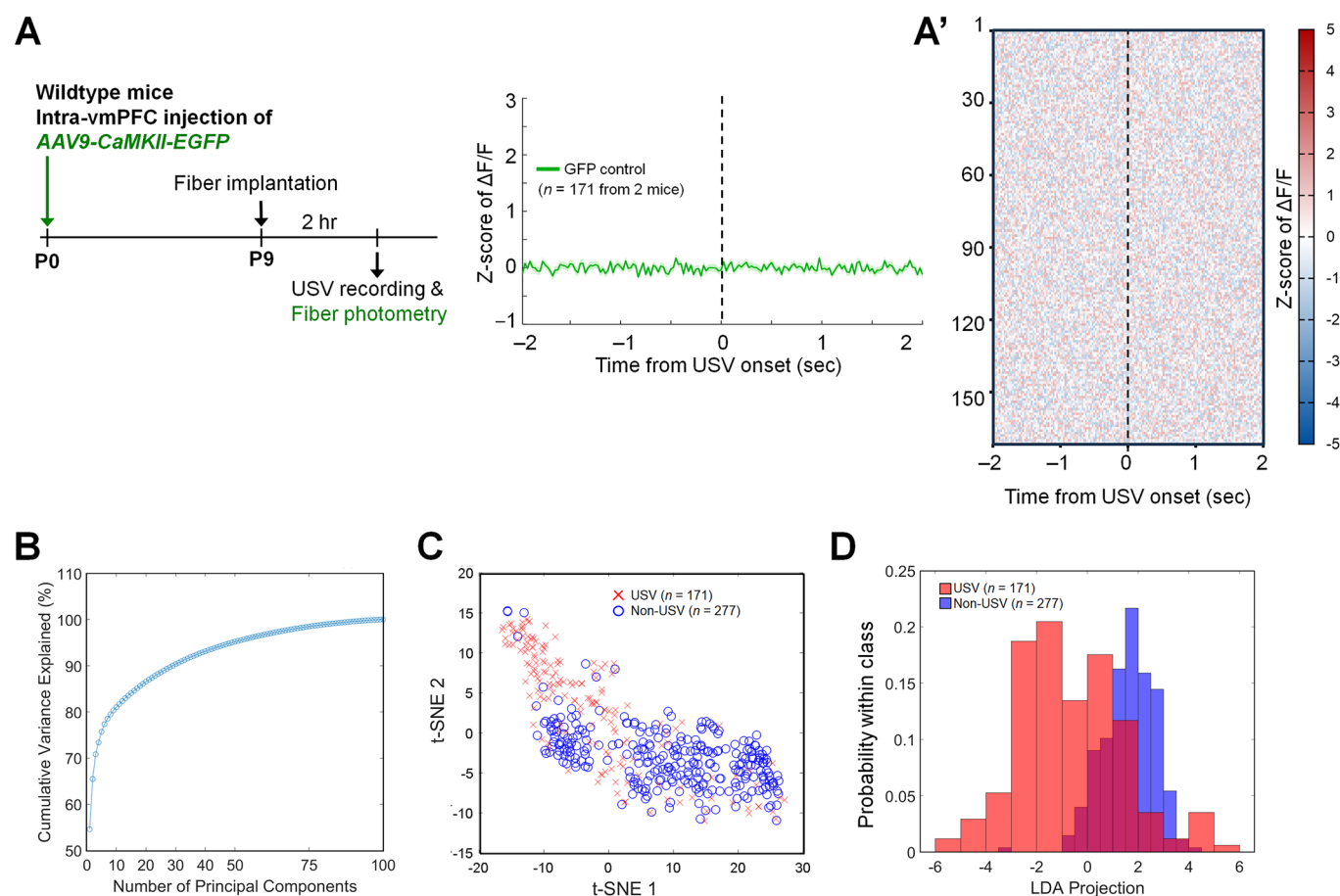
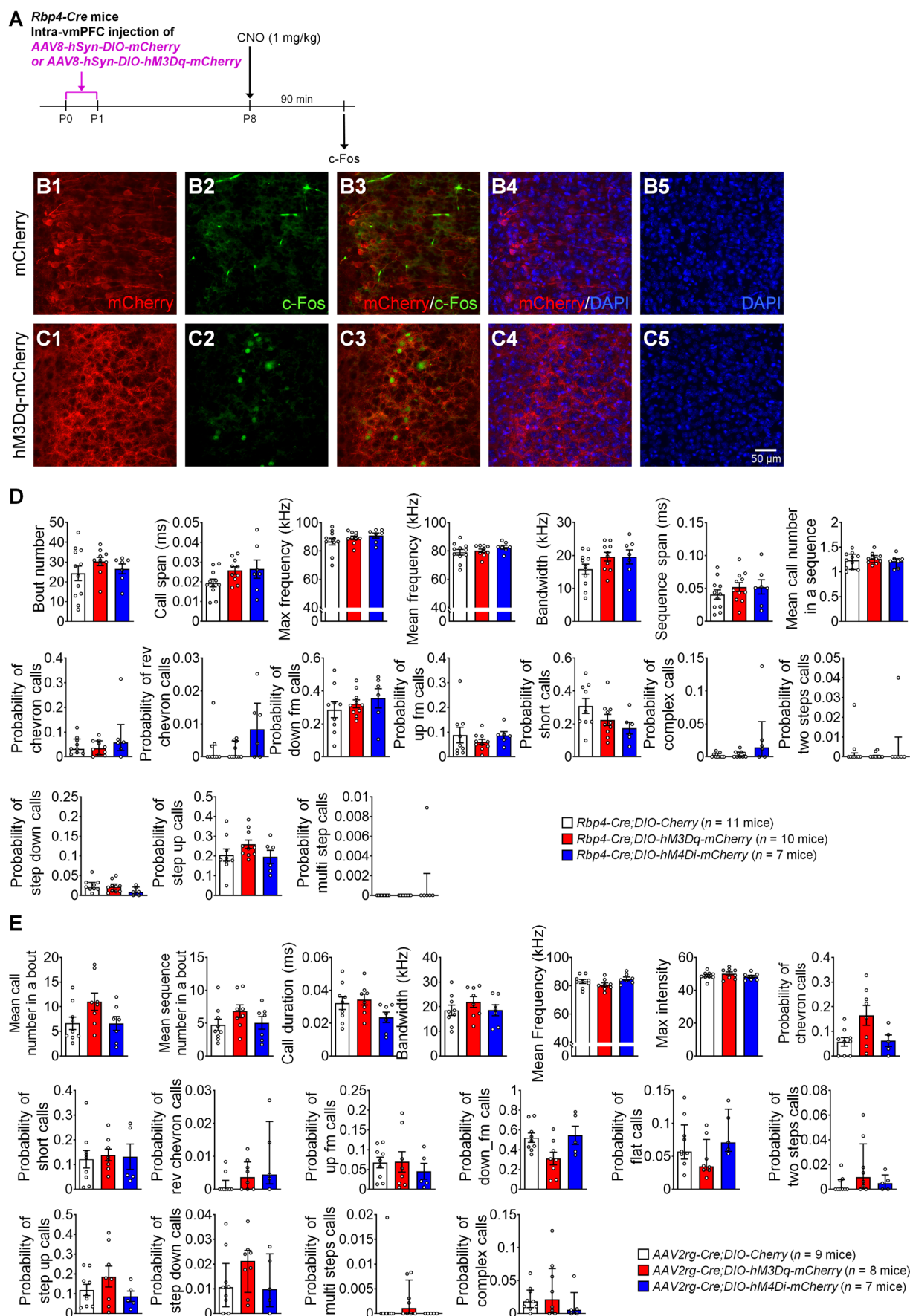


Figure EV3. Dimensionality reduction and discriminative projection of vmPFC trace patterns during USV and non-USV periods.

(A–A') Schematic experimental design and the peri-event GFP signals recorded during fiber photometry. The green line shows the mean value, and the shaded green region indicates the standard error of the mean. The normalized signals of each event are aligned and shown in a heat map. The warmer color represents the stronger signals. (B) Cumulative variance explained by the top 100 principal components (PC) derived from USV and non-USV groups. (C) The t-SNE projection using PC1-PC10, showing the distribution of trace patterns in an alternative nonlinear projection. (D) Histogram of LDA projection scores using PC1-PC10.



◀ Figure EV4. Validation of hM3Dq activation and analysis of USV properties in DREADD-manipulated mice.

(A) The schematic experimental design. (B, C) Increased c-Fos expression was observed in vmPFC neurons expressing hM3Dq-mCherry (C1–C5) compared with neurons expressing the control virus (B1–B5) 90 min after CNO administration. (D) USV acoustic and syntactic parameters were not significantly altered in mice with acute vmPFC DREADD manipulation. (E) USV acoustic and syntactic parameters were not significantly altered in mice with acute manipulation of corticostriatal projection neurons.

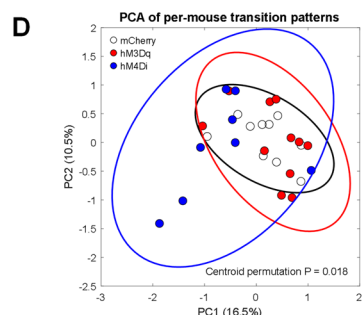
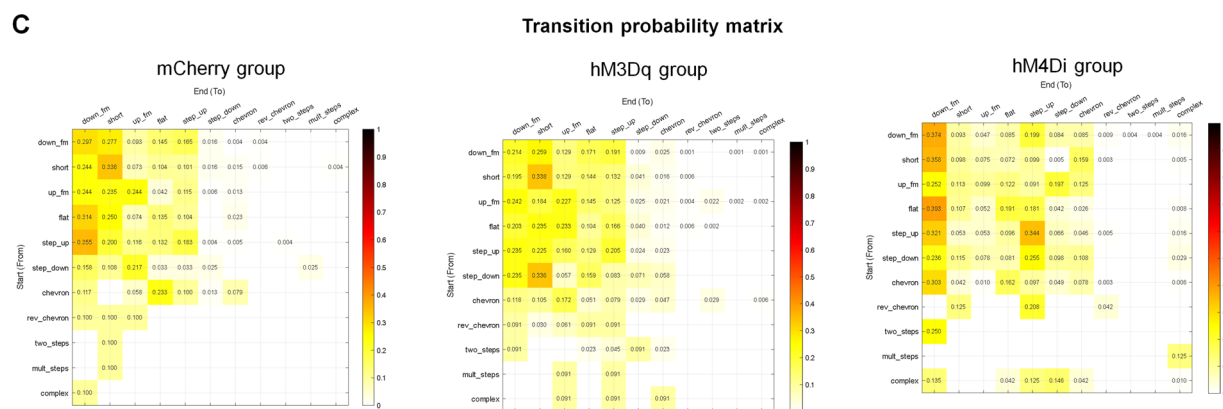
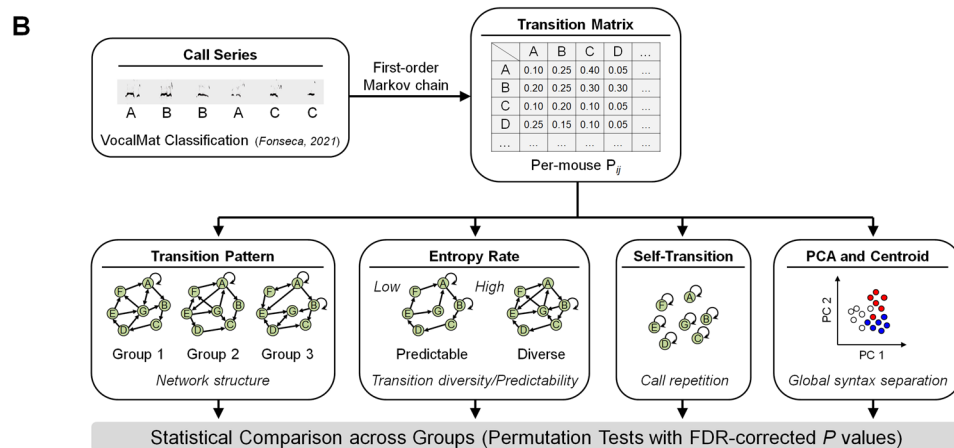
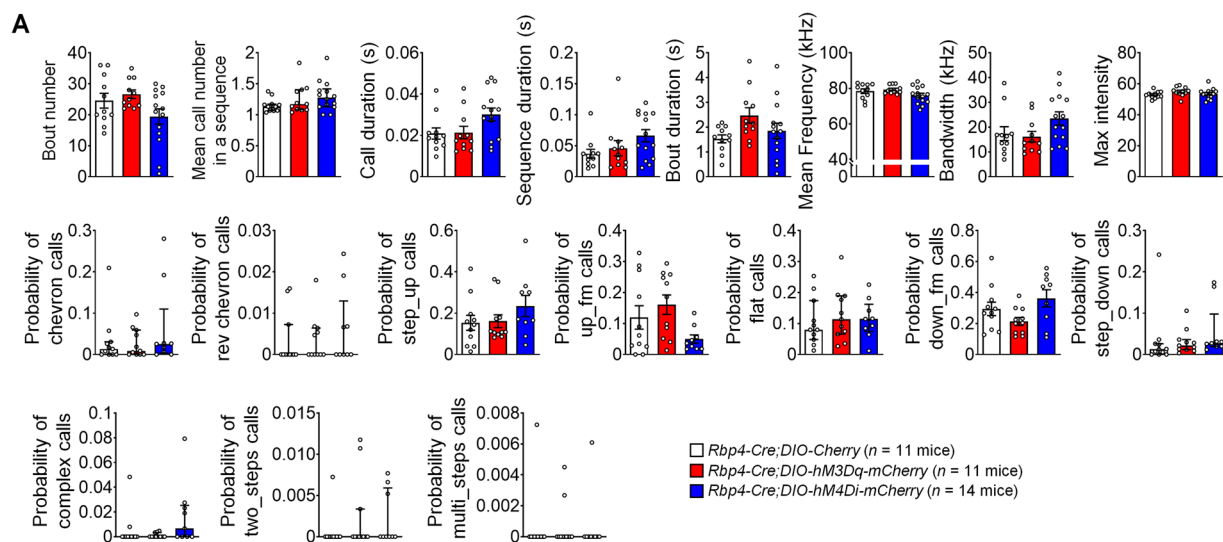


**Figure EV5. Analysis of USV properties and USV syntax in DREADD-manipulated mice.**

(A) USV acoustic and syntactic parameters were not significantly altered in mice with chronic vmPFC DREADD manipulation. (B) Schematic flowchart of Markov chain analysis. (C) Transition probability matrix of each group. (D) Principal component analysis (PCA) of transition patterns across groups. Group ellipses illustrate clustering in PC space.

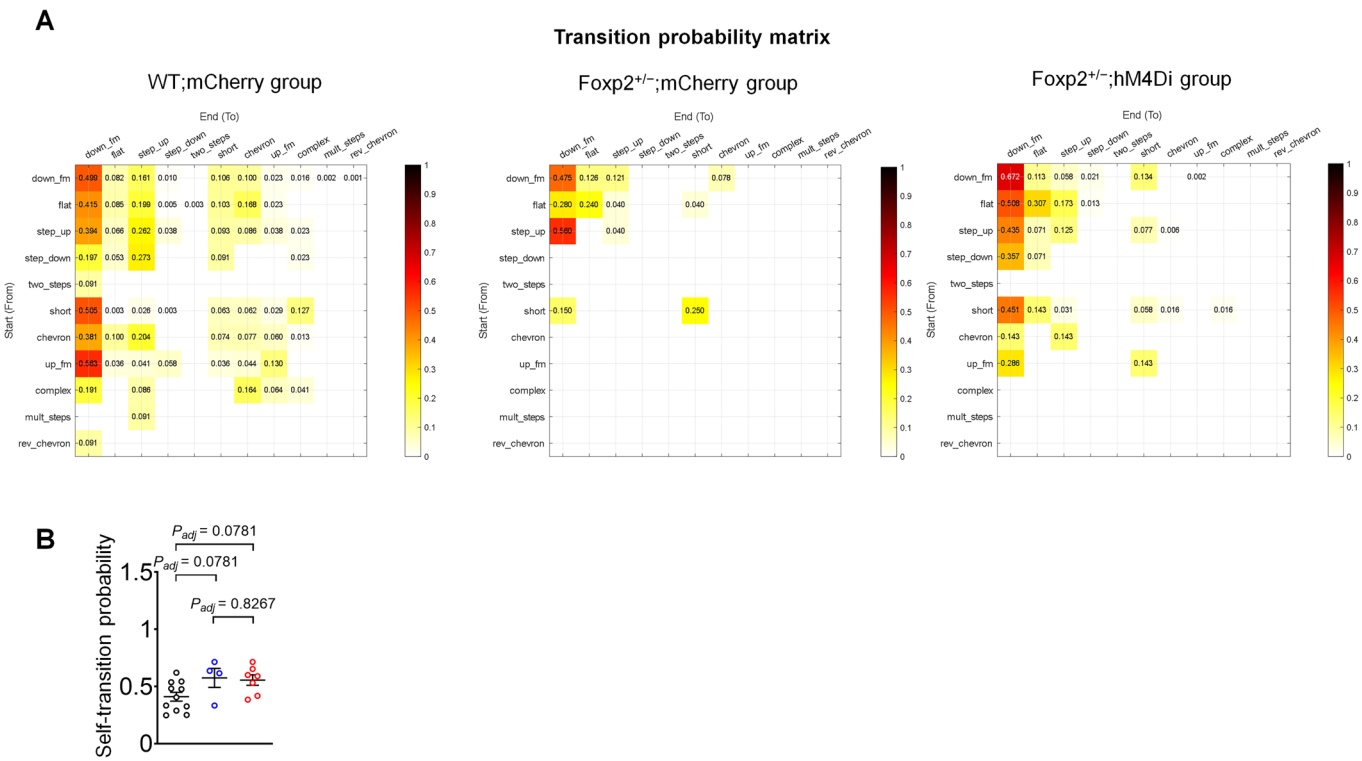


Figure EV6. Analysis of USV properties and USV syntax in DREADD-manipulated mice.

(A) Transition probability matrix of each group. (B) Per-mouse permutation test of USV self-transition probability across groups.