Sex-Dependent Genetic Expression Signatures within Cocaine- and Sucrose-Seeking Ensembles in Mice

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Abstract

Maladaptive reward seeking is a hallmark of cocaine use disorder. To develop therapeutic targets, it is critical to understand the neurobiological changes specific to cocaine-seeking without altering the seeking of natural rewards, e.g., sucrose. The prefrontal cortex (PFC) and the nucleus accumbens core (NAcore) are known regions associated with cocaine- and sucrose-seeking ensembles, i.e., a sparse population of co-activated neurons. Within ensembles, transcriptomic alterations in the PFC and NAcore underlie the learning and persistence of cocaine- and sucrose-seeking behavior. However, transcriptomes exclusively driving cocaine seeking independent from sucrose seeking have not yet been defined using a within-subject approach. Using FosiCreERT2/+/Ai14 transgenic mice in a dual cocaine and sucrose self-administration model, we fluorescently sorted (FACS) and characterized (RNAseq) the transcriptomes defining cocaine- and sucrose-seeking ensembles. We found reward-, sex-, and region-specific transcriptomic changes that refine clinically relevant genetic approaches to decrease cocaine-seeking behavior without altering non-drug reward-based positive reinforcement.

Introduction

Cocaine use disorder (CUD) is characterized by the recurrent seeking of cocaine after periods of abstinence and withdrawal, often leading to relapse (*1*). The absence of pharmaceutical intervention for attenuating cocaine seeking is partially due to a lack of understanding of the discrete molecular alterations driving the rewarding effects of cocaine apart from natural rewards, e.g., sucrose. Cocaine and sucrose seeking involve cross-talking reward-signaling regions: the nucleus accumbens core (NAcore) and prefrontal cortex (PFC) (*2*–*5*). Within the NAcore and PFC, sparse activity-dependent neural circuits, defined as neuronal ensembles (*6*), are linked to cue-associated cocaine and sucrose seeking and are mostly separate despite having an overlapping population of neurons (*7*–*9*). Studies focused on ensemble-specific genetic expression in cocaine seeking often lack within-subject comparisons between different rewards.

Mounting evidence indicates that contingent psychoactive intake facilitates persisting alterations in gene expression within the NAcore and PFC (*10*–*16*). For instance, cellular regulation of cocaine seeking persists through disruption of post-transcriptional modifications associated with cAMP response element-binding protein (CREB), nuclear factor kappa light chain enhancer of activated b cells (NF-kB), and myocyte enhancer factor 2 (MEF2), in addition to epigenetic modifying histone deacetylases (HDAC) and histone demethylases (HDT) (*17*). However, little is known about gene expression profiles specific to ensembles activated during cocaine seeking after self-administration and extinction training.

Sex differences are observed in motivation for cocaine (*18*, *19*), but are not well understood from a genetic perspective. Sex differences also exist in basal gene expression of neurons but are not fully annotated in reward-linked psychiatric disorders like CUD (*20*). The finite number of sex-considerate studies within CUD has, in part, limited the effectiveness of translating preclinical findings to meaningful clinical outcomes. Moreover, improper or absence of sex-considerate statistical analysis and pooling of sexes has led to discrepancies in experimental reproducibility (*21*).

Considering these gaps in our understanding of CUD, we focused on three main questions: 1) Do neuronal ensembles linked to different types of reward differentially express different genes, or do they regulate the expression of the same genes differently? 2) Are differentially expressed genes (DEG) sex-dependent? and 3) are they different in two critical regions of the reward system, the NAcore and PFC? To address these questions, we used targeted recombination in active populations (FosiCreERT2/+/Ai14, or cFos-TRAP2) (*22*) and fluorescent activated cell sorting (FACS) (*15*, *23*) to identify and isolate ensembles in the NAcore and PFC from temporally distinct cocaine- and sucrose-seeking events. We then completed an unbiased RNA-sequencing (RNAseq) to compare significant (false discovery rate (FDR) < 0.1) (*24*, *25*) sex- and region-dependent genetic profiles in cocaine- and sucrose-seeking ensembles in reference to naïve mice. We also assessed ensemble-specific genetic expression in relation to the non-ensemble population. Using this approach, we developed a repository of sex-considerate genetic information giving insight into distinct or intersecting genetic alterations within cocaine- and sucrose-seeking ensembles in the NAcore and PFC.

Results

Cue-Induced Seeking of Cocaine and Sucrose Recruit Reward-Specific Ensembles

To characterize genetic profiles in cocaine- and sucrose-seeking ensembles, we used a dual self-administration (SA) and extinction (EXT) model and reward-specific cued-reinstatements, followed by fluorescently activated cell sorting (FACS) for downstream bulk RNA sequencing (RNAseq; Fig. 1a). cFos-TRAP2 mice underwent alternating daily sessions of intravenous cocaine and oral sucrose SA on a Fixed Ratio 1 (FR1) schedule. SA was contingent on cue-paired interaction with cocaine- or sucrose-reinforced active nose pokes (NP). An additional non-reinforced inactive NP without consequences was also present. Observing all SA sessions regardless of reward type, mice interacted more with the active NP than the inactive NP throughout SA (Fig. 1b) with no differences between sexes (Fig. S1a). Cocaine (Fig. S2a) and sucrose (Fig. S2b) SA sessions showed consistent discrimination between the active NP and the inactive NP. Despite no sex differences observed during cocaine SA (Fig. S3a), females interacted significantly more with the inactive NP than males during sucrose SA (Fig. S3b). This finding is consistent with heightened cocaine preference during sucrose SA sessions for females when compared to males (Fig. S3c). Overall, reward intake was equal for cocaine and sucrose and remained stable throughout SA (Fig. S4a) with no sex differences (Fig. S4b). Following SA, mice underwent EXT in the original SA chamber without reward administration or cue exposure. During EXT, mice decreased interaction and discrimination of NPs previously paired with cocaine or sucrose during SA (Fig. 1c and Fig. S5a) with no sex differences (Fig. S5b,c).

To induce seeking of cocaine and sucrose, mice underwent cocaine- (C-RST) and sucrose-specific (S-RST) cued-reinstatement sessions (Fig. 1d) where mice were exposed to reward-specific cues in the absence of reward administration. Mice sought cocaine during the C-RST session, as demonstrated by increased interaction with the cocaine-paired active NP compared to the inactive NP and to the cocaine-paired active NP during the EXT session from the previous day (Fig. 1d). Immediately following C-RST, 4-hydroxytamoxifen (4-OHT) was administered to tag active cFos-dependent neurons in the ensemble underlying cocaine-seeking behavior with fluorescent tdTomato. In parallel, mice sought sucrose during the S-RST session, as observed by increased interaction with the sucrose-paired active NP compared to the inactive NP and to the sucrose-paired active NP during the previous EXT session (Fig. 1d). Mice were euthanized 60 minutes post-S-RST to capture endogenous cFos expression using immunocytochemistry (ICC) for neurons comprising sucrose-seeking ensembles while the tagged cocaine-seeking neuronal ensemble retained tdTomato expression. Despite differences in sex within SA, no sex differences were found in cocaine- or sucrose-seeking behavior (Fig. S6a).

Immediately following euthanasia, we dissociated neurons from the NAcore and PFC regions using FACS separation of cocaine and sucrose-seeking neuronal ensembles. After debris removal (Fig. S7a), we used an cFos-TRAP2 control mouse with only a neuronal marker (NeuN+) and no ensemble tagging (Fig. 1e, Fig. S7b,c) to identify and sort cocaine- (C; lower right quadrant; tdTomato+) and sucrose-seeking (S; upper left quadrant; cFos+) neuronal ensembles in males (Fig. 1f; Fig. S7d) and females (Fig. 1g; Fig. S7e). We additionally sorted the overlapping ensemble (O; upper right quadrant; tdTomato+/cFos+), comprised of neurons activated during both cocaine and sucrose seeking. To ensure the downstream genetic analysis was specific to ensembles, we isolated neurons referred to as the non-ensemble (non-tagged, bottom left quadrant) that were not tagged in cocaine-seeking, sucrose-seeking, or overlapping ensembles. Additional flow cytometry analysis showed an overall significant main effect in the size of sex- and region-specific ensembles (Fig. S8a-d). After identifying and isolating cocaine-seeking, sucrose-seeking, and overlapping ensembles, we proceeded with transcriptomic characterization.

Reward-seeking ensembles differentially express ensemble-specific genes and genes shared with between ensembles in the male NAcore.

To understand genetic regulation specific to cocaine or sucrose seeking, we sequenced the RNA of lysates from reward-specific seeking ensembles for significant DEG comparison (FDR <0.1; Fig. S9a). Before comparing DEGs between reward-seeking ensembles, a principal component analysis of our samples revealed genetic profiles are defined by region- and sex-specific variation (Fig. S9b,c). Thus, we completed downstream DEG comparisons accordingly (Fig. S9d). DEG data sets for female and male cocaine-seeking, sucrose-seeking, and

overlapping ensembles in addition to non-ensemble neurons were determined in reference to female or male naïve data sets (Fig. S9e; Tab. S1). Without initially considering gene expression values, we compared DEGs in each seeking ensemble with the non-ensemble DEGs and revealed both ensemble-specific or shared genes between the ensembles and non-ensemble neurons (Fig. S9f). To identify DEGs specific to cocaine- or sucrose-seeking ensembles, we excluded DEGs that were specific to or shared with the non-ensemble population for downstream analysis. After excluding non-ensemble genes, we compared DEGs in the cocaine-seeking, sucrose-seeking, and overlapping ensembles amongst each other to determine genes that are specific or shared between reward-seeking ensembles in the NAcore and PFC (Fig. S9g).

Male-specific DEG analysis in the NAcore resulted in 2105 cocaine-seeking, 3282 sucrose-seeking, and 2265 DEGs in the overlapping ensemble that were specific to reward-seeking ensembles apart from the non-ensemble population (Fig. 2a). Following removal of non-ensemble DEGs, comparing DEGs from male reward-seeking ensembles in the NAcore with each other resulted in 427 DEGs specific to the cocaine-seeking ensemble, 1316 DEGs specific to the sucrose-seeking ensemble, 572 DEGs specific to the overlapping ensemble (Fig. 2b, Fig. S10a). Male cocaine-seeking and overlapping ensembles in the NAcore showed a higher ratio of downregulated DEGs compared to the sucrose-seeking ensembles that featured more upregulated DEGs (Fig. 2c).

The extremities of gene expression, i.e., the ten highest expressed and ten lowest expressed DEGs specific to the male cocaine-seeking ensemble in the NAcore, based on log transformation of the fold change (log2FC), showed robust differential expression in comparison to the expression of the same gene in sucrose-seeking and overlapping ensembles. For example, *Mef2c* (myocyte enhancer factor 2C) was upregulated in the male cocaine-seeking ensemble compared to the male sucrose-seeking and overlapping ensembles (Fig. 2d; Tab. S2). Similar to discrete genetic expression patterns in the male NAcore cocaine-seeking ensemble, we found the extremities of DEGs specific to the male sucrose-seeking ensemble within the NAcore showed differences in gene expression when compared to the expression of the same gene in male cocaine-seeking and overlapping ensembles. For example, *Psd* (pleckstrin and sec7 domain-containing) was upregulated for the male sucrose-seeking ensemble compared to the male cocaine-seeking and overlapping ensembles (Fig. 2e; Tab. S3). Additionally, *Phf21a* (phd finger protein 21a) was upregulated for the male overlapping ensemble in comparison to the male cocaine-seeking and sucrose-seeking ensembles (Fig. 2f; Tab. S4). We further characterized 1033 DEGs within the male NAcore shared between cocaine-seeking, sucrose-seeking, and overlapping ensembles and found mostly similar expression between genes for all reward-seeking ensembles (Fig. S11a-c; Tab. S5). Taken together, these data highlight genetic expression underlying cocaine- and sucrose seeking is both shared and distinct in the male NAcore.

Reward-seeking ensembles differentially express ensemble-specific genes and genes shared with between ensembles in the female NAcore.

Female-specific analysis of DEGs in the NAcore resulted in 2182 cocaine-seeking, 4973 sucrose-seeking, and 3225 DEGs in the overlapping ensemble specific to reward-seeking ensembles apart from the non-ensemble population (Fig. 2g). After removing non-ensemble DEGs, comparing ensemble-specific DEGs from each reward-seeking ensemble in the female NAcore with each other resulted in 279 DEGs specific to the cocaine-seeking ensemble, 2065 DEGs specific to the sucrose-seeking ensemble, 540 DEGs specific to the overlapping ensemble (Fig. 2h, Fig. S10b). Contrary to males, female cocaine-seeking and overlapping ensembles in the NAcore showed a higher ratio of DEGs upregulated overall compared to sucrose seeking which features more downregulated DEGs (Fig. 2i).

Extremities of DEGs specific to the female cocaine-seeking ensemble in the NAcore displayed a unique pattern of differential expression compared to the expression of the same gene in sucrose-seeking and overlapping ensembles. For example, *Prokr1* (prokineticin receptor 1) was upregulated in the female cocaine-seeking ensemble compared to the female sucrose-seeking and overlapping ensembles (Fig. 2j; Tab. S6). Additionally, the extremities of female gene expression within the NAcore specific to the sucrose-seeking ensemble showed distinct differential expression compared to the expression of the same gene in female cocaine-seeking and overlapping ensembles. *Psd*, highlighted previously as upregulated during sucrose seeking in the male NAcore, was also upregulated in the female sucrose-seeking ensemble compared to the female cocaine-seeking and overlapping ensembles (Fig. 2k; Tab. S7). Similarly to males, female DEGs in the NAcore overlapping ensemble

showed specific gene expression compared to expression in cocaine- and sucrose-seeking ensembles (Fig. 2l; Tab. S8). *Creb1* (camp responsive element binding protein 1) was downregulated the most in the female overlapping ensemble in comparison to the female cocaine-seeking and sucrose-seeking ensembles. We additionally identified 1440 DEGs within the female NAcore shared between cocaine-seeking, sucrose-seeking, and overlapping ensembles to reveal mostly common genetic expression between all reward-seeking ensembles similar to patterns seen in the male NAcore (Fig. S11d-f; Tab. S9). We thus identified DEGs with gene expression exclusive to and common between cocaine-seeking and sucrose-seeking ensembles in the female NAcore.

Reward-seeking ensembles differentially express ensemble-specific genes and genes shared with between ensembles in the male PFC.

Male-specific DEG analysis within the PFC resulted in 3423 cocaine-seeking, 3365 sucrose-seeking, and 2931 DEGs in the overlapping ensemble that were specific to reward-seeking ensembles apart from the non-ensemble population (Fig. 3a). After exclusion of non-ensemble DEGs, comparing DEGs from male reward-seeking ensembles in the PFC with each other resulted in 695 DEGs specific to the cocaine-seeking ensemble, 732 DEGs specific to the sucrose-seeking ensemble, 503 DEGs specific to the overlapping ensemble (Fig. 3b, Fig. S10c). Furthermore, cocaine- and sucrose-seeking ensembles in the male PFC showed a higher ratio of DEGs upregulated overall compared to overlapping that features more downregulated DEGs (Fig. 3c).

Similar to the NAcore, extremities of DEGs specific to male cocaine-seeking in the PFC displayed robust specificity of differential expression compared to the expression of the same genes in sucrose-seeking and overlapping ensembles. For instance, *Grip1* (glutamate receptor interacting protein 1) was downregulated in the male cocaine-seeking ensemble compared to the male sucrose-seeking and overlapping ensembles (Fig. 3d; Tab. S10). Going further, extremities of male DEGs specific to the sucrose-seeking ensemble within the PFC showed distinct up and downregulated expression compared to the expression of the same gene in male cocaine-seeking and overlapping ensembles. Again, *Psd* was specifically overexpressed in the sucrose-seeking ensemble. Previously described *Creb1* was also downregulated in the male PFC, with the sucrose-seeking ensemble having the lowest expression compared to the male cocaine-seeking and overlapping ensembles (Fig. 3e; Tab. S11). Like the NAcore, some DEGs in the male PFC were specific to the overlapping ensemble (Fig. 3f; Tab. S12). Furthermore, we characterized 1783 DEGs within the male PFC shared between cocaine-seeking, sucrose-seeking, and overlapping ensembles with overall similar genetic expression (Fig. S11g-i; Tab. S13). Analysis in the DEGs in the male PFC highlights both similar and unique gene expression between cocaine- and sucrose-seeking.

Reward-seeking ensembles differentially express ensemble-specific genes and genes shared with between ensembles in the female PFC.

Analysis of female-specific DEGs in the PFC resulted in 2020 cocaine-seeking, 2058 sucrose-seeking, and 2092 DEGs in the overlapping ensemble that were specific to reward-seeking ensembles apart from the non-ensemble population (Fig. 3g). Excluding non-ensemble DEGs and comparing DEGs from female reward-seeking ensembles in the PFC with each other resulted in 629 DEGs specific to the cocaine-seeking ensemble, 602 DEGs specific to the sucrose-seeking ensemble, 643 DEGs specific to the overlapping ensemble (Fig. 3h, Fig. S10d). Female sucrose-seeking and overlapping ensembles in the PFC showed a higher ratio of DEGs upregulated overall compared to cocaine seeking, which features more downregulated DEGs (Fig. 3i).

The extremities of DEGs specific to female cocaine seeking in the PFC displayed a distinct pattern of differential expression compared to the expression of the same gene in sucrose-seeking and overlapping ensembles. For example, *Fblim1* (filamin binding LIM protein 1) was upregulated for the female cocaine-seeking ensemble compared to the female sucrose-seeking ensemble and overlapping ensembles (Fig. 3j; Tab. S14). Similar to males, the extremities of female sucrose seeking specific DEGs within the PFC showed distinct differential expression compared to the expression of the same gene in female cocaine-seeking and overlapping ensembles (Fig. 3k; Tab. S15). *Tenm1* (teneurin transmembrane protein 1) was downregulated in the female sucrose-seeking ensemble compared to the female cocaine-seeking ensemble and overlapping ensembles. Like the NAcore, DEGs within the female PFC overlapping ensemble also displayed discretion in gene expression compared to the cocaine- and sucrose-seeking ensembles (Fig. 3l; Tab. S16). *Cinp* (cyclin dependent 2 kinase

interacting protein) was downregulated in the female overlapping ensemble in comparison to the female cocaine-seeking ensemble and sucrose-seeking ensembles. We additionally characterized 784 DEGs within the female PFC shared between cocaine-seeking, sucrose-seeking, and overlapping ensembles to find most genes also share similar genetic expression (Fig. S11j-l; Tab. S17). Like our results in the male and female NAcore, the PFC is characterized by genes both shared between and specific to cocaine-seeking, sucrose-seeking, and overlapping ensembles further defined by similar or opposite gene expression patterns.

Cocaine- and sucrose-seeking ensembles have both shared and discrete genetic expression between males and females within the NAcore.

We further compared DEGs between sexes from cocaine-seeking, sucrose-seeking, and overlapping ensembles within the NAcore and PFC (Fig. S9h). Within the NAcore, male and female mice more sex-specific DEGs than shared between sexes (Fig. 4a). Comparing cocaine-seeking ensembles in the NAcore resulted in 10 DEGs shared between both sexes, 417 male-specific DEGs, and 269 female-specific DEGs. Comparing sucrose-seeking in the NAcore showed 197 DEGs shared between both sexes, 1119 male-specific DEGs, and 1868 female-specific DEGs. Within the overlapping ensemble in the NAcore, we found 23 DEGs shared between both sexes, 549 male-specific DEGs, and 517 female-specific DEGs.

We found the 10 DEGs shared between sexes have similar and opposite expression patterns in the NAcore (Fig. 4b; Tab. S18). *Rab3d* (member RAS oncogene family) was downregulated in the NAcore for both female and male cocaine-seeking ensembles but not in the sucrose-seeking and overlapping ensembles. In addition, some DEGs shared between sexes have opposite expression levels in the NAcore. Within the 197 DEGs associated with sucrose seeking and shared between sexes in the NAcore, we found upregulation of *Psd* is shared in females and males compared to both sexes in cocaine-seeking and overlapping ensembles (Fig. 4c; Tab. S19). Within the 23 DEGs shared between sexes in the NAcore overlapping ensembles, we found *Camsap3* (calmodulin-regulated spectrin-associated protein family, member 3) was robustly downregulated in females and males in the overlapping ensemble compared to both sexes in the cocaine- and sucrose-seeking ensembles (Fig. 4d; Tab. S20). These data suggest that genetic expression underlying cocaine-seeking, sucrose-seeking, and overlapping ensembles in the NAcore is sex-dependent.

Cocaine- and sucrose-seeking ensembles have shared and discrete genetic expression between males and females within the PFC

Similar to NAcore patterns, comparing reward-seeking ensembles between sexes in the PFC resulted in DEGs both shared by and specific to males and females for cocaine-seeking, sucrose-seeking, and overlapping ensembles (Fig. 4e). Within the cocaine-seeking ensemble in the PFC, we found 21 DEGs shared between both sexes, 674 male-specific DEGs, and 608 female-specific DEGs. Within the sucrose-seeking ensemble in the PFC, we found 45 DEGs shared between both sexes, 687 male-specific DEGs, and 557 female-specific DEGs. Within the overlapping ensemble in the PFC, 21 DEGs were shared between both sexes, 482 were male-specific DEGs, and 622 were female-specific DEGs.

The 21 DEGs shared by males and females in cocaine-seeking ensemble showed both similar and different expression patterns in the PFC (Fig. 4f; Tab. S21). *Gipr* (gastric inhibitory polypeptide receptor) was upregulated in the female cocaine-seeking ensemble in comparison to downregulation in the male cocaine-seeking ensemble. In addition, we characterized 45 sex-shared genes within the PFC for sucrose seeking that displayed mostly differential expression compared to cocaine-seeking and the overlapping ensembles (Fig. 4g; Tab. S22). In addition to being downregulated in the female PFC for sucrose seeking (Fig. 3m), *Tenm1* gene expression was also downregulated in the male sucrose-seeking ensemble in the PFC compared to cocaine-seeking ensembles and overlapping ensembles. We report downregulation of *Rfx7* (regulatory factor x 7) occurred in females and males overlapping ensemble compared to both sexes in cocaine and sucrose-seeking ensembles (Fig. 4h; Tab. S23). Similar to the NAcore, genetic expression in the cocaine-seeking, sucrose-seeking, and overlapping ensembles are contingent on sex in the PFC.

Cocaine- and sucrose-seeking ensembles have similar and unique genetic expression between the male NAcore and PFC

We further compared DEGs between the male NAcore and PFC to determine region-dependent alterations within cocaine and sucrose-seeking ensembles (Fig. S9i). Although most genes were region-specific, we observed genes shared between regions for each reward-seeking ensemble (Fig. 5a). Within the 21 DEGs shared between the male NAcore and PFC in cocaine-seeking ensembles, we found *Tmem245* (transmembrane protein 245) was downregulated in the NAcore and upregulated in the PFC cocaine-seeking ensembles (Fig. 5b; Tab. S24) in comparison to the sucrose-seeking and overlapping ensembles. Furthermore, we found 157 genes shared between the male NAcore and PFC sucrose-seeking ensembles with mostly unique genetic expression compared to cocaine-seeking and overlapping ensembles (Fig. 5c; Tab. S25). We, again, saw overexpression and discretion of *Psd* was again robustly overexpressed in all sucrose-seeking ensembles except for the female PFC. Additionally, we found discrete patterns of expression within the 24 genes associated with the overlapping ensemble (Fig. 5d; Tab. S26). For example, *Trim13* (tripartite motif containing 13) was downregulated in both the NAcore and PFC in males for the overlapping ensemble. Taken together, we suggest genetic alterations underlying cocaine-seeking, sucrose-seeking, and overlapping ensembles are region-dependent in males.

Cocaine- and sucrose-seeking ensembles have similar and reward-specific genetic expression between the female NAcore and PFC

Similar to males, most genes were discrete to the female NAcore or PFC with a lesser portion of DEGs shared between both regions (Fig. 5e). The 9 DEGs shared between the female NAcore and PFC in cocaine-seeking ensembles (Fig. 5f; Tab. S26) had similar direction of expression in contrast to the male DEGs shared between regions which show both bidirectional and similar expression of the same gene. For example, *Olfr1286* (olfactory receptor 1286) was downregulated in both regions for female cocaine-seeking ensembles compared to female sucrose-seeking and overlapping ensembles. Additionally, we found 123 genes shared between the female NAcore and PFC sucrose-seeking ensembles had mostly discrete genetic expression compared to expression of the same gene in cocaine-seeking and overlapping ensembles (Fig. 5g; Tab. S27). Downregulation of *Tenm1* occurred in both regions for female sucrose seeking which was previously seen downregulated for both sexes within the PFC (Fig. 4g). Similar to males, we found discrete patterns of expression within the 30 genes associated with the female overlapping ensemble (Fig. 5d; Tab. S26). Previously described in females for both regions, our findings further show downregulation of *Rfx7*, *Campsap3*, and *Cinp* is shared between the NAcore and PFC for the female overlapping ensembles. Altogether, we report that genetic alterations within female cocaine-seeking, sucrose-seeking, and overlapping ensembles are region-dependent.

Cocaine- and sucrose-seeking networks are more intertwined in the NAcore compared to the PFC.

To further characterize cocaine- and sucrose-seeking DEGs, we utilized a multi-level gene ontology (GO) analysis to identify genetic networks shared between sexes within the cocaine-seeking, sucrose-seeking, and overlapping ensembles. For this, we used previously discussed DEGs shared between females and males for each reward-seeking ensemble in the NAcore (Fig. 4a) and PFC (Fig. 4e), i.e., 10 DEGs for the NAcore cocaine-seeking ensemble, 197 DEGs for the NAcore sucrose-seeking ensemble, 23 DEGs for the NAcore overlapping ensemble, 21 DEGs for the PFC cocaine-seeking ensemble, 45 DEGs for the PFC sucrose-seeking ensemble, and 21 DEGs for the PFC overlapping ensemble.

Analysis of GO pathways in the NAcore resulted in a total of 74 annotated clusters consisting of 1819 significantly enriched pathways (FDR < 0.1; Fig. 6a; Tab. S30,31). 85.4% of pathways were specific to cocaine-seeking, sucrose-seeking, or the overlapping ensembles – 177 cocaine-seeking, 1078 sucrose-seeking, and 332 overlapping. In addition to discrete pathways, 14.6% of pathways were shared between reward-seeking – 27 shared between all reward-seeking ensembles, 73 shared between cocaine and sucrose-seeking ensembles, 18 shared between cocaine-seeking and overlapping ensembles, and 114 shared between sucrose-seeking and overlapping ensembles. Within the PFC, a total of 28 clusters were annotated, consisting of 768 significantly enriched pathways (Fig. 6b; Tab. S32,33). 98.1% of pathways were specific to cocaine, sucrose, or overlapping seeking – 65 cocaine seeking, 473 sucrose seeking, and 216 overlapping seeking – while 1.9% of pathways were shared amongst reward seeking – 1 shared between all reward seeking, 2 shared between cocaine and sucrose seeking, 2 shared between cocaine and overlapping seeking, and 9 shared between sucrose and overlapping seeking. Within a region, most pathways are highly specific to cocaine-seeking, sucrose-seeking, or

overlapping ensembles. However, we observed that the NAcore has more intertwined pathways between seeking ensembles than the PFC, which shows more discrete pathways between rewards.

The largest cluster of pathways within the NAcore was annotated as "post-transcriptional regulation," featuring shared pathways between cocaine-seeking, sucrose-seeking, and overlapping ensembles. We also identified pathways associated with only the cocaine-seeking ensemble, e.g., "nf kappab signaling." Compared to the NAcore, the PFC exhibited more ensemble-specific pathways, with the largest cluster annotated as "induced cellular regulation" featuring only pathways exclusive to sucrose-seeking or the overlapping ensemble. We further identified GO clustered pathways in the PFC associated only with the cocaine-seeking ensemble, e.g., "epigenetic chromatin remodeling." In addition to epigenetic pathways enriched in the PFC, we identified two similar epigenetic-related clusters in the NAcore and the PFC, e.g., "deacetylase/deacylation histone deacetylation." Cocaine-seeking thus disrupts post-translational and epigenetic regulation in female and male reward-signaling regions.

Discussion

Sex-dependent transcriptome profiles underlying cocaine-seeking ensembles are not fully characterized in critical regions of reward circuitry, particularly in comparison to natural reward seeking. We used within-subject cocaine and sucrose dual SA conditioning followed by extinction training and cued reinstatement to induce cocaine and sucrose seeking in cFos-TRAP2 mice (Fig. 1b-d). Through FACS (Fig. 1e-g), we isolated neurons from reward-specific seeking ensembles and the non-ensemble population for downstream genetic profiling in reference to naïve controls. Within two critical reward-signaling regions, the NAcore and PFC, we used an unbiased approach to identify sex- and region-dependent genetic signatures exclusive to or shared between cocaine- and sucrose-seeking ensembles in addition to ensembles activated during both cocaine and sucrose seeking, i.e., overlapping ensembles.

Cocaine-seeking and sucrose-seeking neuronal ensembles recruit both distinct and shared neuronal ensembles in the NAcore and PFC of mice.

Our current study was based on previous results (*8*) showing cocaine- and sucrose-seeking ensembles are both specific and overlapping in the NAcore for both sexes. We expanded on these findings by isolating reward-defined ensembles in the NAcore in addition to the PFC using FACS (Fig. 1f,g). While it is known that different cell types are involved in ensembles (*26*), we limited our FACS procedure to isolate neuronal ensembles exclusively. The flow cytometric results determined that the size of female and male cocaine- and sucrose-seeking ensembles are equivalent in the NAcore and PFC except for the female PFC, where the sucrose-seeking ensemble was larger (Fig. S8a-d). We suggest the difference in ensemble size could be due to variability in the tagging process or possibly linked to the estrous cycle, discussed further. Considering the size differences in ensembles, we note that an equal number of events were sorted for each reward-seeking ensemble for normalization. We further conducted normalization of RNAseq counts within the DESeq2 pipeline to address inconsistencies within the sorting process. The DESeq2 pipeline used here determined DEG significance using adjusted p-values (FDR) with a stringent cutoff (< 0.1) (*25*, *27*).

Genes underlying cocaine and sucrose seeking are similarly and differently expressed in the NAcore or PFC.

In reference to naïve mice, we identified significant ensemble-specific genetic alterations in addition to changes present in the non-ensemble population, i.e., non-active neurons during seeking behaviors (Fig. S9e). We also found genes shared between reward-specific ensembles and the non-ensemble populations, suggesting multifaceted genetic mechanisms that are not exclusive to ensembles (Fig. S9f). Further characterization of reward-defined genes revealed similarity and specificity in cocaine-seeking, sucrose-seeking, and overlapping ensembles (Fig. S9g). Anayzing the extremities of reward-specific gene expression in the NAcore (Fig. 2e-g, l-n) and PFC (Fig. 3e-g, l-n) revealed patterns of exclusive gene expression for cocaine-seeking, sucrose-seeking, or overlapping ensembles for both sexes. Correlating reward-specific genes with previous annotations allowed further understanding of the genes involved in transcriptional, epigenetic, and cellular regulation of cocaine seeking.

Reward-specific transcriptomic comparisons within the NAcore highlighted discrete expressions for each reward-seeking ensemble (Fig. 2), such as *Mef2c* overexpression in the male NAcore. Overexpression of *Mef2c* has previously been linked with increased cocaine responses (*28*). *Mef2c* facilitates phosphorylation of HDACs regulating epigenetic landscapes and is thought to allow cocaine dependence to persist (*29*). Our findings of increased *Mef2c* expression discrete to the male NAcore during cocaine seeking further supports the idea that *Mef2c* facilitates long-term cocaine memory formation underlying cocaine seeking apart from memories linked with natural reward seeking. We also characterized distinct upregulation of *Phf21a* in the male overlapping ensemble. *Phf21a* is an H3K4 histone-binding protein linked with disruptions in intellectual capability due to genetic aberration in neurodevelopment processes (*30*, *31*). Additionally, *Phf21a* has been shown to be associated with noncontingent heroin exposure in the hippocampus of nonhuman primates, a critical region linked with learning and memory formation (*32*). Expression of genes regulating learning like *Phf21a* in both

cocaine- and sucrose-seeking ensembles suggests that cocaine allows reconfiguration of pathways that otherwise regulate natural reward seeking.

In parallel with reward-specific genetic changes in the NAcore, we observed distinct genetic expressions linked with each reward-seeking ensemble in the PFC (Fig. 3). We previously highlighted the upregulation of *Fblim1* in the female PFC. *Fblim1* has been shown to be correlated with genes hypomethylated after cue-reinstatement (*33*). Together, these results support that methylation-directed transcriptional modifications are critical in facilitating an epigenetic landscape underlying the persisting effects of cocaine seeking (*17*). In the male PFC cocaine-seeking ensembles, we observed downregulation of *Grip1*, a glutamate receptor previously linked with increased cocaine seeking without affecting food seeking in the PFC (*34*). In support of this previous annotation, our results also showed downregulated *Grip1* only in the cocaine-seeking ensemble, apart from sucrose-seeking and the overlapping ensembles.

Differentially expressed genes exclusive to cocaine-seeking ensembles should be prioritized for behavioral follow-up experiments, i.e., manipulating target genes in the NAcore or PFC and observing the effect on cocaine and sucrose seeking.

Sex differences are defined in cocaine- and sucrose-seeking behavior, ensembles, and underlying transcriptomes.

We identified considerable sex differences supporting previous findings of sex-dependent characteristics in CUD (*18*). While we did not observe a higher sucrose intake in females shown in a previous study (*8*), females interacted significantly more with the NP associated with cocaine during the sucrose sessions compared to males (Fig. S3b) and showed higher cocaine preference during sucrose self-administration (Fig. S3c). Females could be more susceptible to the maladaptive rewarding effects of cocaine or could show learning delays after cocaine exposure compared to males. Cue-induced seeking behavior was equal in male and female mice (Fig. S1l), which is different from what is seen in female patients, who are more likely to report increased craving in response to cocaine-associated cues than males (*35*). We did not record the estrous cycle in this study to avoid stress-induced responses (*36*), but it is known that the estrous cycle influences cocaine-seeking (*37*) and food rewards (*38*) in female rats.

Sex differences and similarities in gene expression were observed in cocaine-seeking, sucrose-seeking, and overlapping ensembles (Fig. 4). Sex-dependent principal component analysis (Fig. S9c) showed a higher variation between naïve and reward-exposed mice in males than females suggesting that males have more robust genetic alterations in reward-seeking. Our findings revealed genes shared between both sexes within the NAcore have dynamic and reward-dependent genetic expression. For instance, *Rab3d*, a synaptic vesicular GTP-binding protein (*39*), was downregulated in both sexes during cocaine-seeking. Supporting our finding, *Rab3* expression, including isoform *Rab3d*, has previously been associated with increased cocaine responses (*40*). Although *Rab3d* expression was downregulated in cocaine-seeking for both sexes, expression was increased only in females for both sucrose-seeking and overlapping ensembles, suggesting that *Rab3d* has a sex-dependent alternative role in natural reward signaling. In contrast to having similar direction of expression in cocaine-seeking, *Prokr1* displayed sex-specific expression with downregulation in male and upregulation in female cocaine-seeking ensembles. Expression of *Prokr1*, a g-protein coupled receptor (*41*), is linked to self-administration of oxycodone (*42*). Our data suggests that *Prokr1* plays an additional role in facilitating cocaine seeking in the NAcore which is dependent on sex. Furthermore, our reward- and sex-dependent comparisons highlighted genes that were shared between sex in the PFC for cocaine seeking but with dynamic expression, e.g, *Gipr*, upregulated in females and downregulated in males. This supports previous results showing knockdown of *Gipr* leads to increased expression of genes known to regulate cocaine-seeking (*43*), e.g., *Bdnf* (brain-derived neurotrophic factor) (*44*) and *Cart* (cocaine and methamphetamine-regulated transcript) (*45*). Here, we provide evidence that regulation of *Gipr* expression during cocaine-seeking is sex-specific.

In addition to differential genetic expression during cocaine seeking in both females and males, we further identified sex-dependent genetic expression of genes shared between cocaine and sucrose seeking, e.g., *Creb1*. Although under-expression of *Creb1* has been associated with enhancing motivation to attain both cocaine (*46*) and sucrose (*47*), it was not annotated in a within-subject model. We observed overall downregulation of *Creb1*

in all reward ensembles in the male PFC with the lowest expression in the sucrose-seeking ensemble compared to the cocaine-seeking ensemble and overlapping ensemble where expression was similarly underexpressed. Contrary to males, females featured overall *Creb1* downregulation but with the lowest expression in the overlapping ensemble. These findings further detail how *Creb1* is downregulated in the female NAcore to highlight that, despite having overall downregulation, there are sex-contingent differences in the level of downregulation between cocaine and sucrose seeking. Importantly, our findings highlight significant and distinct sex differences in the behavior and genetics underlying cocaine seeking in relation to sucrose seeking.

Genetic expression underlying cocaine-seeking, sucrose-seeking, and overlapping ensembles are region-dependent.

Cocaine-seeking ensembles in multiple brain regions have been shown to regulate reward signaling (*48*). However, it is unclear whether system-wide genetic alterations exist to drive cocaine-seeking and how they relate to sucrose-seeking. In our study, we investigated the transcriptomes in the NAcore and PFC (Fig. 5). Importantly, the number of DEGs specific to cocaine-seeking ensembles in the NAcore was much lower than the number of DEGs in the sucrose-seeking ensemble. In the PFC, the number of DEGs was stable across reward-specific ensembles (Fig. S9g). In addition, we reported more sucrose-seeking DEGs in the NAcore than in the PFC. This suggests that the NAcore is more involved in sucrose-seeking regulation in comparison to the PFC, which shows higher genetic regulation during cocaine seeking than during sucrose seeking.

Additionally, we observed genetic expression within each reward-seeking ensemble with similar or differential expression (Fig. 5). For instance, in the NAcore and PFC, we observed downregulation of *Olfr1286* in females. Previously, it has been shown that *Olfr1286* is downregulated during withdrawal from tobacco (*49*) and morphine (*50*). We hypothesize that *Olfr1286* is also linked with persisting effects from cocaine withdrawals that facilitate cocaine seeking. Contrary to the similarity in the direction of expression for *Olfr1286* in both regions, *Tmem245* was downregulated in male NAcore compared to the upregulation in the male PFC. *Tmem245* has been linked with schizophrenic psychosis (*51*), which is similar to psychostimulant-induced psychosis (*52*). Here, we hypothesize a region-dependent role for *Tmem245* in facilitating psychosis-like behavior during cocaine withdrawal linked with increasing the likelihood of seeking cocaine during periods of abstaining cocaine intake. In addition, we found similar and unique genetic expression between the NAcore and PFC for DEGs specific to the overlapping ensemble. We describe a male-specific region-dependent *Trim13* expression (upregulated in the PFC and downregulated in the NAcore) during cocaine seeking. *Trim13* has previously been annotated to regulate cellular architecture and is linked with differing expression between noncontingent cocaine and methamphetamine exposure in the hippocampi of nonhuman primates (*32*).

Further, we identified genetic expression shared between both regions while sex-dependent for the sucrose-seeking and overlapping ensembles. Upregulation of *Psd* was observed in both sexes for sucrose-seeking but was only shared in both regions for males. *Psd*, also known as *Efa6a*, is a neural-growth regulating gene (*53*) contiguous with the coding region for NF-kB (*54*), a gene regulating cocaine responses (*17*, *55*). Interestingly, *Psd* regulates sucrose seeking with very discrete expression considering the proximity to the NF-kB coding region. Downregulation of *Tenm1* was observed in both sexes for sucrose-seeking but was only shared in both regions for females. The teneurin family, including *Tenm1*, are cell-surface adhesion proteins regulating axonal guidance and have been linked with regulating cAMP-based signaling (*56*) upstream of *Creb1* transcriptional regulation (*57*). Here, we have described dynamic *Creb1* regulation involved in the sucrose seeking and the overlapping ensemble. Additionally, downregulation of *Rfx7* was observed in the overlapping ensemble. The *Rfx* (regulatory factor x) family, including *Rfx7* as the highest expressing isoform within the brain (*58*), is known to regulate methylation of cytosine residues in DNA affecting the epigenetic availability for transcription to occur (*59*). Here, we propose a new region- and sex-shared role for Rfx7 in regulating both cocaine and sucrose seeking.

Gene ontology is shared and discrete between cocaine-seeking, sucrose-seeking, and overlapping ensembles with more specificity in the PFC compared to the NAcore

We have shown evidence of diverse and convergent genetic expression between cocaine- and sucrose-seeking ensembles. We further correlated sex-shared genetic expression with ontological annotations to provide a

holistic perspective on how genetic signatures underlying cocaine and sucrose seeking are related (Fig. 6). In the NAcore, we identified both discrete and shared pathways between cocaine and sucrose seeking within the largest cluster described as “post-transcriptional regulation.” Post-transcriptional regulation is known to be altered during cocaine (*60*) or sucrose exposure (*61*) underlying the persisting genetic perturbations that facilitate cocaine- or sucrose-seeking behavior (*7*). Here, we highlight the complexity of transcriptional regulation within multiple types of reward-seeking in the NAcore. We also report pathway clusters in the NAcore that are cocaine-seeking specific, e.g., “nf kappab signaling.” Our finding supports the previously described role of nuclear factor kappa B (NF-kB) in increasing the preference for cocaine reward (*55*) while not altering sucrose-seeking (*62*). In our analysis of genetic ontology in the PFC, we highlighted there are both specific and shared pathways between cocaine and sucrose seeking with the largest cluster described as “induced cellular regulation.” Supporting our finding, it has been shown that disruptions in cellular regulation are induced by cocaine (*63*) or sucrose exposure (*64*). Considering the lack of specificity for cocaine seeking in this cluster, this finding suggests a large portion of cellular regulation of sucrose seeking is also involved in cocaine seeking. In addition to shared ontology, we identified clusters discrete to cocaine seeking in the PFC, “epigenetic chromatin remodeling.” Further, we identified two similar epigenetic-modifying pathway clusters within the NAcore (“deacetylase histone deacetylation”) and PFC (“deacylation histone deacetylation”) distinct to cocaine seeking for both sexes. Epigenetic histone and DNA modifications regulate genetic expression initially altered during cocaine exposure and are thought to facilitate persisting cocaine seeking (*65–69*). Specifically, little is known about how diacylation plays a role in post-transcriptional regulation, but recent research has revealed new roles of protein deacylation to allow genetic diseases to form and persist (*70*). Thus, we suggest there are epigenetic modifications specific to cocaine seeking apart from sucrose-seeking.

Considerations in RNA sequencing

Although we gained great insight into how cocaine- and sucrose-seeking are genetically related through a bulk RNA sequencing approach, we recognize that using a single-cell RNA sequencing approach allows for a deeper understanding of how the heterogeneous population of reward-associated brain regions is defined by specific transcriptomes (*71*). Recently developed spatial RNA sequencing (*72*) also provides genetic information for single-cell types from a traceable region such as the NAcore or PFC.

Conclusion

We identified, isolated, and characterized specific and shared patterns of gene expression within cocaine-seeking, sucrose-seeking, and overlapping ensembles. This repository of reward-specific genetic information is a valuable resource for further functional validation of target genes to reduce cocaine-seeking without altering natural reward-seeking behavior.

Acknowledgments

This project was supported by NIDA DA046522 (A-CB), NIGMS-NIA P20GM121310 (A-CB), NIGMS IDeA 2P20GM103432 (A-CB, NB). The authors thank the University of Colorado Anschutz Genomics and Microarray Core for performing the RNA sequencing and all the members of the Bobadilla Lab for their help and support.

Materials and Methods

cFos-TRAP2 Mice

Female and male cFos-TRAP2 transgenic mice were obtained by crossing male tamoxifen-inducible TRAP2 Cre-recombinase knock-in mice (Fostm2.1(icre/ERT2)Luo/J, Strain# 030323; The Jackson Laboratory) with female Ai14 loxP-flank regulated Cre-reporter knock-in mice (B6;Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J; Strain# 007914; The Jackson Laboratory). Mice were individually housed on a 12:12 reverse light schedule during the length of dual-reward self-administration, extinction training, and cue-induced reinstatements. Sample size and attrition rate were determined from dual-reward self-administration conditioning previously established methodology (*8*). All procedures were conducted in accordance with the University of Wyoming and Colorado State University IACUC and NIH animal handling procedures.

Dual Reward Self-Administration, Extinction, And Reinstatement Tests

Both sexes were used for dual-reward self-administration of intravenous cocaine or oral sucrose intake. During self-administration acquisition, all mice were on a food-restricted diet (80% of normal food intake) to increase reward-specific operant conditioning responses. Female and male 8-16-week-old mice (20-30g) underwent catheter implantation following a previously established methodology (*8*). All mice underwent dual-reward self-administration of intravenous cocaine infusions and oral intake of sucrose pellets during 2-hour daily sessions (10 days of cocaine reward and ten days of sucrose reward: 20 alternating days in total) within standard mouse modular test chambers. Each chamber included two nose-poke (NP) holes associated with cocaine infusions (0.5 mg/kg/inf; NIDA) or delivery of sucrose pellets (15 mg; Bio-Serv). Each NP was paired with an availability light cue (always on until nose poked; off for ten seconds after NP activation) as well as an internal NP light cue (on for 3 seconds after NP activation). The reward-associated NP not used during a reward-specific session served as an inactive NP negative control (i.e., cocaine-associated NP during a sucrose session acted as an inactive NP with no cues or reward administration and vice versa). Following reward-specific cue conditioning, mice were extinguished of rewards or cues until reaching a 70% reduction of NP association compared to the averaged last three days of reward self-administration. Following the extinction phase, 30-minute reinstatement sessions for each reward were carried out with the reward-associated light cues and without administering the reward. Following intraperitoneal injection of 4-hydroxy tamoxifen (4-OHT, 50 mg/kg) directly after the cocaine reinstatement session, the Cre-regulated TRAP2 mechanism tagged active cocaine-seeking cells with endogenous tdTomato fluorescence. Following sucrose reinstatement sessions, mice were euthanized 60 minutes post-session to capture cFos expression reflective of active cells during sucrose-seeking behavior.

Extraction of Reward-Specific Ensemble RNA

Whole brains were extracted from euthanized mice (5% isoflurane exposure followed by cervical dislocation) and incubated for 5 minutes on ice in cellular stabilizing solution: 45% HEBG medium (2% 50X B27 (Thermo Fisher; 17504044) and 0.25% 100X Glutamax (Thermo Fisher; 35050061) per 1 mL of Hibernate E-Minus Phenol Red (Thermo Fisher; NC1506837)), 45% artificial cerebral spinal fluid (aCSF; 7.13 g/L NaCl (Sigma-Aldrich; S9888-500G), 0.23 g/L KCl (Sigma-Aldrich; P3911-25G), 0.14 g/L MgSO4 (Sigma-Aldrich; 746452-500G), 0.19 g/L CaCl2 (Sigma-Aldrich; C33067-100G), 0.048 g/L NaH2PO4 (Millipore-Sigma; 10049-21-5), 2.1 g/L NaHCO3 (Millipore-Sigma; 144-55-8), 1 L diH2O adjusted for dry reagent volumes), 9.97% RNAlater (Thermo Fisher; AM7020), and 0.03% RNaseOUT (Thermo Fisher; 10777019). Brain sections (1.94-0.64 bregma; 1.5 mm thickness) containing the nucleus accumbens core and prefrontal cortex were cut using a brain matrix (0.5 mm spaced slits) kept cold at -20°C with ice-cold razors. Punches of brain tissue were made using a 1 mm core puncher for each nucleus accumbens bilateral region and 1.5 mm sized punches for the prefrontal cortex. Brain regions from three mice per sex were pooled together for adequate RNA collection. Brain punches were immediately placed in enzymatic dissociation solution (2mg/mL papain (Worthington; LS003120) diluted in 1 mL HEBG with 0.125% 100x Glutamax – heated in 30C water bath for 10 minutes before immediately placing on ice for brain punches) on ice for 20 minutes. Mechanical trituration while incubating in papain solution was carried out using 10x syringe fluctuations with a 23G needle followed by a 27G needle. Caution is taken to not introduce air bubbles during syringe fluctuations that otherwise rupture cell membranes. Tissue was centrifuged for 3 minutes at 1000 rcf at 4°C, and the supernatant was carefully discarded via pipette, leaving ~20 uL medium to

prevent significant cell loss. Cells were slowly resuspended via pipette with 500 uL of 4°C HEBG medium and then filtered through a 40 um cell strainer. Semi-permeabilization was carried out by adding 500 uL of -20°C ethyl acetate (95% ethanol; 5% acetate) to the 500 uL of HEBG medium with cells for a final 50% ethyl acetate concentration. Tubes were slowly inverted by hand for 1 minute briefly before centrifuging for 5 min at 1000 rcf in 4°C. The supernatant was discarded carefully via pipette leaving ~20 uL to prevent cellular loss and cells are resuspended gently via pipette in 500 uL of 4°C HEBG medium with neuronal (NeuN-Alexafluor-405; conc. 5:500; Novus Biologicals; NBP1-92693AF405) and cFos-labeling conjugated antibodies (cFos-Alexafluor-488; conc. 5:500; Novus Biologicals; NBP2-50037AF488). Cells were incubated at 4°C while rotating in the dark for 45 minutes. Cells were centrifuged for 5 minutes at 1000 rcf at 4°C, and the supernatant was carefully discarded via pipette, leaving ~20 uL medium to prevent significant cell loss. Cells were resuspended in 1 mL of 4°C HEBG medium and filtered with a 40 um cell strainer into a sterile 5 mL FACS tube and kept at 4°C. Cells were then sorted for neuronal and reward-specific tags (neuron – immunolabeled NeuN; cocaine – endogenous tdTomato; sucrose – immunolabeled cFos; overlap – tdTomato/cFos) using FACS (FACSMelody; FACSChorus Software 100-um nozzle; BD Biosciences). 80k-100k events per reward-specific population were sorted directly into the RLT buffer (RNeasy Mini Kit; Qiagen; 74104) for downstream RNA extraction using RNeasy Mini Kit. RNA samples were kept at -80°C until downstream RNA sequencing.

RNA Sequencing of Reward-Specific Neurons

RNA samples (average 260/280 ratio of 2.12) underwent library preparation and next-generation RNA sequencing at the University of Colorado Anschutz Medical Campus Genomics and Microarray Core, Colorado, USA. Low-input ribosome depleted (SMART-seq; Takara Bio; library size: 200-500bp) library prep was used for downstream paired-end 2x150bp RNA sequencing at a 50 million read depth using NovaSEQ 6000 (Illumina). Raw fastq output files were used for downstream bioinformatic analyses. Raw fastq files are being uploaded on gene expression omnibus (GEO, accession # TBD).

Bioinformatic Analysis of Reward-Specific Transcriptomes

Raw sample files in fastq form were trimmed for Takara library prep adaptations using the "CutAdapt" R package (*73*). Samples were mapped to mouse transcriptome (grc39) using Linux-based "Salmon" software (*74*). Output counts from mapping were further normalized and analyzed for principal component variation and differentially expressed genes (DEG) utilizing "tximport" (*75*) and "DESeq2" (*27*) R packages. DEG lists are represented in log2(fold change) format. Venn diagrams created using the "venndetail" (*76*) and "ggven" (*77*) R packages were used to compare sex-, reward-, and region-specific genes with a false discovery rate (FDR) lower than 0.1. Heatmap representations of log2FC gene expression values were created using Prism 10 (GraphPad) and ordered sequentially based on the relevant significant specific DEG list (represented by *). Volcano plots were created using the "EnhancedVolcano" (*78*) R package to compare significance (-log(FDR)) and gene expression (log2FC) of sex-, reward-, and region-specific DEG lists. Gene ontology (GO) pathway analysis was sequential. First, output data from gene set enrichment analysis (g:Profiler) of biological processes was inputted into Cytoscape for "EnrichmentMap" (*79*) network analysis to compare sex-shared reward- and region-specific gene sets. Second, the "AutoAnnotate" (*80*) Cytoscape application clustered and labeled gene sets based on the top three words shared between enriched pathways.

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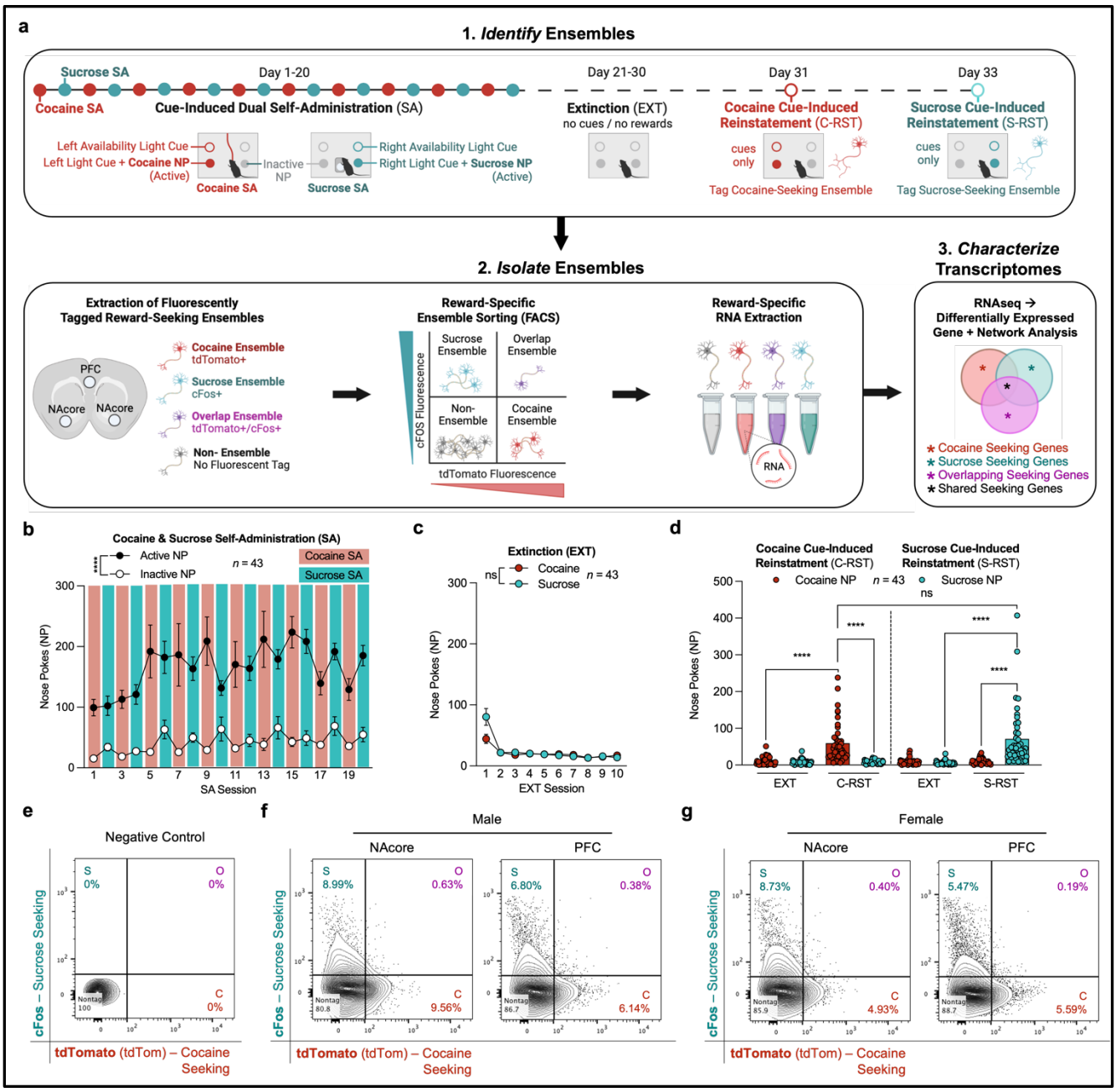


Fig. 1 | Polyreward self-administration of cocaine and sucrose and cell sorting of reward-specific ensembles. a. Behavioral training timeline for inducing cocaine and sucrose seeking for downstream fluorescently activated cell sorting and transcriptomic characterization of reward-specific ensembles from the nucleus accumbens core (NAcore) and prefrontal cortex (PFC) . **b.** During SA, mice interacted significantly more with the cocaine-seeking and sucrose-reinforced active NP (repeated measures two-way ANOVA for SA Active NP vs. SA Inactive NP over 20 sessions; NP $F_{(1, 84)} = 70.85$, $P<0.0001$; session $F_{(5.147, 432.3)} = 3.343$, $P=0.0052$; interaction $F_{(19, 1596)} = 2.004$, $P=0.0063$). **c.** During EXT, mice diminished differentiation between the active NP paired with cocaine or sucrose SA (repeated measures two-way ANOVA for EXT Cocaine NP vs. EXT Sucrose NP over 20 sessions; NP $F_{(1, 84)} = 1.005$, $P=0.3190$; session $F_{(1.758, 147.7)} = 28.98$, $P<0.0001$; interaction $F_{(9, 756)} = 4.886$, $P<0.0001$). **d.** Induction of cocaine-seeking and sucrose-seeking behavior displayed by significantly increased interaction with the active NP in comparison to the inactive NP during both C-RST (paired t-test for C-RST Cocaine NP vs. C-RST Sucrose NP; $P<0.0001$) and S-RST (paired t-test for S-RST Sucrose NP vs. S-RST Cocaine NP; $P<0.0001$) in addition to significantly increased interaction with the active NP in comparison to the level of active NP interaction in the last EXT session before C-RST (paired t-test for C-RST Cocaine NP vs. C-EXT Cocaine NP; $P<0.0001$) or S-RST (paired t-test for S-RST Sucrose NP vs. S-EXT Sucrose NP; $P<0.0001$). There was no difference when comparing levels of interaction with the active NP between C-RST and S-RST sessions (paired t-test for C-RST Cocaine NP vs. S-RST Sucrose NP; $P=0.2399$). **e.** Flow cytometric quadrant plot for negative control absent of antibody for cocaine-seeking and sucrose-seeking ensembles. **f,g.** Flow cytometric quadrant plots for male (**f**) and female (**g**) with percentages of cocaine-seeking neurons (C; bottom-right; tdTomato+/cFos-), sucrose-seeking neurons (S; top left; cFos+/tdTomato-) and overlapping neurons between cocaine-seeking and sucrose-seeking (O; top right: tdTomato+/cFos+) and non-ensemble (bottom-left; tdTomato-/cFos-) "nontag" neurons. Quadrant plots are a representative for male (Fig. S2d) and female replicates (Fig. S2e).

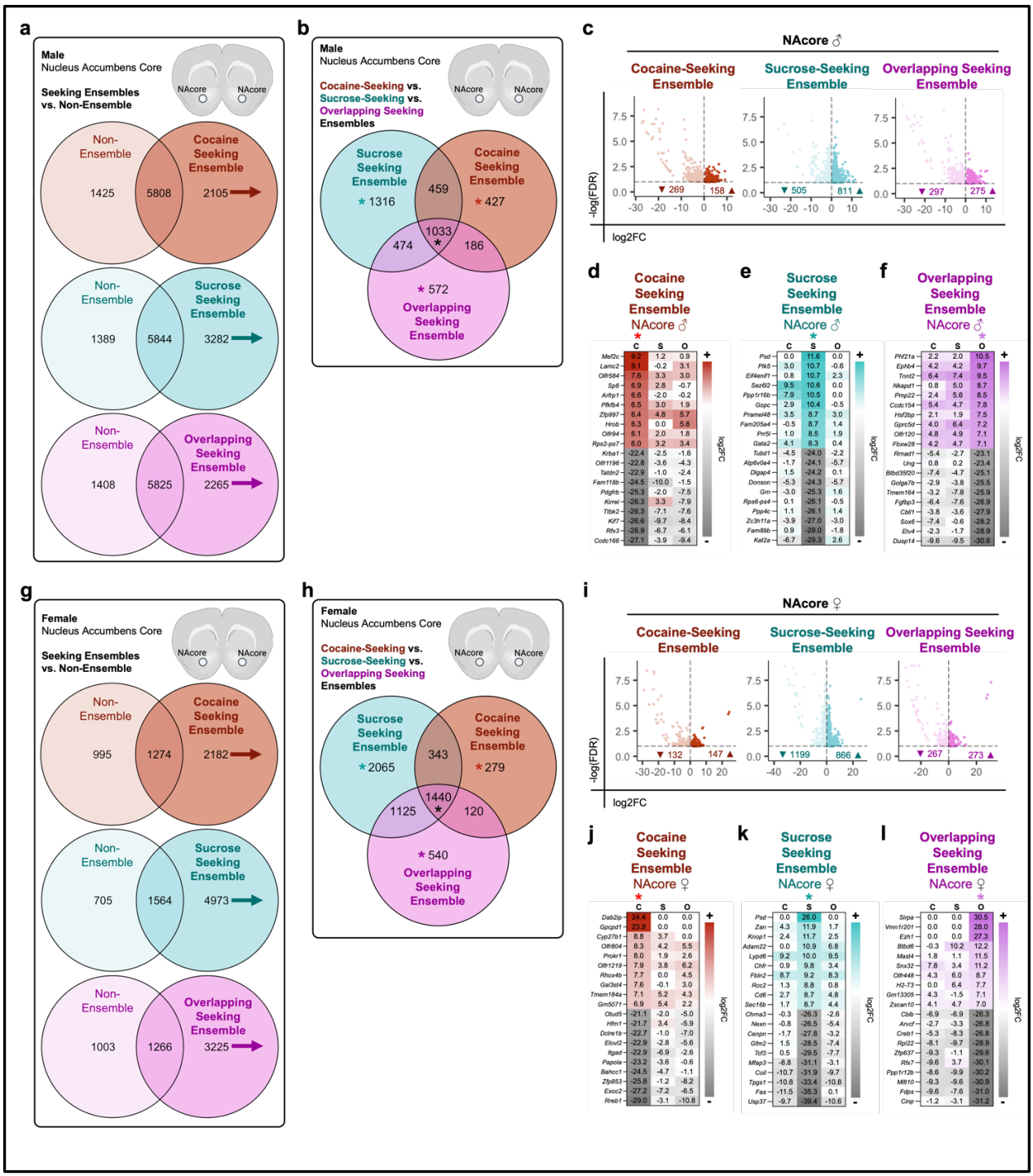


Fig. 2 | Cocaine-seeking and sucrose-seeking ensembles have overlapping and discrete genetic factors within the nucleus accumbens core (NAcore). All DEG data sets are in reference to naive female and male mice. **a,g.** Two-way Venn diagrams comparing differentially expressed genes (DEG) in the male (**a**) and female (**g**) cocaine-seeking, sucrose-seeking, and overlapping ensembles to the non-ensemble population within the NAcore. DEGs moving forward for further reward-specific comparison in fig. 2b (male) and fig. 2h (female) are represented by red (cocaine-seeking), aqua (sucrose-seeking), and purple (overlapping) arrows. **b,h.** Three-way Venn diagram comparing significant DEGs in the NAcore (false discovery rate [FDR] < 0.1) for male (**b**) and female (**h**) cocaine-seeking, sucrose-seeking, and overlapping ensembles with the exclusion of non-ensemble genes. Asterisks denote the DEGs defined as cocaine-seeking (red asterisk), sucrose-seeking (aqua asterisk), overlapping (purple asterisk), or shared (black asterisk) for downstream characterization. **c,i.** Male (**c**) and female (**i**) volcano plots representing ensemble-specific DEGs showing number of up- (upward pointing black triangle) and down-regulated (downward pointing black triangle) DEGs scaled by -$\log_{10}$(FDR) (y-axis; factor representing significance) and $\log_2$(fold-change) (log2FC; x-axis; factor representing gene expression) within the NAcore. **d,e,f,j,k,l.** Male heatmap for the ten highest and ten lowest expressed male cocaine-seeking specific (**d**), male sucrose-seeking specific (**e**), male overlapping specific (**f**), female cocaine-seeking specific (**j**), female sucrose-seeking specific (**k**), and female overlapping specific (**l**) DEGs based on log2FC within the NAcore. Asterisks (*) denote the DEGs (from the comparison in Fig. 2b) used for comparison with log2FC of the same gene in other reward-seeking ensembles.

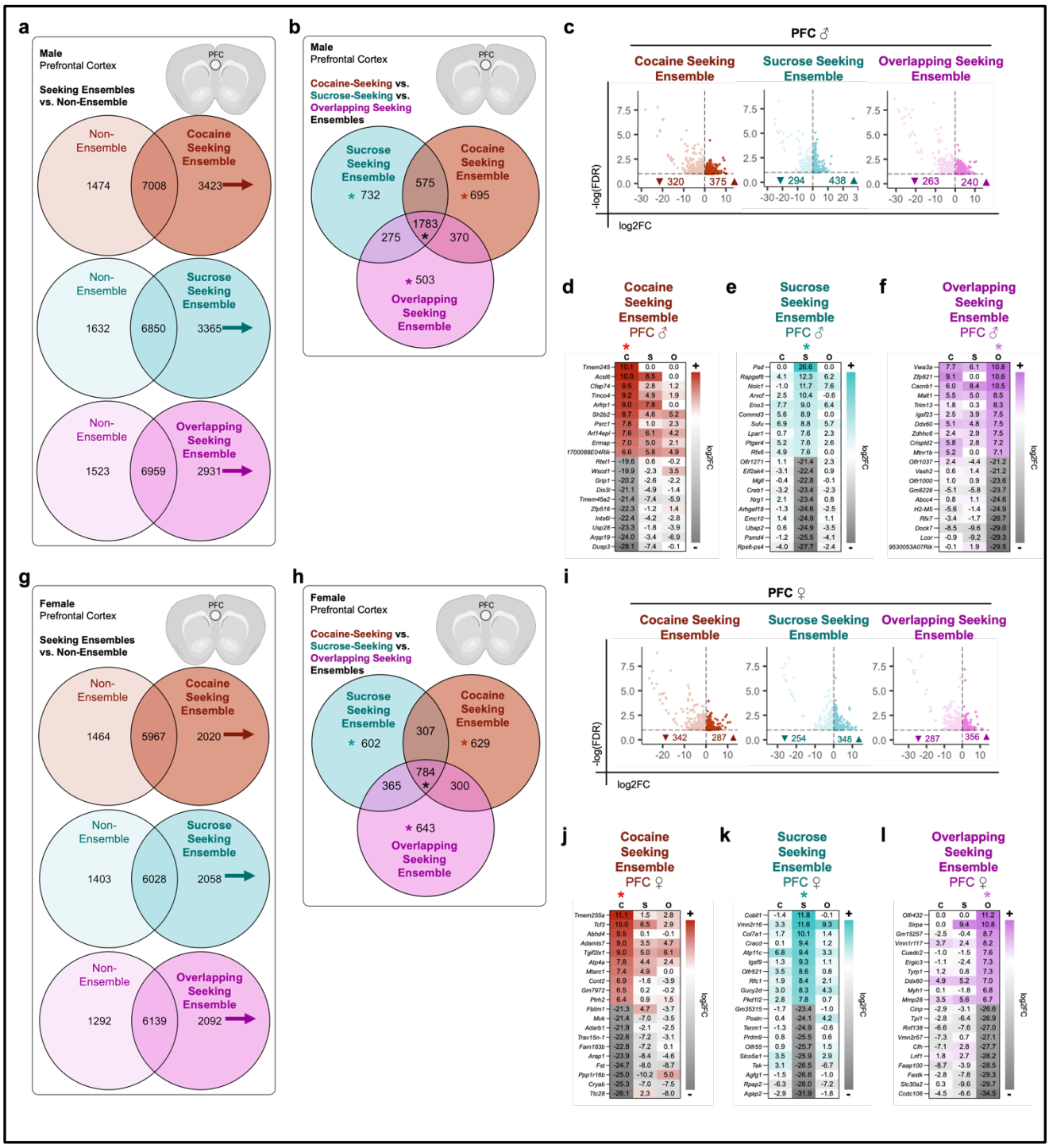


Fig. 3 | Cocaine-seeking and sucrose-seeking ensembles have overlapping and discrete genetic factors within the prefrontal cortex (PFC). All DEG data sets are in reference to naive female and male mice. **a,g.** Two-way Venn diagrams comparing differentially expressed genes (DEG) in the male (**a**) and female (**g**) cocaine-seeking, sucrose-seeking, and overlapping ensembles to the non-ensemble population within the PFC. DEGs moving forward for further reward-specific comparison in fig. 2b (male) and fig. 2h (female) are represented by red (cocaine-seeking), aqua (sucrose-seeking), and purple (overlapping) arrows. **b,h.** Three-way Venn diagram comparing significant DEGs in the PFC (false discovery rate [FDR] < 0.1) for male (**b**) and female (**h**) cocaine-seeking, sucrose-seeking, and overlapping ensembles with the exclusion of non-ensemble genes. Asterisks denote the DEGs defined as cocaine-seeking (red asterisk), sucrose-seeking (aqua asterisk), overlapping (purple asterisk), or shared (black asterisk) for downstream characterization. **c,i.** Male (**c**) and female (**i**) volcano plots representing ensemble-specific DEGs showing number of up- (upward pointing black triangle) and down-regulated (downward pointing black triangle) DEGs scaled by -$\log_{10}$(FDR) (y-axis; factor representing significance) and $\log_2$(fold-change) (log2FC; x-axis; factor representing gene expression) within the PFC. **d,e,f,j,k,l.** Male heatmap for the ten highest and ten lowest expressed male cocaine-seeking specific (**d**), male sucrose-seeking specific (**e**), male overlapping specific (**f**), female cocaine-seeking specific (**j**), female sucrose-seeking specific (**k**), and female overlapping specific (**l**) DEGs based on log2FC within the PFC. Asterisks (*) denote the DEGs (from the comparison in Fig. 2b) used for comparison with log2FC of the same gene in other reward-seeking ensembles.

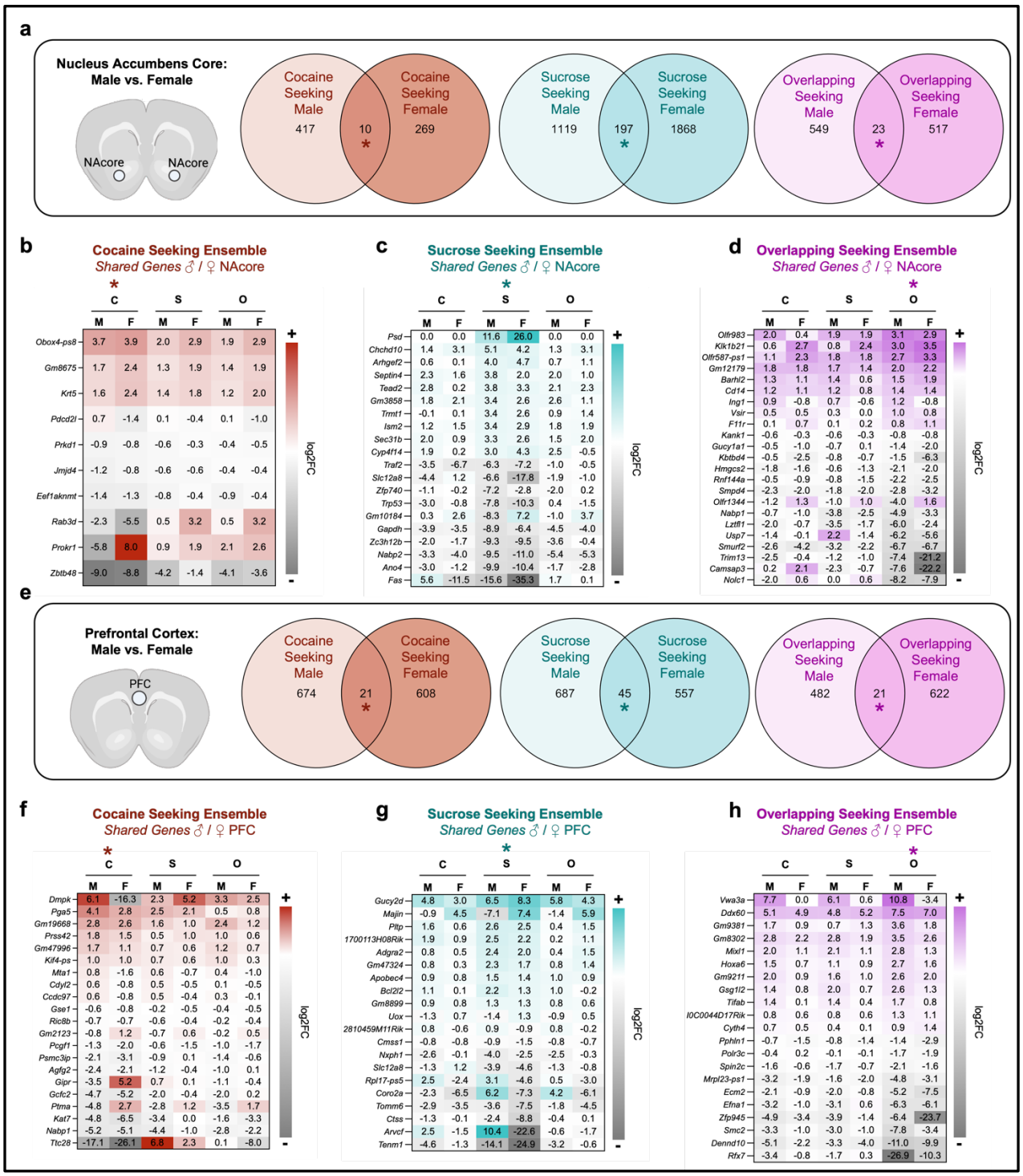


Fig. 4 | Cocaine-seeking and sucrose-seeking ensembles have similar and discrete genetic expressions between males and females within the nucleus accumbens core (NAcore) or prefrontal cortex (PFC). Differentially expressed genes (DEG) used for comparisons are denoted by colored asterisks in the reward-, region-, and sex-specific Venn diagram comparisons from fig. 2b for males (M) and fig. 2h for females (F) for the NAcore and fig. 3b for males and fig. 3h for females for the PFC. **a,e.** Venn diagrams for NAcore (**a**) and PFC (**e**) to compare discrete cocaine-seeking (C), sucrose-seeking (S), and overlapping (O) ensemble DEGs between females and males. **b,f.** Heatmaps for the ten highest and ten lowest NAcore (**b**) and PFC (**f**) DEG log2FC expression for shared cocaine-seeking ensemble DEGs between females and males. **c,g.** Heatmap for the ten highest and ten lowest NAcore (**c**) or PFC (**g**) DEG log2FC expression for shared sucrose-seeking ensemble DEGs between females and males. **d,h.** Heatmap of all NAcore (**d**) or PFC (**h**) DEG log2FC expression for shared overlapping ensemble DEGs between females and males. Asterisks denote the ensemble-specific DEGs used for top ten and bottom ten DEGs based on log2FC for comparison with other reward-specific ensemble-seeking DEG log2FC.

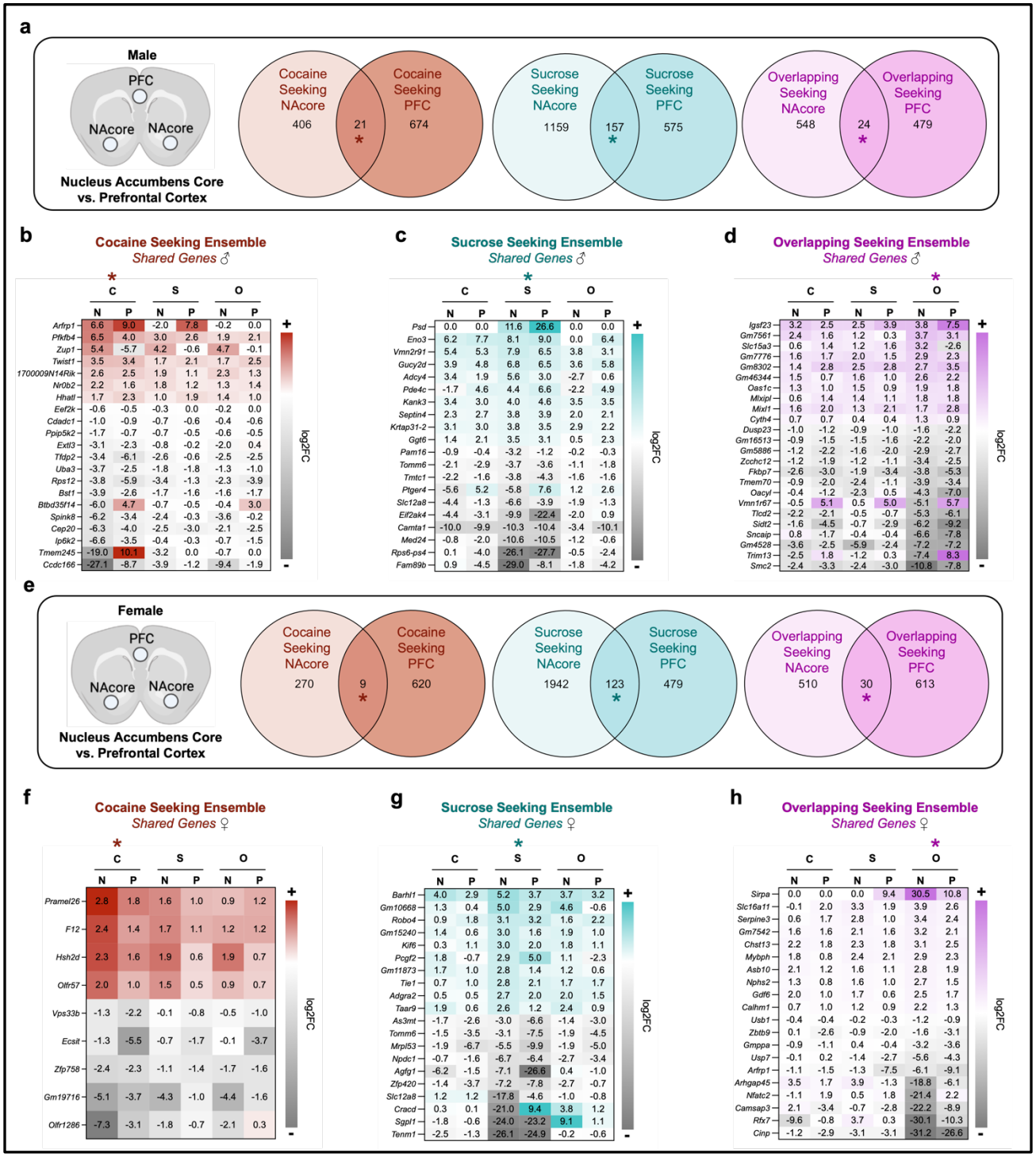


Fig. 5 | Cocaine-seeking and sucrose-seeking ensembles have a similar and discrete genetic expression in the nucleus accumbens core (NAcore; N) and prefrontal cortex (PFC; P). Differentially expressed genes (DEG) used for comparisons are denoted by colored asterisks in the reward-, region-, and sex-specific Venn diagram comparisons from Fig. 2b for males and Fig. 2h for females for the NAcore (N) and Fig. 3b for males and Fig. 3h for females for the PFC (P). **a,e.** Venn diagrams to compare male (**a**) or female (**e**) DEGs between the NAcore and PFC for discrete cocaine-seeking (C), sucrose-seeking (S), or overlapping (O) ensembles DEGs. **b,f.** Heatmap of all male (**b**) or female (**f**) DEGs based on log2FC for discrete cocaine-seeking ensemble DEGs shared between the NAcore and PFC. **c,g.** Heatmap for the ten highest and ten lowest male (**c**) or female (**g**) DEGs based on log2FC for discrete sucrose-seeking ensemble DEGs shared between the NAcore and PFC. **d,h.** Heatmap of all male (**d**) or female (**h**) DEGs based on log2FC for discrete overlapping ensemble DEGs shared between the NAcore and PFC. Asterisks denote the shared DEGs between the male NAcore and PFC used for top ten and bottom ten DEGs based on log2FC for comparison with other reward-specific ensemble-seeking DEG log2FC.

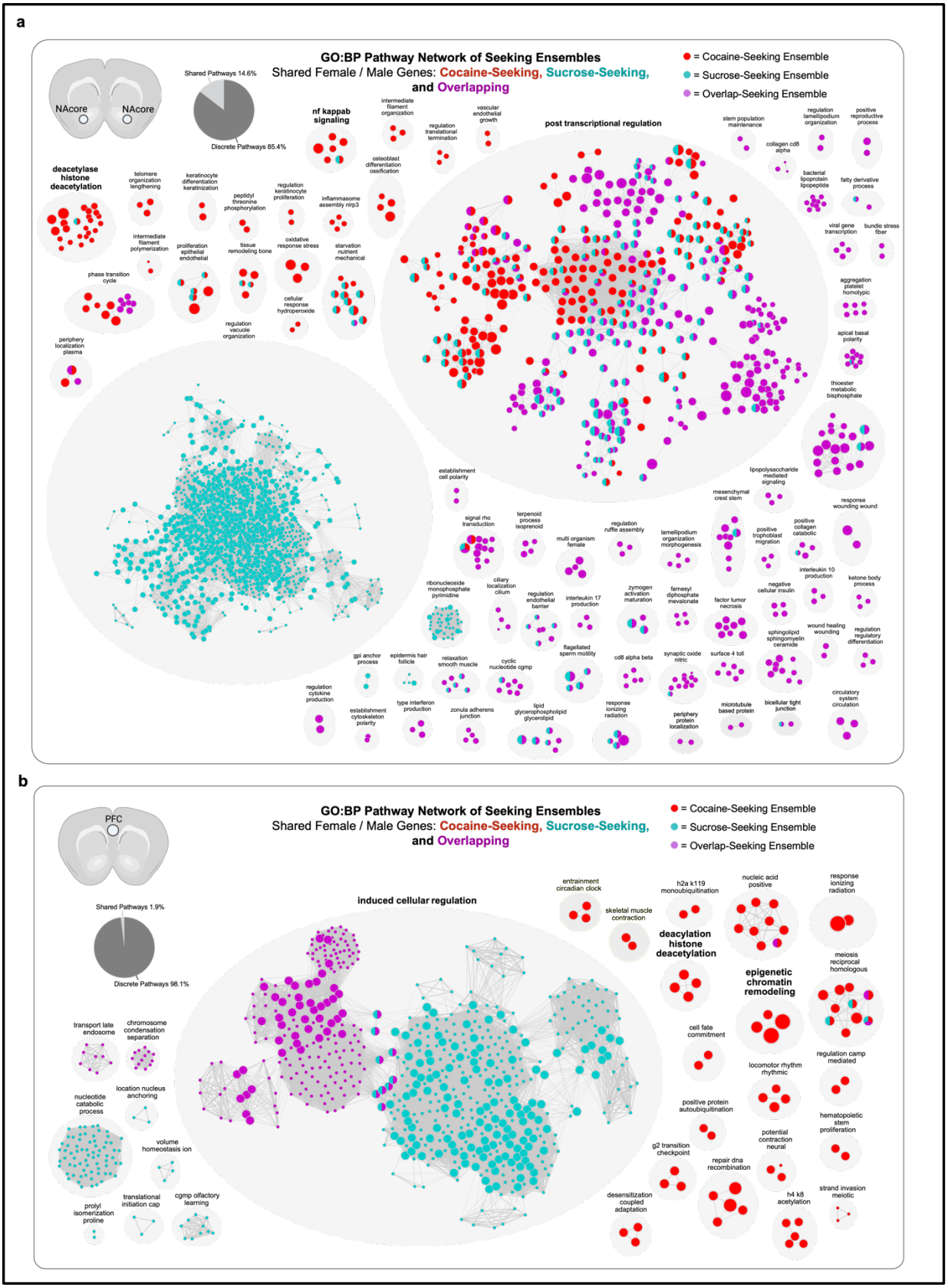
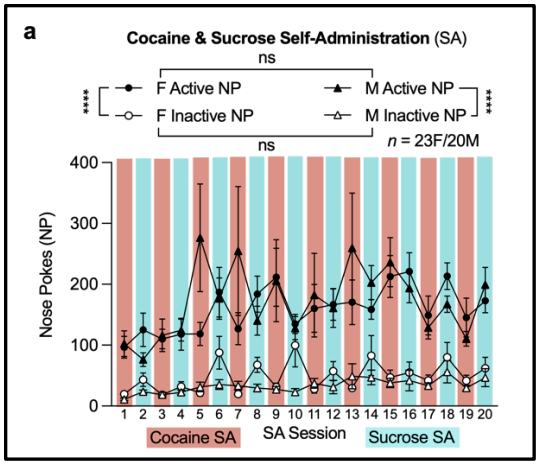
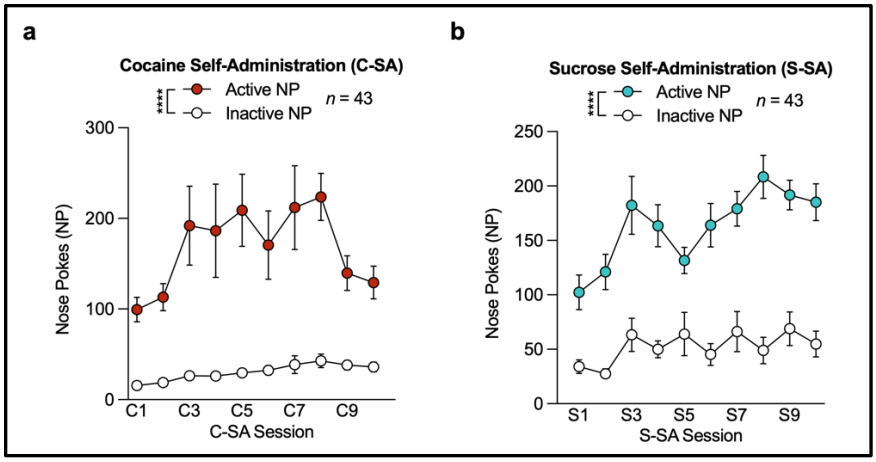


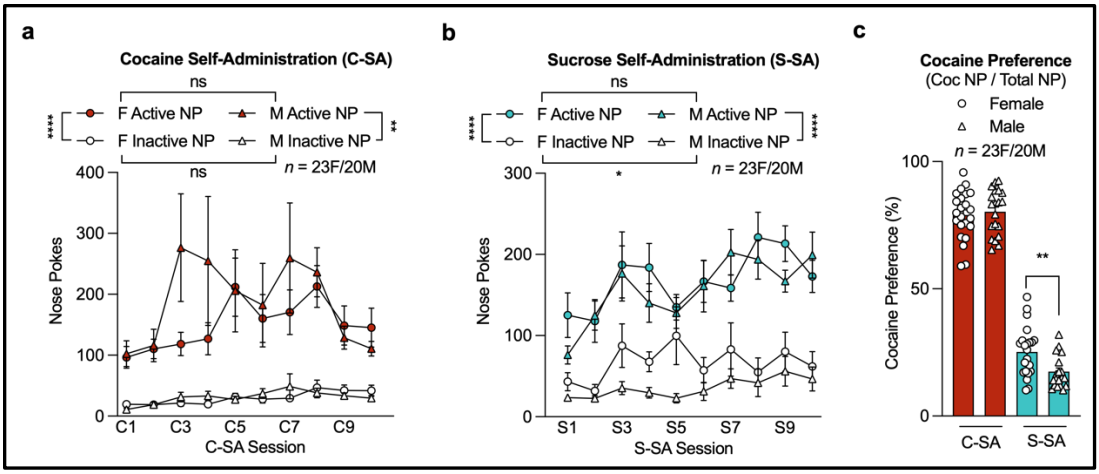
Fig. 6 | Cocaine-seeking and sucrose-seeking ensembles have similar and discrete genetic ontology between males and females within the nucleus accumbens core (NAcore) or prefrontal cortex (PFC). a,b. Genetic ontology (GO) using biological process gene set enrichment analysis (GSEA; via g:Profiler) of DEGs shared between male and females in the NAcore (**a**) and PFC (**b**). Colored circular nodes cocaine seeking = red; sucrose seeking = aqua; overlapping seeking = purple; shared by multiple types of reward seeking or regions = multicolored fractions) represent GO pathways typified by reward. Lines connecting circular nodes represent similarity of genes within two GO pathways. Circular nodes grouped in grey ovals represent similar pathways. Large labels are representative of words that were shared in the GO pathway names. Pie chart in corner represents percentages of the total pathways that are “shared” between multiple reward-seeking ensembles and those that are “discrete” to one type of reward-seeking ensemble.



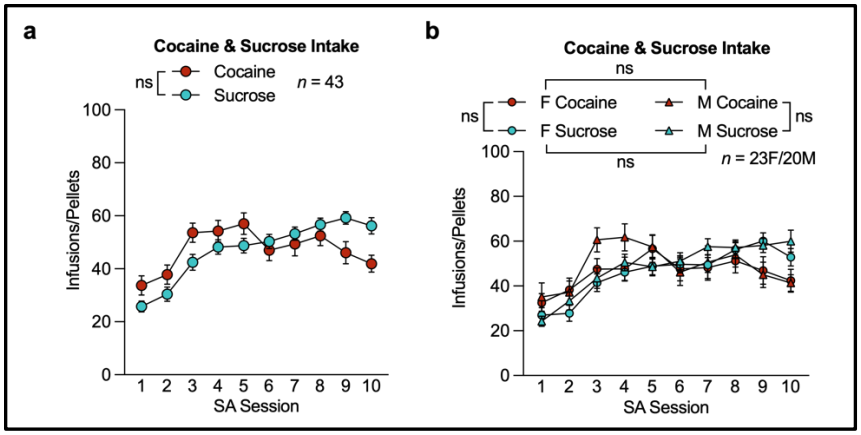
Supplemental Fig. 1 | Supplemental sex-specific overview of self-administration. a. Female (F) and male (M) sex-specific plots for cocaine and sucrose self-administration for females (repeated measures two-way ANOVA for F SA Active NP vs. F SA Inactive NP over 20 sessions; NP $F_{(1, 44)} = 46.21$, $P<0.0001$; session $F_{(7.536, 331.6)} = 3.198$, $P=0.0021$; interaction $F_{(19, 836)} = 1.363$, $P=0.1369$) and males (repeated measures two-way ANOVA for M SA Active NP vs. M SA Inactive NP over 20 sessions; NP $F_{(1, 38)} = 29.03$, $P<0.0001$; session $F_{(2.536, 96.36)} = 2.246$, $P=0.0981$; interaction $F_{(19, 722)} = 1.453$, $P=0.0954$) without an effect between sexes (active NP: repeated measures two-way ANOVA for F SA Active NP vs. M SA Active NP over 20 sessions; NP $F_{(1, 41)} = 0.2039$, $P=0.6540$; session $F_{(4.347, 178.2)} = 2.754$, $P=0.0258$; interaction $F_{(19, 779)} = 1.377$, $P=0.1301$ – inactive NP: repeated measures two-way ANOVA for F SA Inactive NP vs. M SA Inactive NP over 20 sessions; NP $F_{(1, 41)} = 3.758$, $P=0.0595$; session $F_{(5.805, 238.0)} = 2.710$, $P=0.0595$; interaction $F_{(19, 779)} = 1.517$, $P=0.0723$).



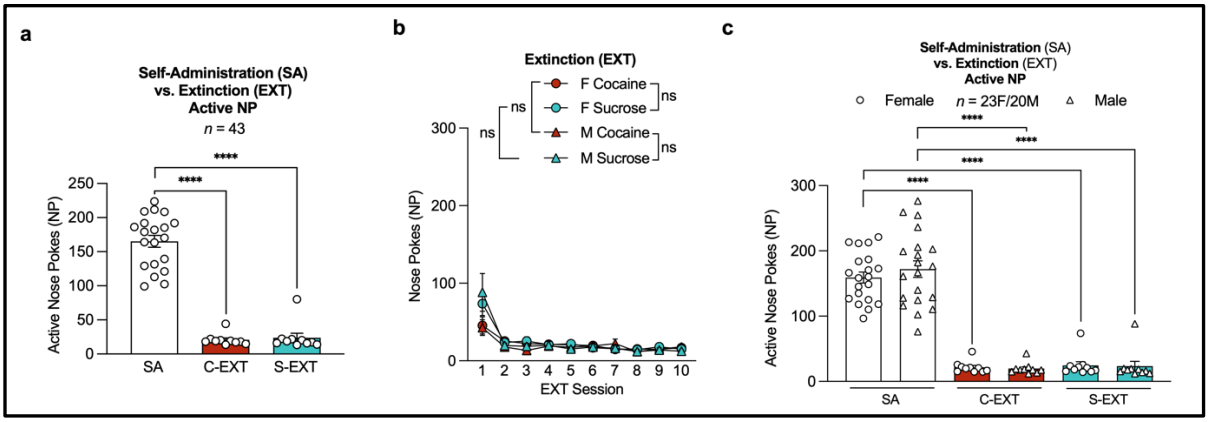
Supplemental Fig. 2 | Supplemental reward intake graphs. a,b. Cocaine (**a,** C-SA) and sucrose (**b**, S-SA) self-administration sessions separated by cocaine (repeated measures two-way ANOVA for C-SA Active NP vs. C-SA Inactive NP over 20 sessions; NP $F_{(1, 84)} = 37.00$, $P<0.0001$; session $F_{(2.079, 174.6)} = 3.373$, $P=0.0348$; interaction $F_{(9, 756)} = 2.141$, $P=0.0243$) or sucrose (repeated measures two-way ANOVA for S-SA Active NP vs. S-SA Inactive NP over 20 sessions; NP $F_{(1, 84)} = 78.17$, $P<0.0001$; session $F_{(4.613, 387.5)} = 5.112$, $P=0.0002$; interaction $F_{(9, 756)} = 2.068$, $P=0.0301$).



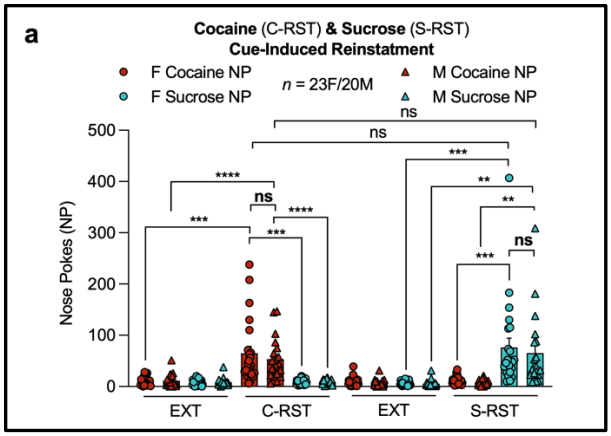
Supplemental Fig. 3 | Supplemental sex-specific self-administration and cocaine preference graphs. a. Sex-specific analysis for C-SA (female: repeated measures two-way ANOVA for F C-SA Active NP vs. F C-SA Inactive NP over 20 sessions; NP $F_{(1, 44)} = 50.16$, $P<0.0001$; session $F_{(4.496, 197.8)} = 2.689$, $P=0.0269$; interaction $F_{(9, 396)} = 1.333$, $P=0.2177$ – male: repeated measures two-way ANOVA for M C-SA Active NP vs. M C-SA Inactive NP over 20 sessions; NP $F_{(1, 38)} = 12.29$, $P=0.0012$; session $F_{(1.511, 57.41)} = 2.513$, $P=0.1305$; interaction $F_{(9, 342)} = 1.682$, $P=0.0919$ – female/male active NP: repeated measures two-way ANOVA for F C-SA Active NP vs. M C-SA Active NP over 20 sessions; NP $F_{(1, 41)} = 0.6792$, $P=0.4146$; session $F_{(2.024, 82.97)} = 2.907$, $P=0.0596$; interaction $F_{(9, 369)} = 1.490$, $P=0.1496$ – female/male inactive NP: repeated measures two-way ANOVA for F C-SA Inactive NP vs. M C-SA Inactive NP over 20 sessions; NP $F_{(1, 41)} = 0.03314$, $P=0.8564$; session $F_{(4.257, 174.5)} = 3.108$, $P=0.0148$; interaction $F_{(9, 369)} = 1.236$, $P=0.2716$). **b.** Sex-specific analysis for sucrose S-SA (female: repeated measures two-way ANOVA for F S-SA Active NP vs. F S-SA Inactive NP over 20 sessions; NP $F_{(1, 44)} = 26.13$, $P<0.0001$; session $F_{(4.082, 179.6)} = 2.383$, $P=0.0520$; interaction $F_{(9, 396)} = 1.425$, $P=0.1752$ – male: repeated measures two-way ANOVA for M S-SA Active NP vs. M S-SA Inactive NP over 20 sessions; NP $F_{(1, 38)} = 77.63$, $P<0.0001$; session $F_{(4.696, 178.5)} = 4.224$, $P=0.0015$; interaction $F_{(9, 342)} = 1.739$, $P=0.0790$ – female/male active NP: repeated measures two-way ANOVA for F S-SA Active NP vs. M S-SA Active NP over 20 sessions; NP $F_{(1, 41)} = 0.3130$, $P=0.5789$; session $F_{(4.001, 164.0)} = 4.628$, $P=0.0014$; interaction $F_{(9, 369)} = 0.9657$, $P=0.4681$ – female/male inactive NP repeated measures two-way ANOVA for F S-SA Inactive NP vs. M S-SA Inactive NP over 20 sessions; NP $F_{(1, 41)} = 4.511$, $P=0.0398$; session $F_{(3.873, 158.8)} = 1.446$, $P=0.2226$; interaction $F_{(9, 369)} = 0.8511$, $P=0.5695$). **c.** Sex-specific analysis of cocaine preference (cocaine NP / sucrose NP * 100) during C-SA and S-SA (unpaired t-test for F S-SA Cocaine Preference vs. M S-SA Cocaine Preference; $P=0.0040$).



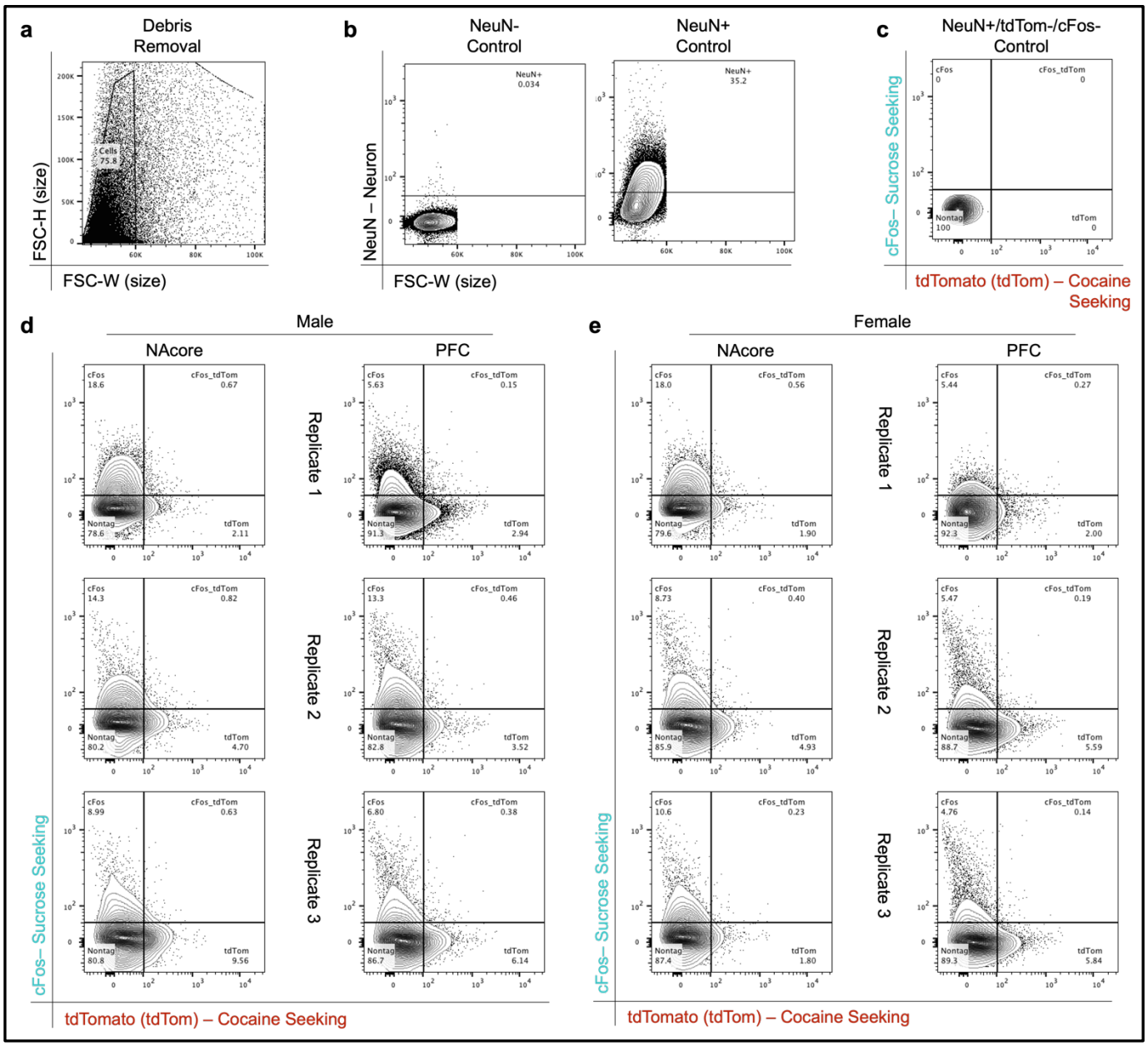
Supplemental Fig. 4 | Supplemental sex-specific reward intake graphs. a. Reward-intake of cocaine (0.5 mg/kg/infusion) injections or sucrose (15 mg) pellets (max = 100 injections/pellets) upon association with active reward-specific nose poke (NP) ports showed no differentiation between (repeated measures two-way ANOVA for Cocaine Infusions vs. Sucrose Pellets over 20 sessions; NP $F_{(1, 84)}$ = 0.005, P=0.9446; session $F_{(4.805, 403.6)}$ = 17.59, P<0.0001; interaction $F_{(9, 756)}$ = 5.347, P<0.0001). **b.** Sex-specific analysis showed no differences between intake of cocaine and sucrose for females (repeated measures two-way ANOVA for F Cocaine Infusions vs. F Sucrose Pellets over 20 sessions; NP $F_{(1, 44)}$ = 0.00045, P=0.9832; session $F_{(4.269, 187.8)}$ = 9.184, P<0.0001; interaction $F_{(9, 396)}$ = 2.065, P=0.0316) and males (repeated measures two-way ANOVA for M Cocaine Infusions vs. M Sucrose Pellets over 20 sessions; NP $F_{(1, 38)}$ = 0.01675, P=0.8977; session $F_{(4.638, 176.2)}$ = 9.083 , P<0.0001; interaction $F_{(9, 342)}$ = 3.911, P<0.0001). Comparing intake of cocaine between sexes showed no difference (repeated measures two-way ANOVA for F Cocaine Infusions vs. M Cocaine Infusions over 20 sessions; NP $F_{(1, 41)}$ = 0.4724, P=0.4957; session $F_{(4.947, 202.8)}$ = 5.182, P=0.0002; interaction $F_{(9, 369)}$ = 0.7462, P=0.6664). Comparing intake of sucrose between sexes showed no difference (repeated measures two-way ANOVA for F Sucrose Pellets vs. M Sucrose Pellets over 20 sessions; NP $F_{(1, 41)}$ = 0.5292, P=0.4711; session $F_{(3.598, 147.5)}$ = 26.71, P<0.0001; interaction $F_{(9, 369)}$ = 0.7441, P=0.6684).



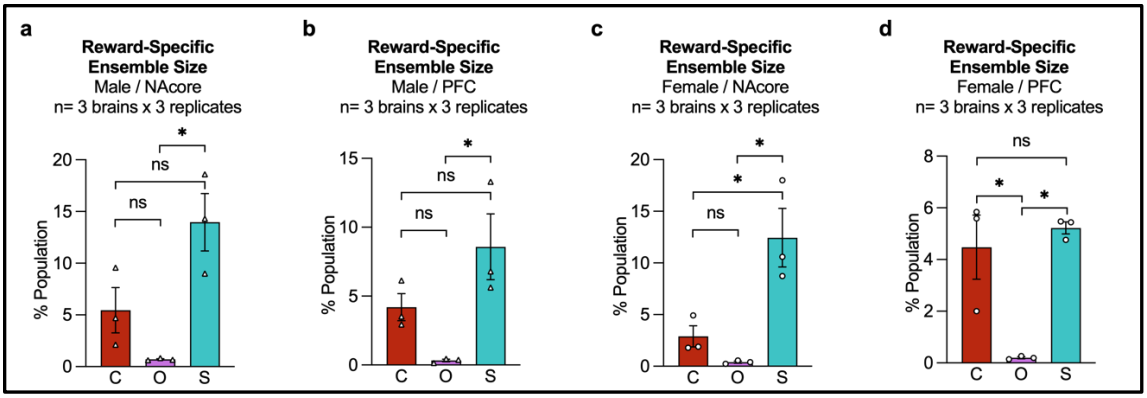
Supplemental Fig. 5 | Supplemental sex-specific extinction graphs. a. During EXT of cocaine (C-EXT) and sucrose (S-EXT), mice showed a decrease in active NP interaction when compared to cocaine (mixed-effect repeated measures two-way ANOVA for SA Active NP vs. C-EXT Active NP over 20 sessions; NP $F_{(1, 84)} = 64.52$, P<0.0001; session $F_{(3.311, 206.5)} = 2.615$, P=0.0466) or sucrose SA (S-SA) sessions (mixed-effect repeated measures two-way ANOVA for SA Active NP vs. S-EXT Active NP over 20 sessions; NP $F_{(1, 84)} = 60.81$, P<0.0001; session $F_{(3.389, 211.4)} = 2.495$, P=0.0536)**. b.** Sex-specific plot for extinction show no differentiation between cocaine and sucrose active NP for females (repeated measures two-way ANOVA for F EXT Cocaine NP vs. F EXT Sucrose NP over 20 sessions; NP $F_{(1, 44)} = 0.4072$, P=0.5267; session $F_{(1.782, 78.39)} = 17.28$, P<0.0001; interaction $F_{(9, 396)} = 2.001$, P=0.0380) and males (repeated measures two-way ANOVA for M EXT Cocaine NP vs. M EXT Sucrose NP over 20 sessions; NP $F_{(1, 38)} = 0.5938$, P=0.4457; session $F_{(1.624, 61.73)} = 12.61$, P<0.0001; interaction $F_{(9, 342)} = 2.916$, P=0.0024) without an effect between sexes for cocaine NP (repeated measures two-way ANOVA for F EXT Cocaine NP vs. M EXT Cocaine NP over 20 sessions; NP $F_{(1, 41)} = 0.2101$, P=0.6491; session $F_{(2.332, 95.61)} = 9.430$, P<0.0001; interaction $F_{(9, 369)} = 0.6856$, P=0.7220) and sucrose NP (repeated measures two-way ANOVA for F EXT Sucrose NP vs. M EXT Sucrose NP over 20 sessions; NP $F_{(1, 41)} = 0.05162$, P=0.8214; session $F_{(1.473, 60.38)} = 19.73$, P<0.0001; interaction $F_{(9, 369)} = 0.4548$, P=0.9041). **c.** When comparing active NP interactions during SA to C-EXT, there was a significant decrease in the active NP during C-EXT for females (mixed-effect repeated measures two-way ANOVA for F SA Active NP vs. F C-EXT Active NP over 20 sessions; NP $F_{(1, 44)} = 48.42$, P<0.0001; session $F_{(5.194, 170.9)} = 2.164$, P=0.0578) and males (mixed-effect repeated measures two-way ANOVA for M SA Active NP vs. M C-EXT Active NP over 20 sessions; NP $F_{(1, 38)} = 23.88$, P<0.0001; session $F_{(1.649, 46.96)} = 1.809$, P=0.1803)**.** Comparing active NP interactions during SA to S-EXT, there was a significant decrease in the active NP during S-EXT for females (mixed-effect repeated measures two-way ANOVA for F SA Active NP vs. F S-EXT Active NP over 20 sessions; NP $F_{(1, 44)} = 45.97$, P<0.0001; session $F_{(5.291, 174.1)} = 2.099$, P=0.0639) and males (mixed-effect repeated measures two-way ANOVA for M SA Active NP vs. M S-EXT Active NP over 20 sessions; NP $F_{(1, 38)} = 22.39$, P<0.0001; session $F_{(1.698, 48.34)} = 1.696$, P=0.1975)**.**



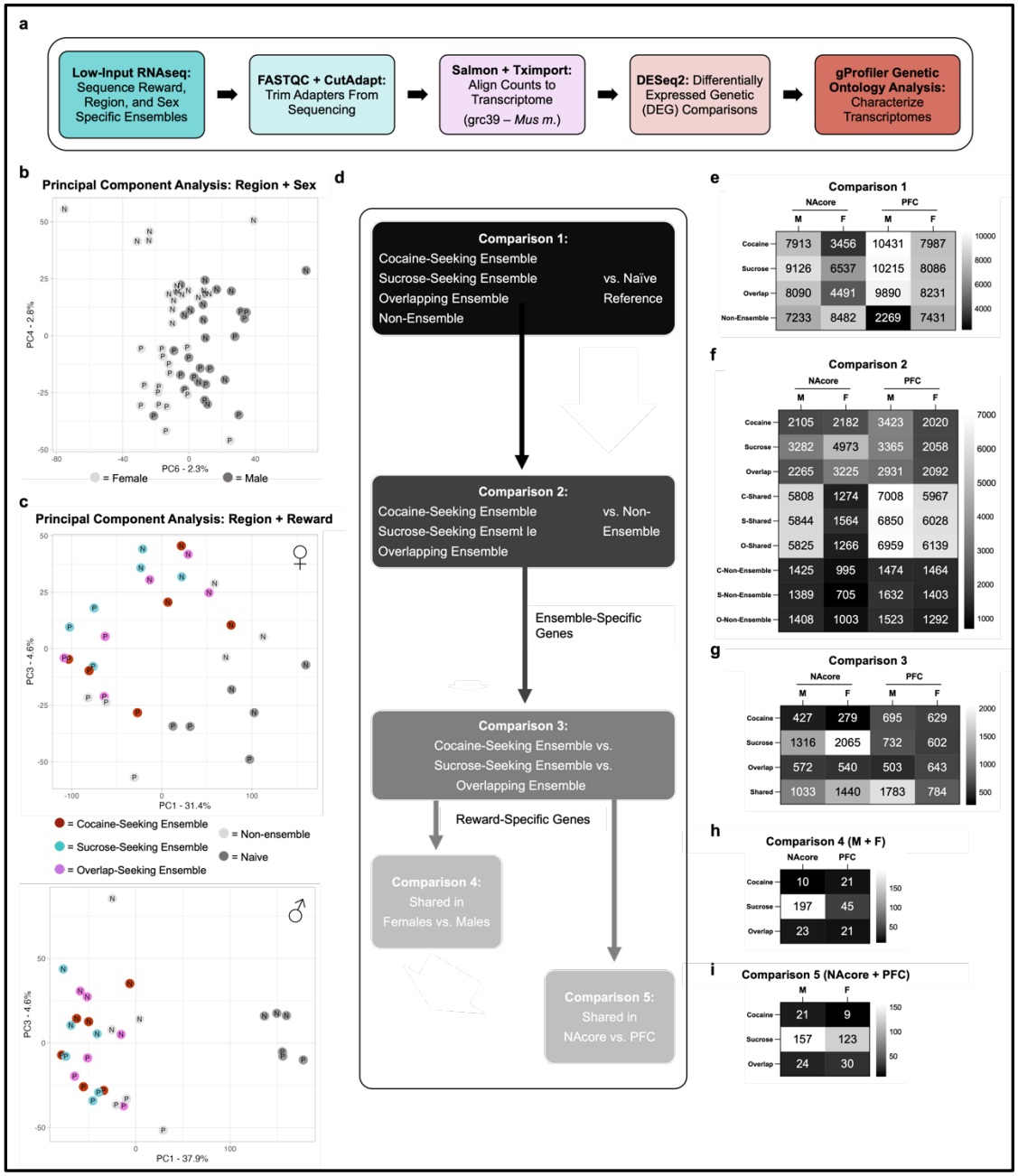
Supplemental Fig. 6 | Supplemental sex-specific reinstatements graphs. a. Sex-specific plot for cocaine reinstatement (C-RST) shows higher interaction with the cocaine NP during C-RST compared to the sucrose NP for females (paired t-test for F C-RST Cocaine NP vs. F C-RST Sucrose NP; P=0.0002) and males (paired t-test for M C-RST Cocaine NP vs. M C-RST Sucrose NP; P<0.0001). There was a significant increase in interactions with the cocaine NP during C-RST compared to the last day of extinction for females (paired t-test for F C-RST Cocaine NP vs. F C-EXT Cocaine NP; P=0.0009) and males (paired t-test for M C-RST Cocaine NP vs. M C-EXT Cocaine NP; P=0.0018). Sex-specific plot for sucrose reinstatement (S-RST) shows higher interaction with the sucrose NP during S-RST compared to the cocaine NP for females (paired t-test for F S-RST Sucrose NP vs. F S-RST Cocaine NP; P=0.0005) and males (paired t-test for M S-RST Sucrose NP vs. M S-RST Cocaine NP; P<0.0001). There was a significant increase in interactions with the sucrose NP during S-RST compared to the last day of extinction for females (paired t-test for F S-RST Sucrose NP vs. F S-EXT Sucrose NP; P=0.0006) and males (paired t-test for M S-RST Sucrose NP vs. M S-EXT Sucrose NP; P=0.0011). Comparing active NP interactions in C-RST and S-RST showed no difference for females (paired t-test for F C-RST Cocaine NP vs. F S-RST Sucrose NP; P=0.3923) and males (paired t-test for M C-RST Cocaine NP vs. M S-RST Sucrose NP; P=0.4379). Comparing the interaction of active NP during C-RST between males and females showed no difference (unpaired t-test for F C-RST Cocaine NP vs. M C-RST Cocaine NP; P=0.4866). Comparing the interaction of active NP during S-RST between males and females showed no difference (unpaired t-test for F S-RST Sucrose NP vs. M S-RST Sucrose NP; P=0.6535).



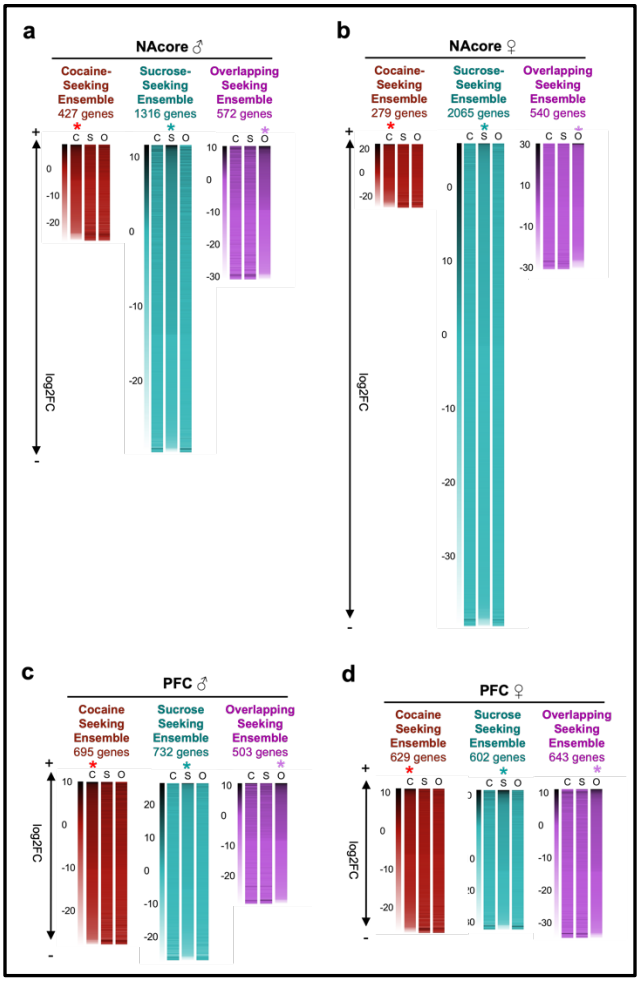
Supplemental Fig. 7 | Supplemental fluorescent-activated cell sorting (FACS) gating strategies and flow cytometric analysis of sample replicates. a. Proof of concept for FACS debris removal from a sample using the size parameters forward scatter width (FSC-W; x-axis) and forward scatter height (FSC-H; y-axis) to filter out unwanted larger and smaller events. **b.** We further gated cells positive for a neuronal marker (NeuN+) in comparison to our negative control (NeuN-) to gate for neurons to use in downstream ensemble-specific quadrant plots. **c.** We used transgenic mice who went through behavioral conditioning paradigm but lacked the induction of tagging scheme as a control for quadrant plot gating strategy from gated NeuN+ cells in b to gate for cocaine-seeking (tdTom; x-axis) and sucrose-seeking (cFos; y-axis) markers. **d.** Male flow cytometric quadrant plots subcategorized into the nucleus accumbens core (NAcore) and prefrontal cortex (PFC) regions for FACS-sorted ensemble specific samples. **e.** Female flow cytometric quadrant plots subcategorized into the nucleus accumbens core (NAcore) and prefrontal cortex (PFC) regions for FACS-sorted ensemble specific samples. For males and females, quadrant analysis allowed separation of cocaine-seeking neurons (bottom-right; tdTom+/cFos-), sucrose-seeking neurons (top left; cFos+/tdTom-) and overlapping neurons between cocaine-seeking and sucrose-seeking (top right: tdTom+/cFos+) in comparison to non-ensemble (bottom-left; tdTom-/cFos-) neurons. Quadrant plots are shown for replicates 1-3 with replicate 3 used as representation in Fig. 1f,g. f-i. Comparisons for quantification of cocaine-seeking (C), sucrose-seeking (S), and overlapping (O) ensemble sizes (% of tagged ensemble-specific neurons per 1,000,000 events).



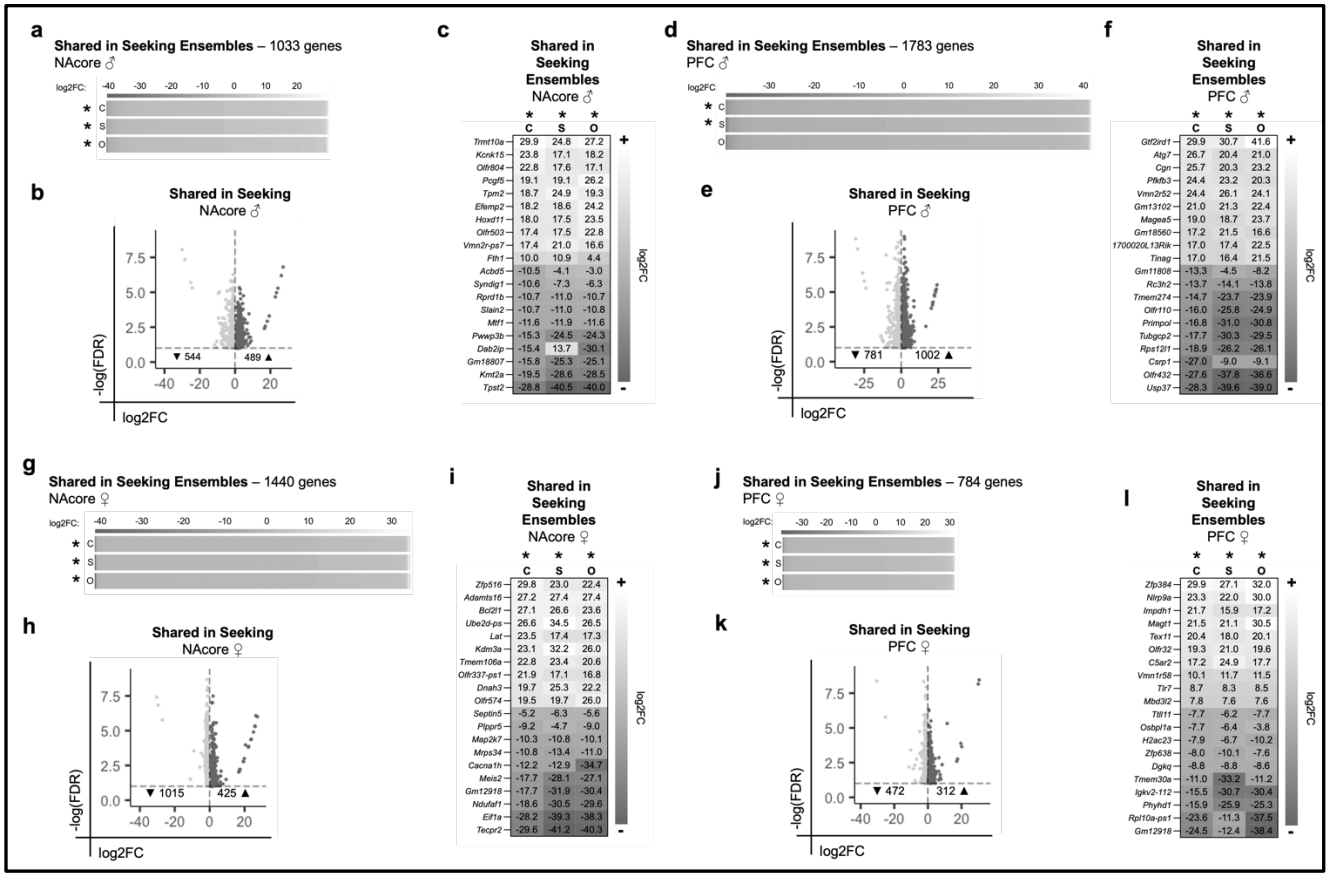
Supplemental Fig. 8 | Supplemental flow cytometric analysis of ensemble sizes. a. Within the male NAcore, there was an overall significant difference in ensemble sizes and replicates (repeated measures two-way ANOVA for M Cocaine NAcore vs. M Sucrose NAcore vs. M Overlap NAcore: Reward vs. Replicate; reward $F_{(2, 4)} = 7.303$, P=0.0462; replicate $F_{(2, 4)} = 0.02303$, P=0.04462), a significant difference between the size of sucrose-seeking and overlapping ensemble (multiple comparisons: repeated measures two-way ANOVA for M Sucrose NAcore vs. M Overlap NAcore; P=0.0419), no significant differences between the size of cocaine-seeking and sucrose-seeking ensembles (multiple comparisons: repeated measures two-way ANOVA for M Cocaine NAcore vs. M Sucrose NAcore; P=0.11475), and no significant difference between the size of the cocaine-seeking ensemble and overlapping ensemble (multiple comparisons: repeated measures two-way ANOVA for M Cocaine NAcore vs. M Overlap NAcore; P=0.4435). **b.** Within the female NAcore, there was an overall significant difference in ensemble sizes (repeated measures two-way ANOVA for F Cocaine NAcore vs. F Sucrose NAcore vs. F Overlap NAcore: Reward vs. Replicate; reward $F_{(2,4)} = 7.354$, P=0.0457; replicate $F_{(2, 4)} = 0.8809$, P=0.4820), a significant difference between the size of sucrose-seeking and overlapping ensemble (multiple comparisons: repeated measures two-way ANOVA for F Sucrose NAcore vs. F Overlap NAcore; P=0.0398), no significant differences between the size of cocaine-seeking and sucrose-seeking ensembles (multiple comparisons: repeated measures two-way ANOVA for F Cocaine NAcore vs. F Sucrose NAcore; P=0.2196), and no significant difference between the size of the cocaine-seeking ensemble and overlapping ensemble (multiple comparisons: repeated measures two-way ANOVA for F Cocaine NAcore vs. F Overlap NAcore; P=0.2808). **c.** Within the male PFC, there was an overall significant difference in ensemble sizes (repeated measures two-way ANOVA for M Cocaine PFC vs. M Sucrose PFC vs. M Overlap PFC: Reward vs. Replicate; reward $F_{(2, 4)} =11.33$, P=0.0225; replicate $F_{(2, 4)} = 0.5409$, P=0.6196), a significant difference between the size of cocaine-seeking and sucrose-seeking ensembles (multiple comparisons: repeated measures two-way ANOVA for M Cocaine PFC vs. M Sucrose PFC; P=0.0493), a significant difference between the size of sucrose-seeking and overlapping ensemble (multiple comparisons: repeated measures two-way ANOVA for M Sucrose PFC vs. M Overlap PFC; P=0.0233), and no significant differences between the size of cocaine-seeking and overlapping ensembles (multiple comparisons: repeated measures two-way ANOVA for M Cocaine PFC vs. M Overlap PFC; P=0.6537). **d.** Within the female PFC, there was an overall significant difference in ensemble sizes (repeated measures two-way ANOVA for F Cocaine PFC vs. F Sucrose PFC vs. F Overlap PFC: Reward vs. Replicate; reward $F_{(2,4)} = 12.38$, P=0.0193; replicate $F_{(2,4)} = 0.6856$, P=0.5546), a significant difference between the size of cocaine-seeking and overlapping ensembles (multiple comparisons: repeated measures two-way ANOVA for F Cocaine PFC vs. F Overlap PFC; P=0.0368), a significant difference between the size of sucrose-seeking and overlapping ensemble (multiple comparisons: repeated measures two-way ANOVA for F Sucrose PFC vs. F Overlap PFC; P=0.0216), and no significant differences between the size of cocaine-seeking and overlapping ensembles (multiple comparisons: repeated measures two-way ANOVA for F Cocaine PFC vs. F Sucrose PFC; P=0.7841).



Supplemental Fig. 9 | Supplemental overview of transcriptomic comparison analysis. a. Bioinformatic workflow used to determine differentially expressed genes (DEG) and genetic ontology for each FACS-sorted reward-specific seeking ensemble transcriptome in comparison to the transcriptome of naïve males (M) and females (F) that did not undergo cocaine or sucrose SA. **b.** Principal component analysis (PCA) plot of principal component 4 (PC4; y-axis) and principal component 6 (PC6; x-axis for females (light grey) and males (dark grey) in the nucleus accumbens core (N) and prefrontal cortex (P). Percent of variation is included on each axis representative of PC6 or PC4. **c.** Principal component analysis (PCA) plot of principal component 3 (PC3; y-axis) and principal component 1 (PC1; x-axis) for females (top panel) and males (bottom panel) in the nucleus accumbens core (N) and prefrontal cortex (P). Points are colored t to represent RNAseq samples (3 replicates per sample) for cocaine-seeking (red circle), sucrose-seeking (aqua circle), and the overlapping ensemble (purple circle) in addition to the non-ensemble (light grey circle) and the naïve (dark grey circle) samples. Percent of variation is included on each axis representative of PC3 or PC1. **d.** Diagram of the approach used to initially determine DEGs (in reference to naïve mice) of cocaine-seeking, sucrose-seeking, and overlapping ensembles in addition to the non-ensemble for further Venn diagram comparisons in Fig. 2-5. **e.** Heatmap representation of total significant DEGs (FDR < 0.1) for cocaine-seeking, sucrose-seeking, and the overlapped ensemble within the nucleus accumbens core (NAcore) or prefrontal cortex (PFC) for females (F) and males (M) when using naïve mice as a reference (comparison 1). **f.** Heatmap representation of female and male significant DEGs exclusive to cocaine-seeking, sucrose-seeking, or the overlapped ensembles when compared to DEGs that are shared with or are exclusive to the non-ensemble population within the NAcore or PFC (comparison 2). **g.** Heatmap representation of female and male significant DEGs exclusive to or shared between cocaine-seeking, sucrose-seeking, and the overlapped ensembles when comparing DEGs between reward-seeking ensembles in the NAcore or PFC (comparison 3). **h.** Heatmap representation of significant DEGs shared between females and males and are exclusive to cocaine-seeking, sucrose-seeking, and the overlapped ensembles when comparing DEGs between reward-seeking ensembles in the NAcore or PFC (comparison 4). **i.** Heatmap representation of significant DEGs shared between the NAcore and PFC and are exclusive to cocaine-seeking, sucrose-seeking, and the overlapped ensembles when comparing DEGs between reward-seeking ensembles (comparison 5).



Supplemental Fig. 10 | Supplemental total heatmaps of reward-specific genes. a. Male heatmap representation of log2(fold change) (log2FC) for discrete cocaine-seeking (C), sucrose-seeking (S), or overlapping (O) DEGs. Asterisks denote the ensemble-specific gene list used for ordering log2FC from low to high for comparison with other reward-specific ensemble-seeking lists. i.e., cocaine-ensemble DEGs, represented by *C, are organized by log2FC from low to high expression and are further compared to the corresponding log2FC in DEGs for male sucrose-seeking and overlapping ensembles. **b.** Female heatmap representation of log2(fold change) (log2FC) for discrete cocaine-seeking (C), sucrose-seeking (S), or overlapping (O) DEGs. Asterisks denote the ensemble-specific gene list used for ordering log2FC from low to high compared with other reward-specific ensemble-seeking lists. i.e., cocaine-ensemble DEGs, represented by *C, are organized by log2FC from low to high expression and are further compared to the corresponding log2FC in DEGs for female sucrose-seeking and overlapping ensembles. **c.** Male heatmap representation of log2(fold change) (log2FC) for discrete cocaine-seeking (C), sucrose-seeking (S), or overlapping (O) DEGs. Asterisks denote the ensemble-specific gene list used for ordering log2FC from low to high for comparison with other reward-specific ensemble-seeking lists. i.e., cocaine-ensemble DEGs, represented by *C, are organized by log2FC from low to high expression and are further compared to the corresponding log2FC in DEGs for male sucrose-seeking and overlapping ensembles. **d.** Female heatmap representation of log2(fold change) (log2FC) for discrete cocaine-seeking (C), sucrose-seeking (S), or overlapping (O) DEGs. Asterisks denote the ensemble-specific gene list used for ordering log2FC from low to high compared with other reward-specific ensemble-seeking lists. i.e., cocaine-ensemble DEGs, represented by *C, are organized by log2FC from low to high expression and are further compared to the corresponding log2FC in DEGs for female sucrose-seeking and overlapping ensembles.



Supplemental Fig. 11 | Supplemental characterization of DEGs shared in all the cocaine-, sucrose-, and overlapping ensembles. Differentially expressed genes (DEG) shared in all ensembles denoted by the black asterisks (*) in fig. 2b for the male NAcore, fig. 2h for the female NAcore, fig. 3b for the male PFC, and fig. 3h for the female PFC are used for region- and sex-specific DEG characterization. **a.** Male heatmap representation of log2(fold change) (log2FC) for DEGs shared in cocaine-seeking (C), sucrose-seeking (S), or overlapping (O) ensembles in the NAcore. **b.** Male volcano plot representing DEGs shared in all ensembles within the NAcore to show the amount of up- (upward pointing black-filled triangle) or down-regulated (downward pointing black-filled triangle) DEGs scaled by -log(false discovery rate) (y-axis; factor representing significance) and log2FC (x-axis). **c.** Male heatmap for the ten highest and ten lowest DEGs based on log2FC for DEGs shared in all ensembles within the NAcore. **d.** Male heatmap representation of log2FC for DEGs shared in cocaine-seeking (C), sucrose-seeking (S), or overlapping (O) ensembles in the PFC. **e.** Male volcano plot representing DEGs shared in all ensembles within the PFC to show the amount of up- (upward pointing black-filled triangle) or down-regulated (downward pointing black-filled triangle) DEGs scaled by -log(false discovery rate) (y-axis; factor representing significance) and log2FC (x-axis). **f.** Male heatmap for the ten highest and ten lowest DEGs based on log2FC for DEGs shared in all ensembles within the PFC. **g.** Female heatmap representation of log2(fold change) (log2FC) for DEGs shared in cocaine-seeking (C), sucrose-seeking (S), or overlapping (O) ensembles in the NAcore. **h.** Female volcano plot representing DEGs shared in all ensembles within the NAcore to show the amount of up- (upward pointing black-filled triangle) or down-regulated (downward pointing black-filled triangle) DEGs scaled by -log(false discovery rate) (y-axis; factor representing significance) and log2FC (x-axis). **i.** Female heatmap for the ten highest and ten lowest DEGs based on log2FC for DEGs shared in all ensembles within the NAcore. **j.** Female heatmap representation of log2FC for DEGs shared in cocaine-seeking (C), sucrose-seeking (S), or overlapping (O) ensembles in the PFC. Asterisks (*) denote the gene list of significant DEGs shared in ensembles used for ordering log2FC from low to high for comparison with specific ensemble-seeking lists. Total ensemble-specific gene counts are next to the heatmap titles. **k.** Female volcano plot representing DEGs shared in all ensembles within the PFC to show the amount of up- (upward pointing black-filled triangle) or down-regulated (downward pointing black-filled triangle) DEGs scaled by -log(false discovery rate) (y-axis; factor representing significance) and log2FC (x-axis). **l.** Female heatmap for the ten highest and ten lowest DEGs based on log2FC for DEGs shared in all ensembles within the PFC.