ARTICLE

Distinct Behavioral Profiles and Neuronal Correlates of Heroin Vulnerability Versus Resiliency in a Multi-Symptomatic Model of Heroin Use Disorder in Rats

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Objective: The behavioral and diagnostic heterogeneity within the opioid use disorder (OUD) diagnosis is not readily captured in current animal models, limiting the translational relevance of the mechanistic research that is conducted in experimental animals. The authors hypothesized that a nonlinear clustering of OUD-like behavioral traits would capture population heterogeneity and yield subpopulations of OUD vulnerable rats with distinct behavioral and neurocircuit profiles.

Methods: Over 900 male and female heterogeneous stock rats, a line capturing genetic and behavioral heterogeneity present in humans, were assessed for several measures of heroin use and rewarded and non-rewarded seeking behaviors. A nonlinear stochastic block model clustering analysis was used to assign rats to OUD vulnerable, intermediate, and resilient clusters. Additional behavioral tests and circuit analyses using c-fos protein activation were conducted on the vulnerable and resilient subpopulations.

Results: OUD vulnerable rats exhibited greater heroin taking and seeking behaviors relative to those in the intermediate and resilient clusters. Akin to human OUD diagnosis, further vulnerable rat subclustering revealed subpopulations with different combinations of behavioral traits, including sex differences. Lastly, heroin cue–induced neuronal patterns of circuit activation differed between resilient and vulnerable phenotypes. Behavioral sex differences were recapitulated in patterns of circuitry activation, including preferential engagement of extended amygdala stress circuitry in males and cortico-striatal drug cue-seeking circuitry in females.

Conclusion: Using a nonlinear clustering approach in rats, the analysis captured behavioral diagnostic heterogeneity reflective of human OUD diagnosis. OUD vulnerability and resiliency were associated with distinct neuronal activation patterns, posing this approach as a translational tool in assessing neurobiological mechanisms underpinning OUD.

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Opioid use disorder (OUD) inflicts worldwide social and personal harm, with over 16 million individuals affected (1) and approximately 1 million a year seeking treatment (2). Greater understanding of the neurobiological mechanisms underlying OUD is necessary to advance treatment options. A consequential impediment to attaining this goal is the behavioral heterogeneity inherent in a diagnosis of OUD based on DSM-5 criteria. Different behavioral traits, or diagnostic criteria, interact and combine in divergent ways to form an OUD diagnosis, resulting in individuals having the same diagnosis with distinct symptom profiles. Animal models necessary for mechanistic neurobiological experimentation fail to recapitulate the multidimensional diagnosis of OUD and largely fail to capitalize the preclinical biological discoveries into useful treatments. We hypothesized that a nonlinear clustering protocol applied to multiple behaviors commonly used in mechanistic animal studies of substance use disorder (SUD) would better recapitulate the multidimensional diagnosis of SUD and behavioral diversity conferring vulnerability. We further hypothesized that this approach would yield heroin-conditioned cue circuit activation differences between OUD vulnerable and resilient subpopulations.

Typically, preclinical studies use individual behavioral traits or summated linear relationships between a few traits

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to predict and explore underpinning neurobiological causes of SUD (3, 4). These models either do not capture a multisymptomatic diagnosis, or when doing so rely on linear interactions that do not capture the complex multidimensional interactions between symptoms that create subpopulations of SUDs patients. Here we describe a rat model and analysis workflow that clusters behaviors using nonlinear modeling (5) on data from over 900 male and female outbred heterogeneous stock rats, a line that emulates the complex within-species genetic and behavioral heterogeneity found in outbred mammalian populations, including humans (6). Rats were clustered as OUD vulnerable, resilient, or intermediate. We further assessed variability within the OUD vulnerable subpopulation and identified distinct subclusters that, akin to human OUD, varied in combinations of traits conferring vulnerability. Also, by correlating Fos protein synthesis (an indicator of neuronal activity) stimulated by heroin-conditioned cues across different brain nuclei, we identified distinct patterns of connectivity between vulnerable and resilient rats.

METHODS

The Medical University of South Carolina Institutional Animal Care and Use Committee and the Italian Ministry of Health approved all experimental procedures. All procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and the European Community Council Directive for Care and Use of Laboratory Animals.

Subjects

Heterogeneous stock rats (NMcwiWFsm:HS) were bred and shipped from Wake Forest University to either Medical University of South Carolina (MUSC; Charleston) or the University of Camerino (UCAM; Camerino, Italy). Litters were equally represented at both sites in an effort to create genetically comparable testing cohorts. Final analyses comprised 917 rats (male: N=465; MUSC, N=271; UCAM, N=194; female: N=452; MUSC, N=258; UCAM, N=194). Yoked saline control rats underwent testing procedures identical to those for their counterparts.

Drugs

Heroin hydrochloride, supplied by the National Institute on Drug Abuse, was dissolved in 0.9% sterile saline.

Behavioral Testing

The behavioral testing procedure is illustrated in Figure 1A. Rats underwent long-access heroin self-administration to quantify consumption and escalation of use. Extinction assessed perseverance of seeking in the absence of reward. Estimates of clinical laboratory-induced craving included both heroin-prime and cued reinstatement. Motivation for reward was assessed using a progressive ratio test. Additional behavioral traits associated with SUD were also examined before and after the heroin self-administration protocol, including the elevated plus maze and open field test to assess stress- and anxiety-like behaviors, and analgesic threshold was evaluated using the tail-flick test. The tail-flick test consisted of two phases: baseline (1 mg/kg saline injection, s.c.) and test (0.75 mg/kg heroin, or saline given to control rats). Additionally, a forced swim test was administered at Wake Forest University prior to shipment to assess stress-coping strategy.

Heroin Self-Administration and Extinction and Reinstatement Procedures

Rats were outfitted with an indwelling jugular catheter prior to training. During self-administration, presses on the active lever resulted in an infusion of heroin (20 μg/kg, 100-μL infusion over 3 seconds) and presentation of a tone and light cue (Figure 1B). Saline-control animals received a noncontingent infusion of saline (100 μL) every 20 minutes, accompanied by a tone and light cue presentation. There were four 12-hour sessions per week, for a total of 12 training sessions. A progressive ratio test occurred on day 36 (Figure 1A), and then self-administration was reestablished. A 6-hour extinction-prime session occurred (Figure 1B), and rats received a heroin priming injection (0.25 mg/kg, s.c.; saline to control rats) after 4 hours of extinction. Daily extinction training sessions then commenced prior to a test for cue-induced reinstatement (Figure 1B). Estrous cycle phase was identified in a subset of MUSC rats (N=36) following the tests for heroin-prime and cued reinstatement.

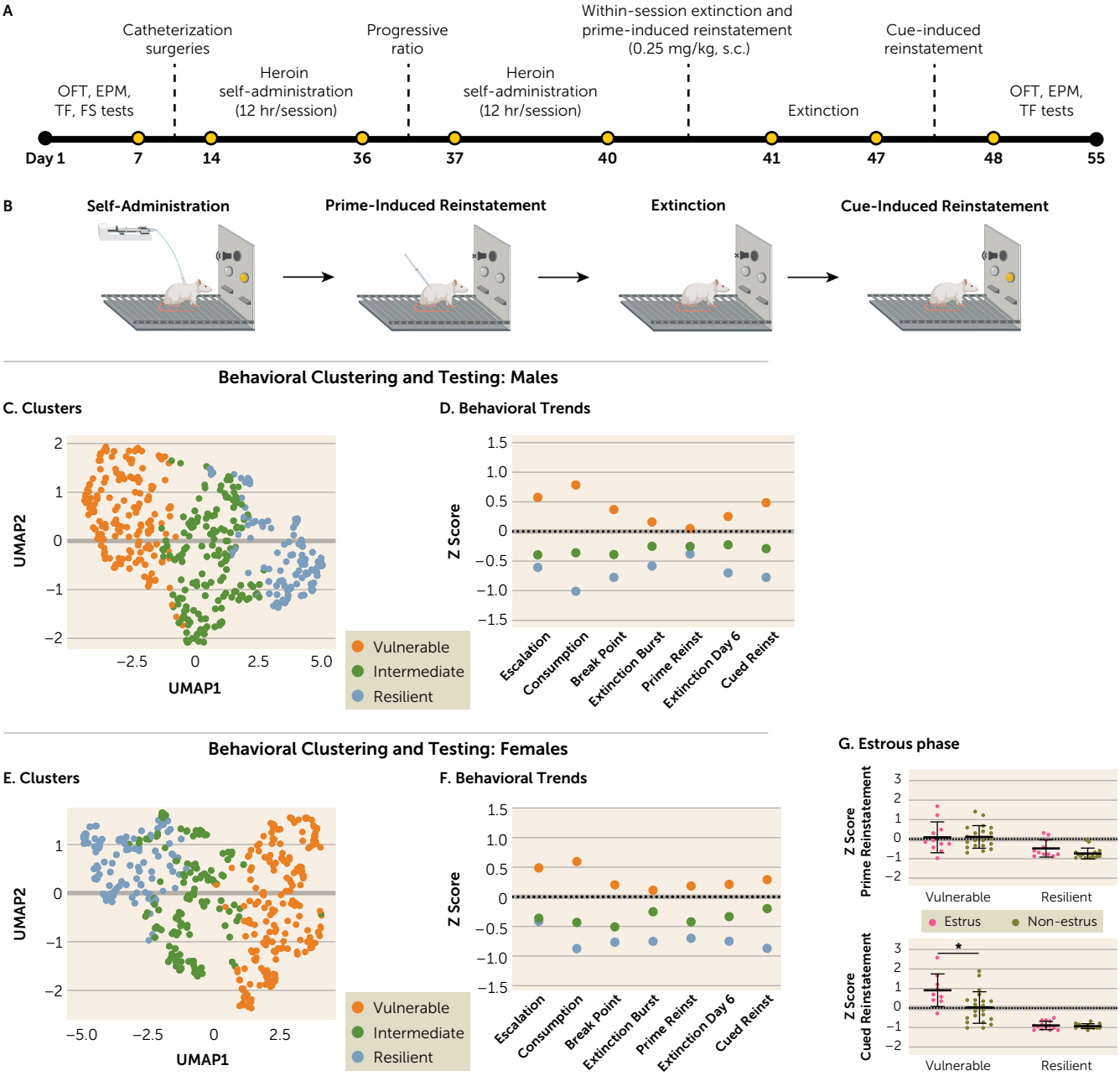
Additional Behavioral Testing

Punished heroin-taking behavior. We hypothesized that vulnerable rats would continue seeking heroin more than the resilient rats in the presence of an adverse stimulus. Three weeks after all testing, a subset of MUSC rats (males, N=15; females, N=14) reestablished heroin self-administration training for 3 days prior to a test session under self-administration conditions where there was a 50% chance of foot shock delivery (0.40 mA, 0.5 seconds) with each infusion earned.

Ultrasonic vocalizations. We examined the possibility that different withdrawal-induced affective states were associated with vulnerable and resilient rats. Four hours prior to extinction on day 41 (Figure 1A), a subset of MUSC rats (males, N=22; females, N=21; saline, N=8) were assessed for acute heroin withdrawal-induced ultrasonic vocalizations, a preclinical test for assessing affective state (7, 8).

Fos protein quantification. A subset of MUSC vulnerable and resilient rats (females, N=13; males, N=18; saline, N=11) underwent nine additional extinction training sessions and a second cued reinstatement test ~3 weeks after testing was completed. Fos protein expression in regions of interest were manually quantified.

FIGURE 1. Experimental timeline and animal OUD phenotype behavioral data[a]



[a] As illustrated in the timeline in panel A, rats underwent behavioral testing prior to jugular catheterization surgery (OFT=open field test; EPM=elevated-plus maze; TF=tail flick test; FS=forced swim test), and again at the end of heroin experience (excluding the forced swim test). Heroin self-administration then commenced and continued for 3 weeks (12 hours per session, 4 days a week), followed by a progressive ratio test and 3 additional days of self-administration training. A within-session extinction-prime reinstatement test then occurred, followed by extinction training and a test for cued reinstatement. Panel B illustrates the behavioral paradigm and testing chamber configuration (images created with BioRender). Active lever presses during self-administration training resulted in presentation of a tone and light cue for 5 seconds and a heroin infusion. Active lever presses had no consequence during the within-session extinction-prime test or during extinction training. During cued reinstatement, active lever presses resulted in tone and light cue presentation, but no heroin infusion. Panel C is a uniform manifold approximation and projection (UMAP) representation of male rats separated into distinct OUD vulnerability clusters using stochastic block model network-based clustering analysis (vulnerable, N=182; intermediate, N=168; resilient, N=115). The graph in panel D shows the median value for heroin taking, extinction, and seeking behavioral measures in male rats. Panel E is a UMAP representation of the distinct cluster formation of OUD vulnerability in female rats following stochastic-block model network-based clustering analysis (vulnerable, N=204; intermediate, N=132; resilient, N=116). The graph in panel F shows the median value for heroin taking, extinction, and seeking behaviors between clusters in female rats. Panel G shows the mean and standard deviation for behavior during the reinstatement tests in female rats. Rats (vulnerable, N=31; resilient, N=27) in the estrus phase of the estrous cycle exhibited potentiated responding during the cued reinstatement test relative to non-estrus-phase rats ($t=2.72$, $df=28$, $p=0.01$). Estrous phase cycle did not affect behavior in vulnerable rats during heroin-primed reinstatement ($t=0.08$, $df=29$, $p=0.94$) or in resilient rats (heroin-primed reinstatement: $t=1.88$, $df=25$, $p=0.07$; cued reinstatement: $t=0.57$, $df=25$, $p=0.57$).

*$p<0.05$.

Statistical Analysis

To capture behaviors across OUD phases (heroin use, extinction, and seeking), seven behaviors were selected for analyses as the dependent variables. Heroin use behaviors included escalation of intake (μg/kg) (average consumption on days 1–3 subtracted from days 10–12) and total consumption across the first 12 training sessions; extinction behaviors included active lever presses made during the first 2 hours of the extinction-prime test (extinction burst) and during the last day of extinction training (extinction day 6); and seeking measures included break point (maximum active lever presses expended to receive an infusion) from the progressive ratio test, active lever presses during heroin-prime reinstatement, and cued reinstatement.

Normality was evaluated using a Kolmogorov-Smirnov test. Raw data were assessed for group differences using a two-way analysis of variance (ANOVA) with sex and site as the independent variables and a Bonferroni post hoc test, with several differences found (see Table S1 in the online supplement). Accordingly, data were z-score transformed within site and assessed for sex (independent variable) differences using the Mann-Whitney test (see Figure S1 in the online supplement). Sex differences were present for every behavior, and data were standardized within sex and site prior to being recombined for clustering analyses. Sexes were then analyzed as independent groups for all subsequent analyses.

We previously described a nonlinear network-based clustering analysis (9) and provided the R package "mlsbm" (5). A rat-rat similarity network was constructed from the standardized data to assess behavioral similarities between rats, and then a Bayesian stochastic block model was applied to define clusters within the data set. Cluster number was set to 3 (vulnerable, intermediate, resilient) in order to identify subpopulations of rats potentially most susceptible and least susceptible to developing OUD and in alignment with a previous study where cluster number was determined according to Bayesian information criterion (see Figure S2 in the online supplement) (9). Behavioral differences between clusters (independent variable) were analyzed using either an ANOVA and Bonferroni post hoc test or a Kruskal-Wallis test and Dunn's post hoc test.

Repeated-measures ANOVAs (with cluster and session as independent variables) and Bonferroni post hoc test were used to assess behavior during heroin reacquisition. Unpaired Student's t or Mann-Whitney tests were used for punishment test, ultrasonic vocalizations, and Fos protein analyses (with cluster as independent variable for all) and estrous cycle phase (with phase as independent variable). To account for experimental training conditions shared across all animals that could affect Fos protein expression (exploratory behavior, testing context, etc.), Fos was standardized to saline control rats prior to analyses. The Spearman correlation coefficient was used for behavioral correlations with a Bonferroni test to correct for multiple comparisons and Fos protein correlations, which employed a false discovery rate of $q=0.01$ to correct for multiple comparisons. Differences in group composition were evaluated with a chi-square test. Subclustering analyses were performed using an agglomerative hierarchical clustering strategy. The Euclidian distance between subjects with a threshold of 0.7*max(linkage) was used to create subclusters, and a minimum of 15 rats per subcluster was necessary to be included in analyses. Multiple comparisons across different analyses were not corrected for. Unless stated otherwise, analyses were performed using GraphPad Prism, version 9.5.1, with a p threshold of 0.05 for statistical significance.

RESULTS

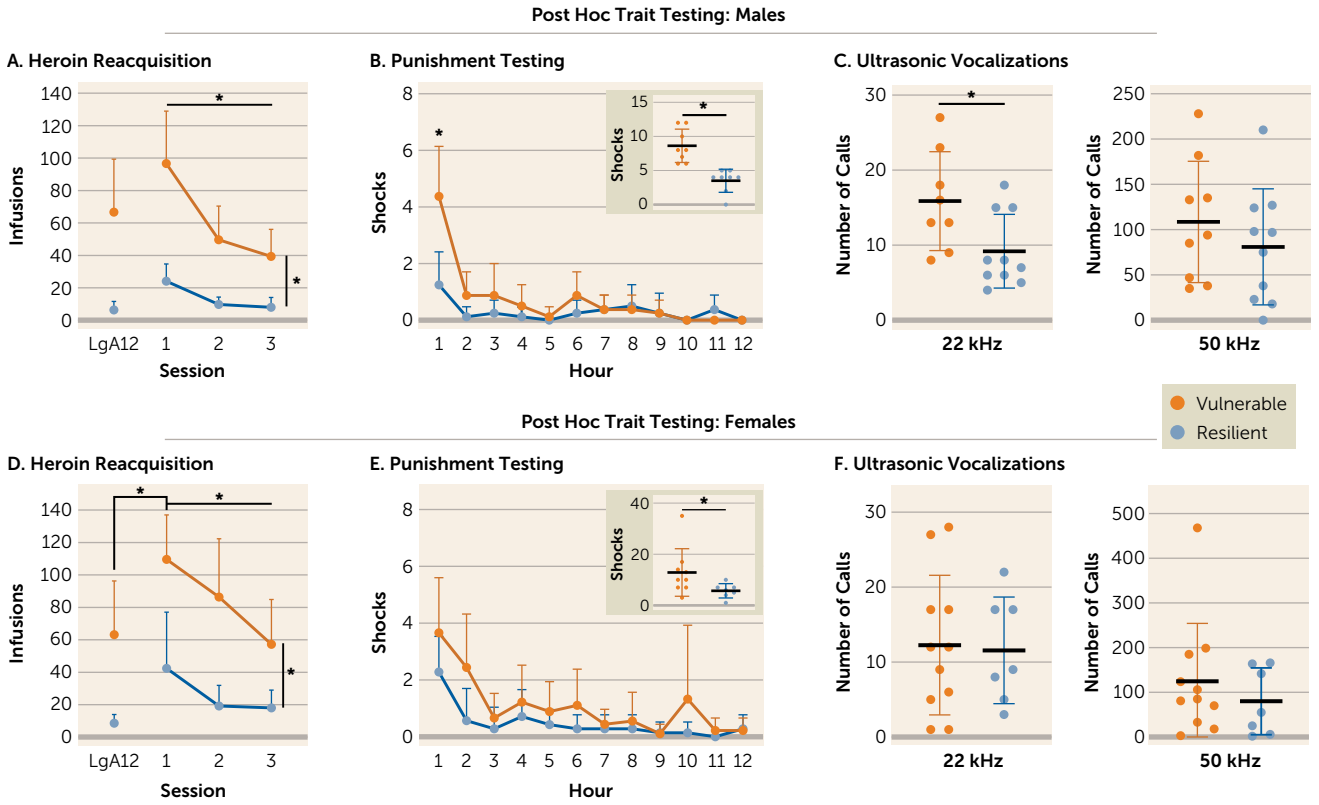
Raw Data

Substantial site and sex differences existed in the raw data for the OUD-like traits and the behaviors quantified prior to and following heroin experience (see Table S1 and Figures S3–S7 in the online supplement). Female rats exhibited an overall more OUD vulnerable phenotype than male rats (see Figure S5 in the online supplement) (10). Despite standardization of experimental procedures across sites, MUSC rats showed augmented levels of heroin-related behaviors compared to UCAM rats (see Figure S5 in the online supplement). The source of these site differences presumably results from variables we did not control, such as shipping and aspects of animal housing protocols and handling (see the Supplemental Methods section of the online supplement for a detailed description). Because these uncontrolled environmental factors were not quantified and the study was designed to examine environmental factors that were experimenter controlled and more related to opioid use and relapse, we z-scored the data within site and sex for further analysis (see Figures S8 and S9 in the online supplement). However, in future studies it would be of interest to determine which environmental differences between sites may have contributed to behavioral differences. After standardization, linear regression revealed ubiquitous covariance across variables for both sites, necessitating an alternative analysis strategy to better define OUD vulnerability (see Figure S10 in the online supplement).

OUD-Like Trait Clustering Analysis

Stochastic block model clustering was used to model the multidimensional nature of the diagnostic criteria for OUD. Three statistically distinct clusters (see Figure S2 in the online supplement) were established as vulnerable, resilient, and intermediate (Figure 1C,E) and were distributed equivalently between the sexes (see Figure S11 in the online supplement). The median value of behavioral traits was higher in the vulnerable compared to resilient rats in both sexes, although the distribution of individuals showed substantial overlap between the clusters (Figure 1D,F; see also Figures S8 and S9 in the online supplement). Vulnerable, but not resilient, rats in estrus showed potentiated cue

FIGURE 2. Post hoc behavioral testing[a]



[a] Panel A shows the mean number of infusions earned the last day of heroin self-administration training (LgA12=long access day 12, or training day 35) and heroin reacquisition in a subset of male rats (N=8 per cluster) (in all panels, error bars indicate standard deviation). Vulnerable male rats maintained higher levels of heroin taking during reacquisition (cluster-by-session interaction: $F=5.29$, $df=3, 42$, $p=0.004$; all post hoc tests, $p<0.01$). There was a 50% chance of foot shock delivery with each heroin infusion earned during the punishment task; as shown in panel B, vulnerable male rats endured more shocks while maintaining heroin-taking behavior compared to resilient rats (inset, Mann-Whitney $U=0$, $p=0.0002$), with differences centralized to the first hour of testing (cluster-by-hour interaction: $F=7.29$, $df=11, 154$, $p<0.001$; significant post hoc test, $p=0.02$). Panel C shows the mean number of acute withdrawal-induced ultrasonic distress vocalization calls. The number of calls was higher in vulnerable male rats compared to resilient male rats (22 kHz: Mann-Whitney $U=14.5$, $p=0.02$), with no difference in appetitive state calls (50 kHz: $t=0.92$, $df=17$, $p=0.37$) (vulnerable, N=8; resilient, N=10). Panel D shows the mean number of infusions earned the last day of heroin self-administration training (LgA12) and heroin reacquisition in a subset of female rats (vulnerable, N=9; resilient, N=7). A main effect of cluster, but not cluster-by-session interaction, was present, indicating that vulnerable female rats in general maintained higher levels of heroin taking during reacquisition (cluster: $F=36.02$, $df=1, 14$, $p<0.0001$; cluster-by-session interaction: $F=1.43$, $df=3, 42$, $p=0.25$) and, as shown in panel E, endured more shock-heroin pairing across the test session relative to resilient rats (inset, Mann-Whitney $U=11$, $p=0.03$). However, phenotypes differed in cumulative shocks endured only across the test session, not across the independent hours of testing (cluster-by-hour interaction: $F=1.56$, $df=11, 154$, $p=0.12$). As shown in Panel F, the mean number of acute withdrawal-induced ultrasonic vocalization calls did not differ significantly between female phenotypes (22 kHz: $t=0.17$, $df=16$, $p=0.87$; 50 kHz: Mann-Whitney $U=30$, $p=0.48$) (vulnerable, N=11; resilient, N=7).
*$p<0.05$.

reactivity compared to non-estrus-phase rats. Estrous phase had no effect on behavior during heroin-primed reinstatement (Figure 1G).

Non-OUD Traits

Stress-coping strategy or anxiety-like behavior in basal conditions were not associated with OUD vulnerability in either sex, as measured by the forced swim test and elevated plus maze (see Figures S12, S13C,F, and S14C,F in the online supplement). Novelty-induced locomotion was augmented in male but not female vulnerable compared to resilient rats (see Figure S13A,B in the online supplement), consistent with our previous finding (10) that exploratory behavior can predict OUD vulnerability in male but not female rats ($r^2=0.18$, $p<0.001$, and $r^2=0.09$, $p=0.08$, respectively; see Figure S14A,B in the online supplement). Analgesic threshold was not associated with OUD vulnerability in either sex (see Figures S15 and S16 in the online supplement). However, after heroin use, female vulnerable rats had a lower analgesic threshold compared to resilient rats (see Figure S16E in the online supplement), implying heroin-induced alterations in pain processing.

Additional Trait Testing After Completing the Heroin Self-Administration Protocol

Punishment training. After ~3 weeks of forced abstinence, vulnerable and resilient rats readily reacquired heroin self-administration, although vulnerable animals to a greater extent (Figure 2A,D; see also Figure S17A,C in the online supplement). Furthermore, both female subpopulations exhibited potentiated heroin taking on the first day of reacquisition relative to the last day of heroin self-administration

training, indicative of a deprivation effect described for alcohol (11) but not previously shown for opioids (Figure 2D). During the punished heroin-taking test, vulnerable male rats endured ~3 times the number of infusion-shock pairings, and females ~2 times, before attenuating heroin-taking behavior to levels of resilient rats (Figure 2B,E; see also Figure S17B,D in the online supplement).

Ultrasonic vocalizations. The effect of acute heroin withdrawal on emotional state was quantified using ultrasonic vocalizations (8). Ultrasonic vocalizations indicative of a positive affective state (50 kHz) did not differ between clusters in male or female rats (Figure 2C,F). Male vulnerable rats showed potentiated ultrasonic vocalizations associated with a negative affective state (22 kHz) compared to resilient rats. Female clusters did not differ in negative affect.

Behavioral Phenotype Subclustering

Clusters were probed for the presence of subpopulations to assess behavioral heterogeneity conferring OUD vulnerability and resiliency. Distinct subclusters existed only in the vulnerable phenotype for both male (N=3 subclusters) and female (N=4 subclusters) rats (Figure 3A,B). For each sex, subclusters corresponded to augmented responding when behavior was 1) reinforced with heroin (break point, consumption, escalation, primed reinstatement); 2) not reinforced by heroin (break point, extinction burst, extinction day 6, cued reinstatement); or 3) in both behavioral categories (Figure 3C,D). Female rats in the nonreinforced subcluster exhibited further heterogeneity, with one group having potentiated responses during acute heroin withdrawal (break point, extinction burst) and the other after more protracted withdrawal (extinction day 6 and cued reinstatement) (Figure 3D). Overall, males were biased toward the heroin reinforced subcluster, and females toward the heroin nonreinforced subclusters (Figure 3E). These data emulate behavioral heterogeneity in OUD diagnosis and emphasize sex differences in vulnerability.

Cue-Induced Fos Protein Expression

While group differences were not present, cue-induced Fos protein expression relative to saline rats differed between vulnerable and resilient rats in the prelimbic (PrL) and infralimbic cortex and the anterior paraventricular nucleus of the thalamus (aPVT; see Figures S18–S20 in the online supplement). We were surprised to find differences in brain nuclei with opposite functions. Much of the animal and human literature shows activation of PrL and infralimbic cortex during cued seeking (12) and of the aPVT in suppressing seeking (13, 14). We further evaluated this apparent contradiction by examining correlated Fos protein between nuclei, which revealed both shared and distinct patterns between the vulnerable and resilient phenotypes (see Table S2 in the online supplement). We subdivided region-of-interest connectivity into subcircuits that in animal and human imaging studies (15–18) are generally associated with cue reactivity, stress, and behavioral inhibition (Figure 4B–D,F–H). Commensurate with vulnerable rats reinstating more than resilient rats (Mann-Whitney U=18.50, $p<0.0001$; vulnerable, mean=42.18, SD=25.96; resilient, mean=6.07, SD=12.23; see Figure S19M in the online supplement), vulnerable rats showed greater levels of correlated neuronal engagement (Figure 4A,E), most notably in regions of interest in the extended amygdala associated with stress responses (Figure 4C,G) (19). It was also notable that the ventral pallidum (VP), which contains subpopulations of neurons contributing to all three subcircuits, was highly interconnected within all subcircuits in vulnerable but not resilient rats. To gain insight into possible sex differences contributing to vulnerability, vulnerable males and females were assessed separately (see Table S3 in the online supplement). Strikingly, the sexes shared almost no common subcircuitry (Figure 5A,E). Female rats exhibited engagement of top-down processes, including the classic cue reactivity pathways (20), with correlated neuronal activation between the PrL, nucleus accumbens core (NAcc), and VP (Figure 5F). Males engaged subcortical cue reactivity regions, specifically the NAcc and VP (Figure 5B). Strikingly, only males showed engagement of the extended amygdala stress circuit (Figure 5C). Since the sexes exhibited equivalent cued reinstatement ($t=0.27$, $df=15$, $p=0.79$; male, mean=44.26, SD=17.59; female, mean=40.7, SD=31.40), these data suggest sexually dimorphic circuits contributing to cue reactivity.

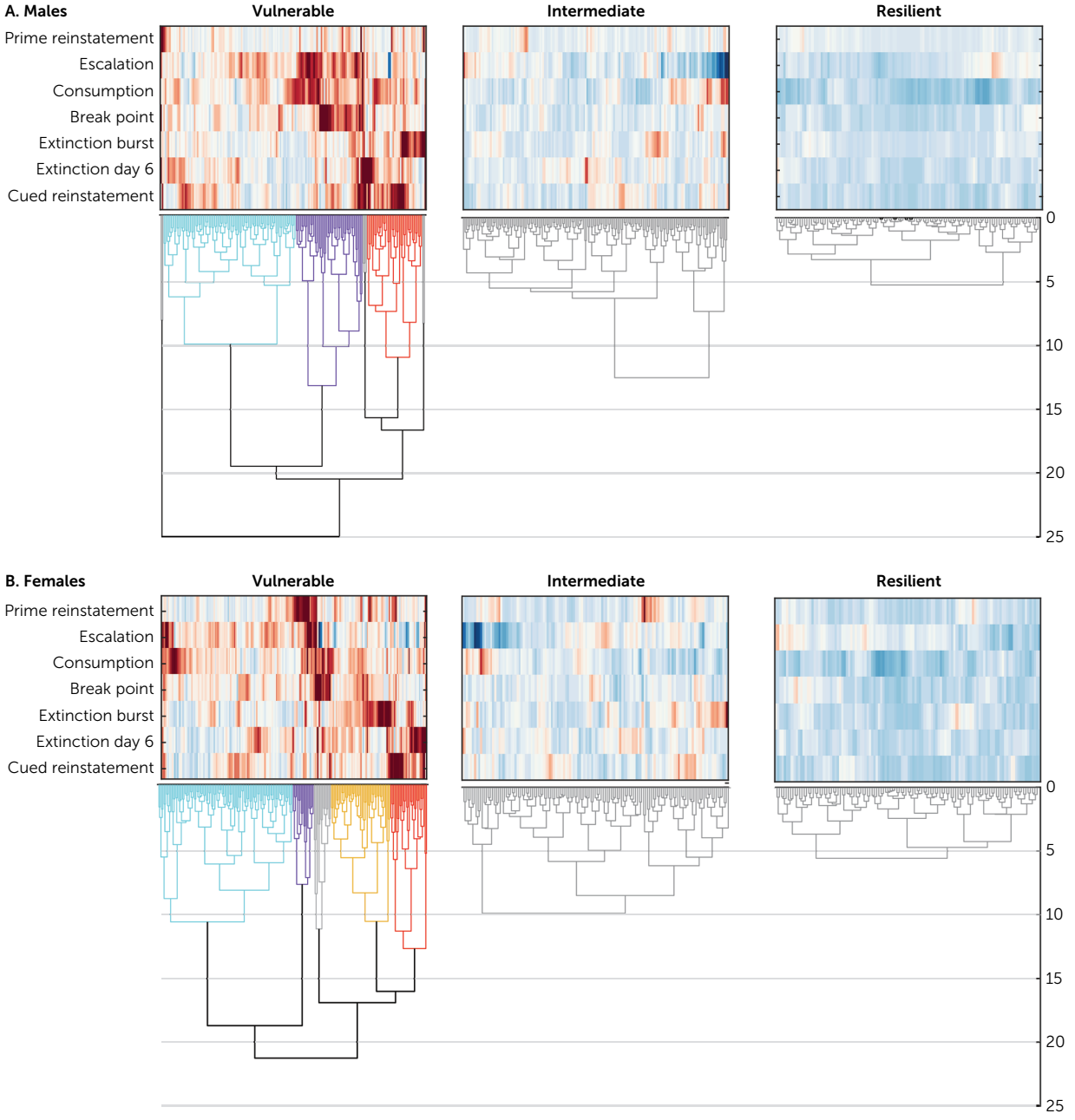
DISCUSSION

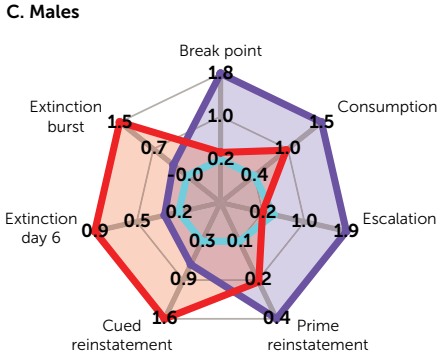
OUD has increased in all demographic groups over the past two decades, regardless of age, sex, or socioeconomic status (21). To capture the diversity of OUD subpopulations and different combinations of DSM-5 diagnostic symptoms, we used genetically heterogeneous rats and a nonlinear network-based clustering of seven rat behavioral traits. Using this approach, we succeeded in separating vulnerable from resilient rats and further subclustering vulnerable into subpopulations of rats possessing different combinations of OUD-like behaviors. We validated this approach by showing distinct neurocircuit activation by heroin cues between vulnerable and resilient subpopulations. We propose that this statistical workflow and the behavioral findings are akin to the multidimensional diagnosis of OUD patients and are ideal for identifying subpopulation heterogeneity in brain mechanisms underpinning OUD vulnerability and resilience.

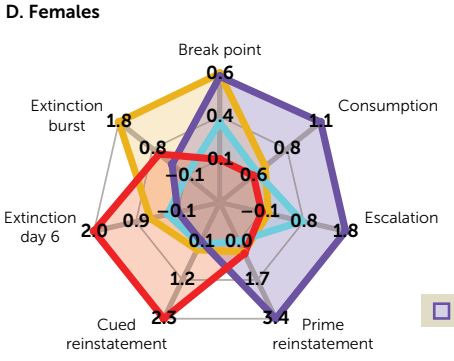
OUD Vulnerability Clustering

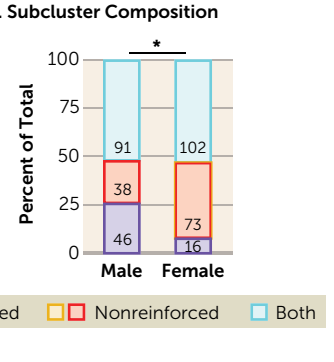
The nonlinear clustering approach identified a vulnerable subpopulation of rats exhibiting greater levels of heroin use, resistance to extinction, and seeking compared to rats in the intermediate and resilient clusters for both sexes. Akin to diagnostic criteria for OUD, behavioral heterogeneity was present in the traits that confer OUD-like vulnerability in both sexes, with distinct subpopulations exhibiting

FIGURE 3. Behavioral heterogeneity within OUD vulnerable clusters[a]



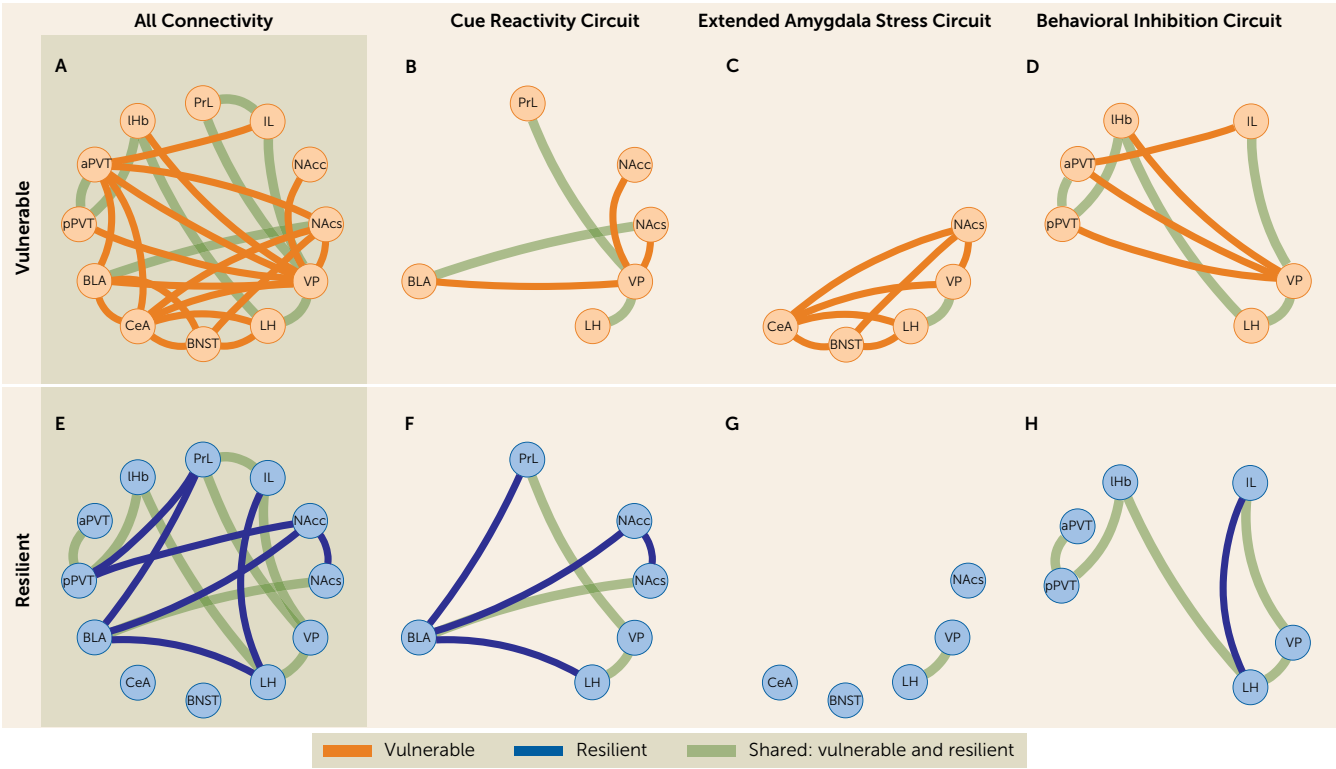






[a] Hierarchical analysis showed that only the vulnerable group among male and female rats (panels A and B) was composed of distinct behavioral subclusters (males: vulnerable, N=182; intermediate, N=168; resilient, N=115; females: vulnerable, N=204; intermediate, N=132; resilient, N=116; clusters with fewer than 15 animals were excluded from further analysis). Spatial representation of vulnerable subcluster behavioral heterogeneity is

FIGURE 4. Correlated neuronal activation patterns between heroin cue–induced c-Fos protein regions of interest in OUD vulnerable and resilient rats[a]



[a] Light green line color illustrates connectivity shared between vulnerable and resilient phenotypes; orange and blue line colors indicate connectivity distinct for vulnerable and resilient clusters, respectively. As shown in panels A and E, rats in the vulnerable cluster exhibited more correlated neuronal activation compared to resilient rats. Connectivity was further broken down into three different functional circuitry categories: regions of interest (ROIs) associated with cue reactivity (panels B and F), extended amygdala stress response (panels C and G), and behavioral inhibition (panels D and H). Although both phenotypes engaged ROIs associated with cue reactivity, the vulnerable group recruited several ROIs that mediate stress response and behavioral inhibition. Data for each ROI were standardized to saline control rats, and correlations shown are $p<0.05$ with $q=0.1$ (vulnerable, N=17; resilient, N=14; saline, N=11). PrL=prelimbic cortex; IL=infralimbic cortex; NAcc=nucleus accumbens core; NAcs=nucleus accumbens shell; VP=ventral pallidum; LH=lateral hypothalamus; BNST=bed nucleus of the stria terminalis; CeA=central amygdala; BLA=basolateral amygdala; pPVT=posterior paraventricular nucleus of the thalamus; aPVT=anterior paraventricular nucleus of the thalamus; lHb=lateral habenula.

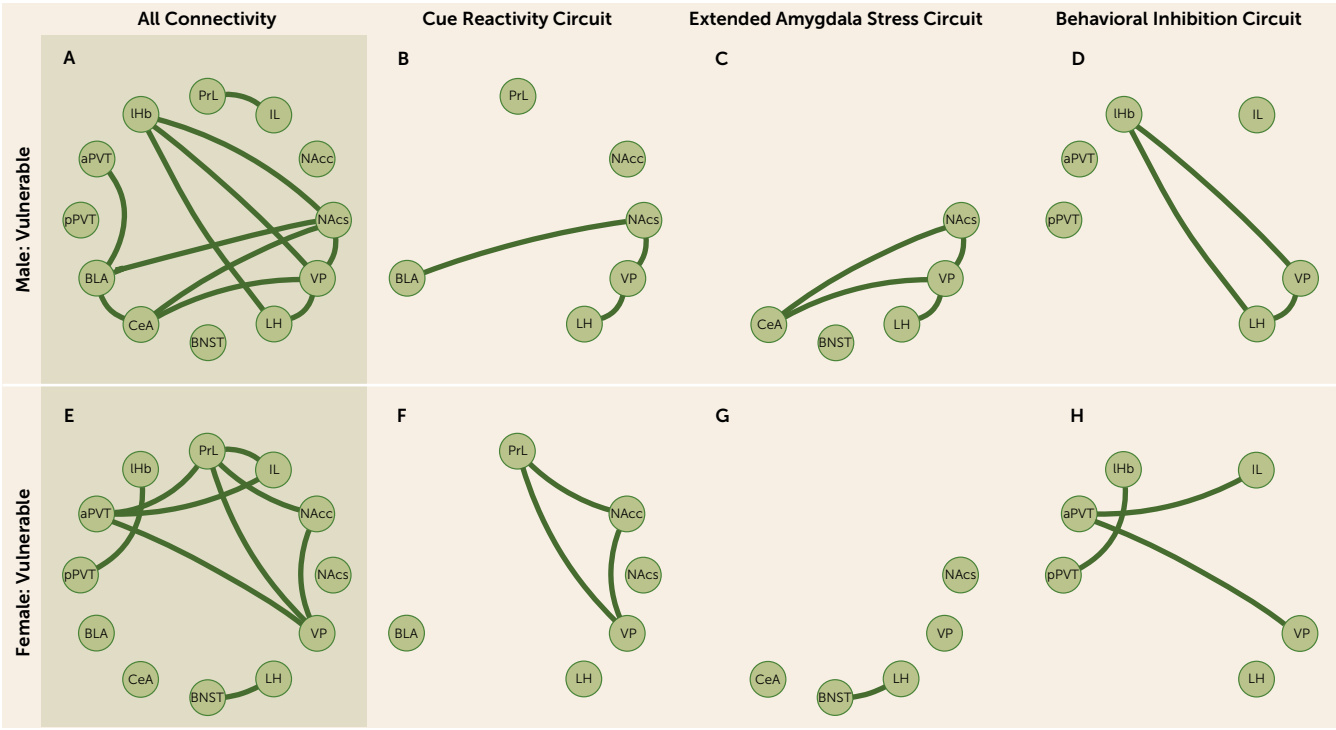
behavioral vulnerability in heroin-reinforced drug seeking, nonreinforced drug seeking, or a combination of both. Male vulnerable subclusters were biased toward heroin-reinforced seeking, and females toward heroin nonreinforced seeking. Female rats in the nonreinforced subpopulation were further subdivided into a subpopulation more motivated to respond during early (i.e., progressive ratio and extinction burst) versus late extinction conditions (i.e., extinction day 6), with the latter also exhibiting high cue reactivity during cued reinstatement, suggesting differential sensitivity to contextual and discrete drug-associated cues.

Other Measures Associated With Vulnerability

In addition to the seven traits used for stochastic block model clustering, hallmark features of OUD were identified in subsequent testing done in a portion of rats. Vulnerable rats reacquired heroin self-administration and required more infusion-shock pairings to attenuate heroin-taking behavior. Male vulnerable rats exhibited more distress than their resilient counterparts during acute heroin withdrawal, with no differences observed in females. However, male rats were more prone to emit stress-induced ultrasonic vocalizations (22), suggesting that alternative withdrawal assays may be more applicable for female rats. Circulating ovarian hormones affected behavioral heterogeneity in female rats. Behavioral responding is potentiated during the estrus phase of the cycle due to estradiol regulation of mesolimbic dopamine (23), and vulnerable estrus-phase rats showed potentiated cued seeking relative to non-estrus-phase rats. No differences were observed during prime reinstatement,

presented as spider plots, where the value at each node represents the average z score for the indicated behavior for each subcluster. As shown in panels C and D, both sexes contained subclusters comprising rats that showed augmented behavior when reinforced by heroin (purple), not reinforced by heroin (red/yellow), and by both circumstances (blue). As shown in panel E, the composition of the subclusters differed between the sexes, with vulnerable male rats biased toward heroin reinforced behaviors and female rats toward heroin nonreinforced behaviors (combined yellow and red subclusters in females: $\chi^2=25.53$, df=1, $p<0.001$); the numbers within the bars indicate the number of rats within each subcluster.
*$p<0.05$.

FIGURE 5. Sexually dimorphic correlated neuronal activation patterns between regions of interest in male and female OUD vulnerable rats[a]



[a] Panels A and E show correlated activity patterns for male and female vulnerable rats, respectively. Connectivity patterns were further broken down into functional circuit categories: regions of interest (ROIs) associated with cue reactivity (panels B and F), extended amygdala stress response (panels C and G), and behavioral inhibition (panels D and H). Male rats showed pronounced subcortical activity patterns, specifically engaging the extended amygdala stress circuitry. In contrast, female rats engaged top-down cortical processing, with minimal engagement of stress circuitry. Data for each ROI were standardized to saline control rats, and correlations shown are $p<0.05$ with $q=0.1$ (females, N=10; males, N=7; saline, N=11). PrL=prelimbic cortex; IL=infralimbic cortex; NAcc=nucleus accumbens core; NAcs=nucleus accumbens shell; VP=ventral pallidum; LH=lateral hypothalamus; BNST=bed nucleus of the stria terminalis; CeA=central amygdala; BLA=basolateral amygdala; pPVT=posterior paraventricular nucleus of the thalamus; aPVT=anterior paraventricular nucleus of the thalamus; lHb=lateral habenula.

suggesting estradiol involvement in modulating discrete environmental cues, but not heroin interoceptive cues.

Correlated Fos Activity Patterns in OUD Vulnerable Versus Resilient Rats

Little is known about the neurobiological mechanisms mediating SUD resilience. Decline in prefrontal cortex gray matter volume (24) and engagement of top-down cortical processes within the dorsal striatum (25) confer SUD resiliency. Resilient OUD rats exhibit hypofunction of neuronal activation in the medial prefrontal cortex (see Figure S19 in the online supplement), suggesting that resiliency may be mediated by prefrontal cortex neuroplasticity. Resilient rats appear to be recruiting circuitry and regions engaged in negative affective states and avoidance, such as the PrL-BLA pathway that mediates anxiety-like behavior and fear memory acquisition (26) and the infralimbic cortex, a region contributing to suppressing drug seeking (27). This profile of correlated activation suggests that OUD resiliency is in part governed by circuits that inhibit behavioral responding to cued seeking.

Commensurate with greater cue-induced heroin seeking, vulnerable rats engaged more complex circuitry, similar to what is observed in human imaging studies in OUD (16). Key features of connectivity distinctions between clusters include a majority of connectivity in vulnerable rats centered on the aPVT, a region involved in mediating anxiety and stress response (28) and suppression of reward seeking (13); the VP, which exhibits cell-specific regulation over drug seeking and drug refraining (i.e., withholding of seeking) (29); and the central amygdala, part of the extended amygdala that mediates stress response and stress-induced reinstatement (30). In an effort to disentangle the differences in circuit activation, we isolated nuclei generally found to be activated in animal and human imaging studies during a cue reactivity test, stress responding with a focus on the extended amygdala, and behavioral inhibition (16–19). Most striking was the involvement of the extended amygdala stress circuit in vulnerable but not resilient rats. Importantly, stress facilitates relapse in animal models and humans (31), which can account for the robust cued relapse in vulnerable but not resilient rats. Surprisingly, the vulnerable rats also showed greater overall connectivity in behavioral inhibition circuitry, notably in the interconnectivity between the lateral habenula, PVT, and VP, all structures experimentally implicated in a cell-, region-, or circuit-specific manner to

promote or induce avoidance behaviors (14, 29, 32). It is possible that following chronic heroin self-administration in vulnerable rats, these brain nuclei have undergone adaptations rendering them less capable of inhibiting cued seeking (33), or that a shift in cell-specific opposing regulation of drug seeking versus avoidance occurred (34).

Sex Differences

In parallel with behavioral sex differences frequently observed in this study, we found minimal overlap in vulnerability circuitry in male or female rats. Approximately 40% of female rats were in the non–heroin reinforced subcluster, and females heavily engaged the PrL→NAcc→VP series circuit that mediates cue reactivity in animals and humans (20). In contrast, male rats predominantly engaged extended amygdala circuitry involved in stress responding (30). Vulnerable males also exhibited withdrawal-induced distress, posing the possibility that male rats were experiencing more distress following prolonged heroin abstinence, resulting in stress circuitry contributing to cued reinstatement. Females, however, showed lower levels of distress across training. Together, these data suggest sexual dimorphism in neuroadaptations governing vulnerability in rats, with male vulnerability driven more by stress circuitry and female vulnerability by cue responsivity. Importantly, the literature supports nuanced sex differences translating preclinical to human OUD studies (23), although a more focused approach is necessary in preclinical and clinical research to sufficiently disentangle SUD sex differences (35).

CONCLUSIONS

Medication-assisted treatment is the current standard of OUD treatment (36). However, diagnostic heterogeneity is an important consideration for treatment, as individual symptom clusters are likely driven by distinct neurobiological mechanisms and genetics. By using a nonlinear clustering model that allowed us to capture aspects of the behavioral heterogeneity and diagnostic diversity in human OUD, we identified distinct behavioral profiles and sexually dimorphic neurobiological circuitry associated with OUD resiliency versus vulnerability in rats. Further subclustering revealed variability in traits conferring vulnerability, with notable differences in subcluster composition between the sexes. This study constitutes a first step in applying nonlinear clustering of SUD vulnerability traits and provides a preclinical database for continued studies of the neurobiological mechanisms and genetic vulnerabilities contributing to OUD phenotypes. Additionally, the neurobiological underpinnings of sex differences in human SUDs are not well characterized, and despite rates of OUD steadily increasing among females (37), females remain underrepresented in clinical OUD research (<15% of participants) (16). The marked behavioral and circuit sex differences we found highlight the use of nonlinear clustering in animal models to discern mechanisms of sex differences in behavioral phenotypes contributing to human OUD.

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