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Stress-induced escalation of alcohol self-administration, anxiety-like behavior, and elevated amygdala Avp expression in a susceptible subpopulation of rats

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Abstract

Comorbidity between alcohol use and anxiety disorders is associated with more severe symptoms and poorer treatment outcomes than either of the conditions alone. There is a well-known link between stress and the development of these disorders, with post-traumatic stress disorder as a prototypic example. Post-traumatic stress disorder can arise as a consequence of experiencing traumatic events firsthand and also after witnessing them. Here, we used a model of social defeat and witness stress in rats, to study shared mechanisms of stress-induced anxiety-like behavior and escalated alcohol self-administration. Similar to what is observed clinically, we found considerable individual differences in susceptibility and resilience to the stress. Both among defeated and witness rats, we found a subpopulation in which exposure was followed by emergence of increased anxiety-like behavior and escalation of alcohol self-administration. We then profiled gene expression in tissue from the amygdala, a key brain region in the regulation of stress, alcohol use, and anxiety disorders. When comparing "comorbid" and resilient socially defeated rats, we identified a strong upregulation of vasopressin and oxytocin, and this correlated positively with the magnitude of the alcohol self-administration and anxiety-like behavior. A similar trend was observed in comorbid witness rats. Together, our findings provide novel insights into molecular mechanisms underpinning the comorbidity of escalated alcohol self-administration and anxiety-like behavior.

KEYWORDS
alcohol use disorder, anxiety disorders, comorbidity, social defeat stress, vasopressin, witness stress

1 | INTRODUCTION

High comorbidity between alcohol use disorder (AUD) and anxiety disorders (ANX) has been observed in epidemiological studies.[1,2] It has been reported that among individuals with AUD, the prevalence of co-occurring ANX was 35.8% for men and 60.7% for women.[2] Comorbid AUD and ANX is a significant public health problem with more severe symptoms and poorer treatment outcomes compared to either of the conditions alone.[3] Few, if any, evidenced-based treatments are available for this patient population, making it an area of large unmet medical needs.

Substantial evidence links stress exposure to the development of AUD and ANX. The prototypic example is post-traumatic stress disorders (PTSD), which results from exposure to traumatic events,[4] and is highly co-morbid with AUD (reviewed, e.g., in[5]). Social stress, which is among the most prevalent stressors experienced by humans, is a significant risk factor for AUD[6–8] and ANX.[9] Importantly, observing others in fear or pain is a form of psychological stress which can also lead to the development of these disorders. Professionals with high rates of occupational exposure to traumatic events, including firefighters, aid workers, and trauma nurses, present a high risk of developing AUD[10,11] and ANX.[12] One study in war veterans further found the perceived threat to be more important for the development of PTSD than having experienced the traumatic event firsthand,[13] suggesting the importance of psychological stress.

Stress is an established risk factor for psychiatric disorders, but among individuals that are directly exposed to, or witness a traumatic event, only a minority will develop a psychiatric disorder.[14] This highlights the importance of individual variation in susceptibility and resilience to develop a maladaptive stress response such as anxiety and increased alcohol consumption. Some individual risk factors have been identified, such as sequence variation in the gene encoding FK506-binding protein 51 (FKBP5).[15] Less is currently known about shared neurobiological substrates of AUD and ANX or about similarities and differences between mechanisms mediating effects of physical versus psychological stress.

In rodents, social stress has been studied using social defeat stress (SDS),[16–18] which mimics some aspects of societal stress including human aggression and chronic subordination.[19] The SDS paradigm is based on social hierarchy and dominance where the "defeated" animal is exposed to attacks and subsequent subordination by a conspecific. Numerous studies report a causal role of SDS in the development of anxiety-like behavior,[20–22] which then can persist for several weeks after the stress exposure.[16] Similar to what is observed after traumatic stress in humans, SDS can generate a range of individual responses, giving some face validity to this paradigm and making it a particularly useful model for studying the mechanisms that underlie the susceptibility to develop stress-induced psychiatric disorders.[16,23]

Compared to the studies on stress-induced anxiety-like behaviors, the findings regarding the effect of stress on alcohol consumption are less consistent, and effects vary depending on type of stressor and method used to assess alcohol intake.[24] SDS has been shown to induce either decrease,[25] increase[26,27] or not change alcohol consumption.[28,29] Caldwell et al. described alcohol consumption to increase with time following exposure to mild SDS, suggesting that temporal parameters are important in stress-induced alcohol intake.[30] The consumption was further increased 1 week following stress, indicating possible long-term neuroadaptations.

To disentangle the physical and psychological elements of SDS that drive the behavioral consequences of this procedure, the model has been further developed by introducing a second rodent that witnesses the social defeat episode but is protected from its physical element.[31,32] Witness stress was found to induce similar anxiety- and depression-like behaviors as those seen in rats exposed to SDS.[31,33] These behaviors persisted for up to 1 month after the stress exposure and were associated with long-lasting molecular changes.[33] Although clinical studies have reported an increased risk to develop AUD in individuals exposed to observational stress, the effect of witness stress on alcohol intake in rodents has to our knowledge not been previously evaluated.

Here, we hypothesized that both social defeat and witness stress can induce co-occurring anxiety-like behavior and escalation of alcohol self-administration, an important feature of AUD.[34] To examine this hypothesis, rats exposed to social defeat and witness stress were evaluated for both behaviors, using the elevated plus maze (EPM) and operant alcohol self-administration, respectively. To identify neural substrates of individual differences in susceptibility and resilience, we capitalized on the large variance in behavioral outcomes after social defeat and witness stress. To identify the long-term neuroadaptations associated with the comorbid and resilient characteristics, respectively, we analyzed gene expression changes in the amygdala (AMG), a key brain region in the regulation of fear and emotion.[35,36]

2 | MATERIALS AND METHODS

2.1 | Animals

Adult male Wistar rats (200–225 g, Charles River, Germany) were pair-housed under a reverse light cycle (lights off at 7 a.m.), in a humidity- and temperature-controlled environment with free access to food and water. Behavioral experiments took place during the dark phase, and rats were habituated to the facility and handled prior to experiments. Procedures were conducted in accordance with the National Committee for animal research in Sweden and approved by the Local Ethics Committee for Animal Care and Use at Linköping University.

2.2 | Overview of behavioral testing

Five batches of rats underwent the SDS paradigm (N = 216). After habituation, rats were trained to self-administer 20% alcohol for approx. 2–3 months. Rats underwent 10 days of SDS, for

10 min/day, and all rats were kept single-housed for the remainder of the experiment. Body weight (BW; batches 1–5) and blood samples (batches 1–4) were collected before the start of and after the last SDS session. Following 1 week of rest, anxiety-like behavior was assessed on the EPM. After another week of rest, rats were then tested for alcohol SA for 1 week. Rats from batches 2–5 were used for gene expression analysis. An overview of this timeline is given in Figure 1.

2.3 | Social defeat and witness stress model

The SDS model is an adaptation of the established mouse model and the vicarious stress model.[16–18,32,37] Pair-housed rats were habituated and trained to self-administer 20% alcohol and divided into SDS and witness (WIT) groups or control (N = 32–46/batch, n = 10–16/group). For 10 days, SDS rats then spent 10 min/day with a larger male Wistar rat ("aggressor") in the aggressor's custom-built home cage (80 cm × 40 cm × 60 cm). A former cage mate witnessed the events through a perforated divider, and the behavior was scored by an observer (Figure S1). After 10 min, the WIT rat was returned to its home cage, and the SDS rat was placed on the other side of the perforated divider. Over the next 9 days, the process was repeated, always meeting a new aggressor. Control rats were given 10 min of social interaction with their former cage mates each day. After the last day of SDS, rats were returned to their housekeeping room and kept single-housed.

Aggressors were larger male Wistar rats (>600 g) that had lived for 3–4 months in their custom-built home cage together with an ovariectomized female. One week prior to the SDS, aggressors were screened for 10 min/day over 3 days, and the most aggressive rats that did not inflict any injury upon the screening rats were kept for the experiment. Aggressors were kept for no more than two batches of SDS. One hour prior to any session, females, food, and enrichment were removed from the home cage.

2.4 | Ovariectomy

A dorsal approach was used. The fat lobe containing the ovary and the distal part of the uterus was identified and pulled out through a midline incision. The fat lobe was clamped just below the junction between the ovary and the uterine horn, and the remaining free end was tied with a ligature. The abdominal wall was closed using a resorbable suture, and the procedure was repeated on the opposite side. The skin was closed with a resorbable suture. Rats were anesthetized with isoflurane and received 0.03 mg/kg Temgesic (buprenorphine) 30 min prior to surgery and 5 mg/kg Rimadyl (carprofen) every 24 h for 3 days post-op.

2.5 | Alcohol self-administration

Operant training and testing were performed in operant chambers housed in sound-attenuating cubicles (Med Associates Inc., St Albans, VT, USA; 30.5 × 29.2 × 24.1 cm). Rats were trained to self-administer 20% alcohol under a fixed ratio 1 (FR1) schedule during a 30 min session without sucrose/saccharin fading as described previously.[38] The sessions were conducted under FR2 after stable FR1 baseline. Operant alcohol self-administration was calculated as the average reinforcers of the last 3 days during baseline and testing. We have previously demonstrated that the number of reinforcers correlates with blood alcohol levels; for instance, 20 reinforcers correlate to ~50 mg/dl.[38]

2.6 | Anxiety-like behavior

Anxiety was measured using the EPM (Med Associates Inc.) as previously described.[39,40] Rats were recorded for 5 min in the arena, and time spent in each arm was scored by two observers. Data are

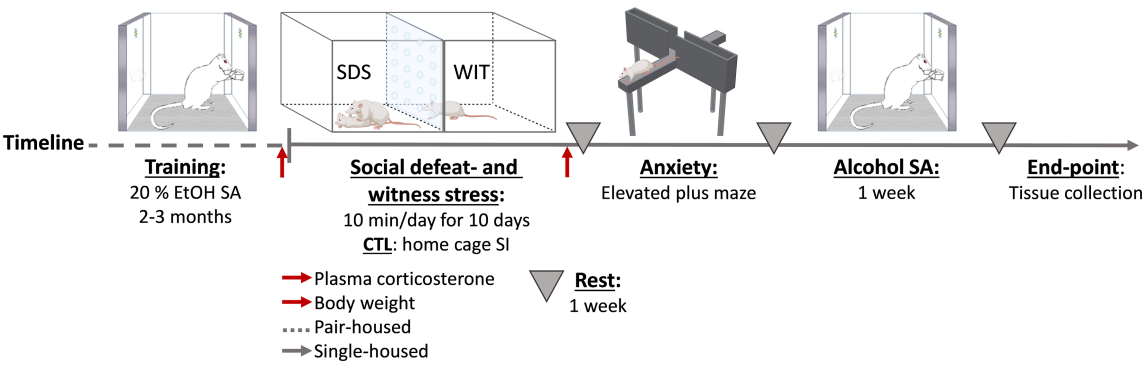

FIGURE 1 Experimental timeline. After 2–3 months of baseline self-administration (SA), rats underwent social defeat or witness stress for 10 days. Controls were given 10 min of social interaction in their home cage. Using the elevated plus maze (EPM), anxiety-like behavior was assessed 1 week after the last stress session. Alcohol self-administration was measured 1 week after EPM. Finally, amygdala samples were collected 1 week after the end of the behavioral testing to investigate long-term gene expression changes. CTL: control; EtOH: ethanol; SDS: social defeat stress; SI: social interaction WIT: Witness. Figure was created with BioRender.com

presented as percentage time spent in the open arm: time spent in open arm/ (time spent in the open arm + time spent in the closed arm) *100.

2.7 | Plasma corticosterone analysis

Blood samples were collected from tail veins of rats in batches 1–4, at baseline and 10 min after last SDS session, into heparin-coated tubes and centrifuged for 5 min at 2,000 x g to separate the plasma. Plasma was transferred into new tubes and stored at −80°C until further analysis. The corticosterone was extracted by adding five parts of ethyl acetate (Thermo Fisher Scientific Inc. Waltham, MA, USA) to each plasma sample. The organic solvent layer was first transferred to a water-prefilled tube and then to second tube. This procedure was repeated two times before samples were dried in a vacuum concentrator. Samples were re-dissolved in Assay buffer from the DetectX Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI, USA). Thereafter, the manufacturer's instructions were followed.

2.8 | Behavioral characterization

To identify rats with "comorbid" anxiety-like behavior and escalated alcohol intake, as well as rats resilient to the stress, we Z-score normalized the two relevant outcome variables: the Δ reinforcers (reinforcers after stress – reinforcers before stress) and the percentage time spent in the open arm. When plotting the population distribution, a unimodal distribution was seen for both variables; we therefore used a thresholding approach to identify susceptible and resilient rats, respectively. The threshold was set to 0.5 standard deviation (σ) from the population mean. This corresponded to a minimum Δ reinforcers of ∼5.2 for escalated alcohol SA and a maximum of ∼17.8% time spent in the open arm for anxiety-like behavior. To reliably identify a resilient population, a second threshold was set to 0.0 σ (the population mean), corresponding to a maximum Δ reinforcers of ∼1.4 and a minimum of ∼31.4% time spent in the open arm. A comorbidity index was created by min-max normalizing (0–100) and summing up the anxiety-like behavior and alcohol self-administration data for downstream correlations. Animals above 0.5 σ for alcohol self-administration and below 0.5 σ for anxiety-like behavior were classified as comorbid, and animals below 0.0 σ for alcohol self-administration and above 0.0 σ for anxiety-like behavior were classified as resilient.

A factor analysis was performed on batches 1–4 (containing data for all 4 parameters), to characterize the contributions of individual behaviors and parameters on the population. Δ reinforcers (reinforcers after stress – reinforcers before stress), percentage time spent in the open arm in the EPM, Δ corticosterone (corticosterone level after last stress session – baseline corticosterone level), and Δ BW (after stress – baseline BW) were Z-score normalized, and orthogonal factors were extracted using normalized varimax rotation.

2.9 | Gene expression analysis

Gene expression analysis was performed using our custom code set NanoString panel (NanoString Technologies Inc., Seattle, WA, USA), containing 383 genes[41]; selected hits were confirmed with qPCR. In brief, AMG (N = 39; CTL n = 9, resilient SDS n = 8, comorbid SDS n = 7, resilient WIT n = 8, and comorbid WIT n = 7) was dissected freshly and flash frozen in isopentane. RNA was extracted using a Qiagen RNA/DNA All Prep Mini kits (Qiagen, Venlo, Netherlands) following manufacturer's protocol, and gene expression was analyzed at KIgene (Karolinska Institute, Stockholm, Sweden). Data were analyzed using nSolver (NanoString Technologies Inc.) and were normalized to six housekeeping genes (*Gapdh*, *Gorasp2*, *Hprt1*, *Sdha*, *Tuba1a*, and *Ywhaz*). Background thresholding was performed (average of negative controls + 2 standard deviations), and genes for which at least half of the samples did not exceed the threshold were excluded. Finally, student's t-test was performed between groups. We used NanoString to identify targets that were then confirmed using an independent method (qPCR); therefore, nominal p-values were used in this analysis.

2.10 | qPCR

RNA was converted to cDNA using TaqMan cDNA synthesis Kit and qPCR was performed using TaqMan Fast Advanced Master Mix, analyzed on a 7900 PCR with SDS 2.4.2 software (Thermo Fisher Scientific). To measure *Oxt* and *Avp*, we used inventoried TaqMan Gene expression assay probes (Life Technologies, Carlsbad, CA). Gene expression was measured with respect to *Gapdh* using $2^{-\Delta\Delta Ct}$ analysis.[42]

2.11 | Statistical analysis

One-way analysis of variance (ANOVAs) was carried out using Statistica 13.0 (TIBCO Software, Palo Alto, CA, USA). Homogeneity of variance was assessed using Levene's test. Data that violated assumptions of parametric tests were analyzed with a non-parametric Kruskal–Wallis ANOVA. Data not showing any violations were analyzed using parametric ANOVA. When appropriate, *post hoc* comparisons were performed using Newman–Keuls test. Factor analysis and G-test (as crosstab()) were performed using the Python FactorAnalyzer and Researchpy packages, respectively. Correlations were carried out in GraphPad Prism 8.4.3 (GraphPad Software, Inc., San Diego, CA, USA). The accepted level of significance for all tests was $p < 0.05$. Data are presented as

averages ± SEM, with the *n* reported above each bar, unless otherwise stated.

3 | RESULTS

3.1 | Persistent comorbid anxiety and escalation of alcohol self-administration following stress

3.1.1 | Defining susceptible and resilient populations for stress-induced comorbidity

Alcohol addiction and anxiety are frequently comorbid, and both are promoted by stress. Our overarching question was whether shared mechanisms mediate a susceptibility for co-occurring escalated alcohol self-administration and high anxiety-like behavior following stress exposure. Accordingly, our first objective was to define populations of rats with escalated alcohol self-administration (Figure 2A) and high anxiety-like behavior (Figure 2B). We used a two-threshold approach and found that both social defeat and witness stress significantly increased the proportion of rats with both those characteristics ("comorbid rats") compared to controls not exposed to SDS (Figure 2C; CTL *n* = 3, SDS *n* = 13, WIT *n* = 9; G-test: comorbid x resilient x other; log-likelihood ratio = 12.7; *p* = 0.013; Cramer's V = 0.18). Rats with an alcohol self-administration below 0.0 σ (the population mean) and with an anxiety level above 0.0 σ were considered resilient (Figure 2D; CTL *n* = 26, SDS *n* = 13, WIT *n* = 15). We also found that a higher number of rats show stress-induced anxiety-like behavior than stress-induced escalation of alcohol self-administration (Figure 2C; anxious: CTL *n* = 17, SDS *n* = 32, WIT *n* = 26; escalated: CTL *n* = 12, SDS *n* = 24, WIT *n* = 19).

Thus, similar to what is found clinically, our data showed that both social defeat and witness stress induce co-occuring escalated alcohol self-administration and high anxiety-like behavior in a subpopulation of rats, suggesting a translational validity of our model. We then proceeded to analyze in detail the pattern of behavioral consequences following the physical and the psychological stressor. To increase the power of the analysis, only the extreme, that is, comorbid

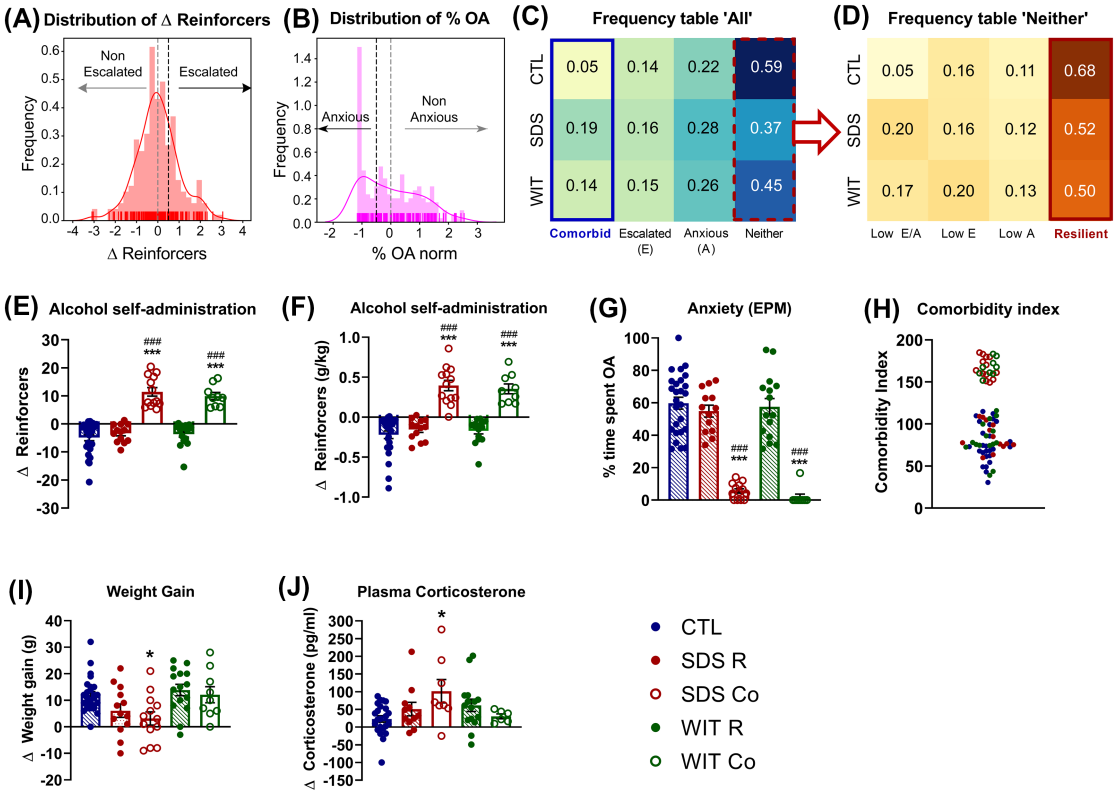

FIGURE 2 Behavioral characterization. Panels A and B show the population distribution of Z-score normalized Δ reinforcer A and % OA B values. A two-threshold approach was used to define "escalated" and "anxious" rats. A Rats above the threshold set at 0.5 σ above the population mean were considered as "escalated," whereas rats below the threshold set at 0.0 σ (i.e., the population mean) were considered as "non-escalated." B Rats below the threshold set at 0.5 σ were considered as "anxious," whereas rats above the threshold set at 0.0 σ were considered as "non-anxious." C-D Frequency tables showing the percentage of rats with the respective behavioral characteristics. E-F Bar graphs showing the level of stress-induced alcohol consumption, expressed as □ reinforcers E, and Δ reinforcers in g/kg F for each group. G Bar graph presenting the anxiety-like behavior as measured by % time spent in the open arm. H Comorbidity Index. I-J Bar graphs indicating weight gain I and stress-induced corticosterone levels J. Closed circles: resilient animals; open circles: comorbid animals; * compared to CTL; # compared to respective resilient group; *$p < 0.05$; ***$p < 0.001$; ### $p < 0.001$. Co: comorbid; CTL: control; EPM: elevated plus maze; OA: open arm; R: resilient; SDS: social defeat stress; WIT: witness

and resilient subpopulations were used for these analyses. The same approach was used in the subsequent molecular analysis.

3.1.2 | Alcohol self-administration

Both SDS and WIT comorbid rats showed a significant escalation of alcohol self-administration (11.4 and 9.9 reinforcers, respectively) that persisted 3 weeks after the last social defeat session (Figure 2E; non-parametric Kruskal–Wallis one-way ANOVA: main effect of group, $H_{(4,76)} = 46.43$, $p < 0.001$). Multiple comparison analysis indicated a significant increase in operant alcohol self-administration in the comorbid SDS and WIT groups compared to control and their respective resilient groups ($p < 0.001$ for all). This corresponds to an average increase of 0.4 and 0.35 g/kg for the comorbid SDS and comorbid WIT, respectively (Figure 2F; one-way ANOVA: $F_{(1.71)} = 31.4$; $p < 0.001$; Newman–Keuls *post hoc* test: comorbid SDS vs. CTL $p < 0.001$; comorbid SDS versus SDS R $p < 0.001$; WIT Co vs. CTL $p < 0.001$; WIT Co vs. WIT R $p = 0.001$). No differences existed in operant alcohol self-administration at baseline (Figure S2), and no correlation was observed between alcohol self-administration during baseline and Δ reinforcers in SDS and WIT rats. A negative correlation was observed in the CTL group ($r^2 = 0.22$; $p = 0.015$; Figure S3).

3.1.3 | Anxiety-like behavior

Anxiety-like behavior was assessed 2 weeks after the last SDS session in the EPM. Comorbid subpopulations of both SDS and WIT rats demonstrated a strong anxiety-like behavior, with almost no exploration in the open arms. Resilient subpopulations did not differ from controls, spending >50% of time in the open arm, on average (Figure 2G; non-parametric Kruskal–Wallis one-way ANOVA: $H_{(4,76)} = 47.14$; $p < 0.001$. Multiple comparison analysis indicated a significant decrease in % time spent in the open arm in comorbid SDS and comorbid witnesses (comorbid SDS vs. CTL $p < 0.001$, comorbid SDS vs. resilient SDS $p < 0.001$, comorbid WIT vs. CTL $p < 0.001$, comorbid WIT vs. resilient WIT $p < 0.001$). No correlation was observed between alcohol self-administration during baseline and anxiety-like behavior after stress in either group (Figure S3).

3.1.4 | Comorbidity index

A comorbidity index was created from the anxiety and alcohol self-administration data to account for both behaviors in downstream correlations with gene expression. Both comorbid SDS and WIT rats have a higher index than CTL (Figure 2H).

3.1.5 | Weight gain

BWs were measured at baseline and after the last SDS session. One-way ANOVA showed a significant effect of group ($F_{(1.71)} = 4.5$; $p = 0.003$). Newman–Keuls' *post hoc* analysis showed a significant decrease in weight gain in the comorbid SDS rats compared to CTL ($p = 0.016$) but not resilient SDS. While the resilient SDS rats were not significantly different from CTL, there was a strong trend (Figure 2I; $p = 0.061$).

3.1.6 | Plasma corticosterone

Plasma corticosterone levels were measured at baseline and after the last SDS session during (dark phase). Only comorbid SDS had significantly increased levels compared to controls but with a large individual variability (Figure 2J; one-way ANOVA: $F_{(1.58)} = 2.89$; $p = 0.03$; Newman–Keuls *post hoc* test: comorbid SDS vs. CTL $p = 0.037$).

3.1.7 | Anxiety and escalated alcohol self-administration load on the same underlying factor

We used factor analysis to identify underlying dimensions in the data in an unbiased manner and confirmed that anxiety-like behavior and escalation of alcohol self-administration in the comorbid rats load on the same factor. This factor explained the highest proportion of the variance in our data, 35%. The increase in plasma corticosterone and decreased weight gain, respectively, loaded on two additional, separate factors; these accounted for a smaller proportion of the variance, or approx. 11% and 14%, respectively (Figure 3A).

(A)

Factor	1	2	3
Δ Reinforcers	-0.41	0.23	-0.06
% Open arm	0.41	-0.01	0.16
Δ Body weight	-0.05	0.28	-0.1
Δ Corticosterone	0.09	-0.1	0.27
Eigen value	1.28	0.97	0.94
% expl. variance	35	14.28	11.04

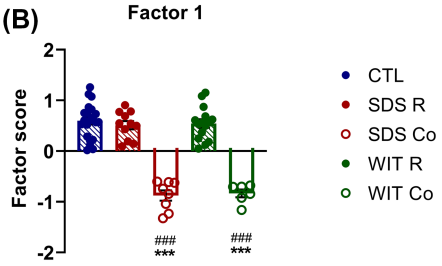

FIGURE 3 Factor analysis: Results from the factor analysis (varimax) demonstrates that factor 1 is largely driven by escalation of alcohol self-administration (Δ reinforcers) and anxiety-like behavior (reduction of % open arm A. Bar graph showing factor scores for factor 1 in each group B. * compared to CTL; # compared to respective resilient group; ***$p < 0.001$; ### $p < 0.001$; co: comorbid; CTL: control; R: resilient; SDS: social defeat stress; WIT: witness

Plotting the factor scores for factor 1 back onto our groups, we confirmed that the variance is driven by the comorbid subpopulations (Figure 3B; one-way ANOVA: $F_{(1.58)}$ = 58.8; p < 0.001; Newman–Keuls post hoc test: comorbid SDS vs. CTL p < 0.001; comorbid SDS vs. resilient SDS p < 0.001; comorbid WIT vs. CTL p < 0.001; comorbid WIT vs. resilient WIT p < 0.001). A four-component Varimax-rotated factor analysis shows the overall distribution of animals in this experiment (Figure S4).

3.2 | Association between amygdala gene expression, stress type, and behavioral characteristics

Having defined susceptible and resilient populations, our next objective was to search for molecular mechanisms associated with susceptibility for developing comorbidity and to examine whether these mechanisms are shared or distinct depending on the nature of the stressor. Because we and others have previously found the amygdala to be critically involved in the association between anxiety- and alcohol-related behaviors,[43,44] our analysis focused on this structure. We generated amygdala gene expression profiles associated with resilient and comorbid patterns of behavior induced by SDS or WIT, using a NanoString panel containing 383 pre-selected genes. All groups were exposed to a similar amount of alcohol during baseline alcohol self-administration (Figure S2). Additionally, AMG was collected 1 week after the last alcohol self-administration session, to avoid any acute effect of alcohol on gene expression. Gene expression levels of comorbid and resilient SDS and WIT groups were compared to controls. An informal Venn diagram summarizes the identified changes in gene expression (Figure 4), and a table containing all significantly different genes is given in Table 1. Complete NanoString analysis is available online (https://doi.org/10.17632/6x5bkz42k7.1). We found 15 genes that were significantly different in the comorbid SDS and 17 genes in the resilient SDS. Of these, two genes were common between the groups (*Fosb* and *Oprm1*), suggesting that the majority of genes identified here are not triggered by stress itself but may be relevant for the observed behaviors, that is, escalation of alcohol self-administration and anxiety-like behavior. In the comorbid WIT, we identified 19 genes that were significantly different and in the resilient WIT we identified 13 genes. Of these, three genes were common between the WIT groups (*Dicer1*, *Ikbkg*, and *Sec24a*). Resilient SDS and WIT had one commonly downregulated gene (*Sema3d*), whereas the comorbid SDS and WIT had four similarly regulated genes (*Avp*, *Esr1*, *Fosb*, and *Sec24a*). Of these four genes, *Avp* and *Esr1* were specific to the comorbid group. However, while not significant, *Esr1* was also downregulated in resilient SDS and WIT.

Among the genes identified here, *Avp* was the only gene that was specific to the comorbid groups (student's *t*-test: comorbid SDS vs. CTL: p = 0.022; fold change (FC) = 36.6; comorbid WIT vs. CTL: p = 0.049; FC = 23.6). We therefore proceeded with *Avp* for qPCR validation and further analysis. We also included *Oxt* as this was strongly increased in comorbid SDS and showed a strong trend to be increased in the comorbid WIT (student's *t*-test: comorbid SDS vs. CTL: p = 0.019; FC = 51.2; comorbid WIT vs. CTL: p = 0.07; FC = 46.2). qPCR analysis showed a significant increase in *Avp* and *Oxt* in the comorbid SDS and a trend in the comorbid WIT rats (Figure 5A,E, respectively; non-parametric Kruskal–Wallis one-way ANOVA; *Avp*: main effect of group, $H^{4,\ 37}$=14.15, p = 0.007; *Oxt*: main effect of group, $H^{4,\ 37}$=15.18, p = 0.004). Multiple comparison analysis indicates a significant upregulation of *Avp* and *Oxt* in the comorbid SDS rats (p = 0.046; p = 0.021, respectively). *Avp* expression was strongly correlated with the expression of *Oxt* (r^2 = 0.90, p < 0.001), suggesting that they may have a common regulation (Figure S5).

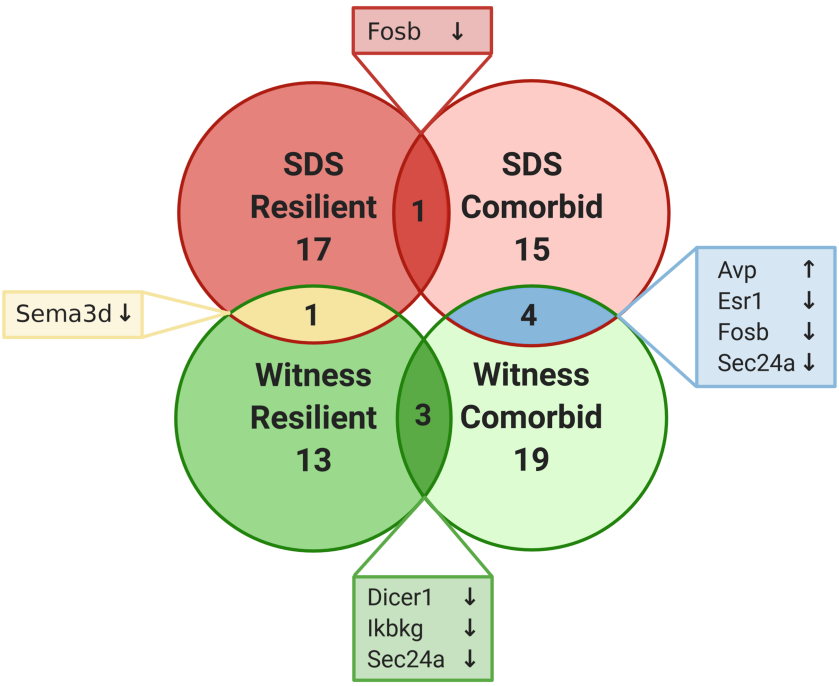

FIGURE 4 Informal Venn diagram showing the number of significant gene expression changes in the comorbid social defeat stress (SDS), comorbid witness, resilient SDS and resilient witness group compared to controls. Figure was made in www.biorender.com

TABLE 1 List of genes with significant expression changes compared to control group

	SDS R		SDS S		Witness R		Witness S	
Gene	*p*-val	FC	*p*-val2	FC2	*p*-val3	FC3	*p*-val4	FC4
Abcd3	0.0473	↓ 0.88	ns	-	ns	-	ns	-
Adra1b	ns	-	0.0220	↓ 0.80	ns	-	ns	-
Adra1d	ns	-	0.0124	↓ 0.69	ns	-	ns	-
Avp	ns	-	0.0217	↑ 36.59	ns	-	0.0491	↑ 23.56
Calb2	0.0309	↑ 1.26	ns	-	ns	-	ns	-
Camk1g	ns	-	ns	-	ns	-	0.0321	↓ 0.83
Chrm1	ns	-	ns	-	0.0176	↑ 1.25	ns	-
Chrm3	0.0381	↑ 1.15	ns	-	ns	-	0.0017	↑ 1.18
Chrm4	0.0104	↓ 0.69	ns	-	ns	-	0.0374	↓ 0.73
Chuk	ns	-	ns	-	ns	-	0.0321	↓ 0.90
Comt	0.0210	↓ 0.89	ns	-	ns	-	ns	-
Crhr1	ns	-	ns	-	ns	-	0.0120	↑ 1.24
Dagla	0.0500	↑ 1.07	ns	-	ns	-	ns	-
Ddc	ns	-	ns	-	0.0214	↓ 0.74	ns	-
Dicer1	ns	-	ns	-	0.0073	↓ 0.88	0.0403	↓ 0.92
Dpf2	0.0243	↓ 0.89	ns	-	ns	-	ns	-
Esr1	ns	1	0.0116	↓ 0.55	ns	-	0.0111	↓ 0.57
Faah	ns	-	ns	-	0.0467	↑ 1.20	ns	-
Fosb	0.0319	↓ 0.72	0.0109	↓ 0.61	ns	-	0.0395	↓ 0.70
Gabrb3	ns	-	0.0374	↓ 0.85	ns	-	ns	-
Gabrd	0.0229	↑ 1.18	ns	-	ns	-	0.0218	↑ 1.19
Grik1	ns	-	ns	-	0.0373	↓ 0.86	ns	-
Grin2c	ns	-	ns	-	ns	-	0.0323	↓ 0.73
Grm5	ns	-	0.0052	↓ 0.85	ns	-	ns	-
Hdac1	ns	-	ns	-	0.0338	↓ 0.91	ns	-
Ikbkg	ns	-	ns	-	0.0253	↓ 0.84	0.0252	↓ 0.83
Jun	ns	-	ns	-	0.0159	↑ 1.20	ns	-
LOC684293	ns	-	0.0437	↓ 0.86	0.0363	↓ 0.85	ns	-
Mapk14	ns	-	ns	-	0.0253	↑ 1.11	ns	-
Mtor	ns	-	0.0249	↓ 0.90	ns	-	ns	-
Myo16	0.0437	↓ 0.90	ns	-	ns	-	ns	-
Nfkbia	ns	-	0.0395	↓ 0.80	ns	-	ns	-
Notch1	ns	-	ns	-	ns	-	0.0173	↓ 0.79
Nrxn1	ns	-	ns	-	ns	-	0.0429	↓ 0.91
Oprm1	0.0431	↓ 0.87	ns	-	ns	-	ns	-
Oxt	ns	-	0.0194	↑ 51.23	ns	-	ns	-
Per1	ns	-	ns	-	ns	-	0.0231	↓ 0.79
Pias3	0.0154	↑ 1.11	ns	-	ns	-	ns	-
Prkcd	0.0143	↓ 0.70	ns	-	ns	-	0.0202	↓ 0.75
Rab3c	0.0450	↑ 1.15	ns	-	ns	-	ns	-
Sec24a	ns	-	0.0483	↓ 0.92	0.0140	↓ 0.90	0.0125	↓ 0.88
Sema3d	0.0247	↓ 0.85	ns	-	0.0143	↓ 0.82	ns	-
Slc17a6	0.0174	↑ 1.70	ns	-	ns	-	0.0091	↑ 1.70
Slc6a13	ns	-	ns	-	0.0315	↓ 0.64	ns	-
Slc6a7	ns	-	0.0363	↓ 0.80	ns	-	ns	-

TABLE 1 (Continued)

	SDS R		SDS S		Witness R		Witness S	
Gene	*p*-val	FC	*p*-val2	FC2	*p*-val3	FC3	*p*-val4	FC4
Smad9	ns	-	0.0403	↓ 0.82	ns	-	ns	-
Syp	0.0209	↓ 1.06	ns	-	ns	-	0.0022	↑ 1.09
Tac1	ns	-	0.0466	↓ 0.55	ns	-	ns	-

Abbreviations: R, resilient; S, Susceptible; SDS, social defeat stress; WIT, witness; FC, fold change.

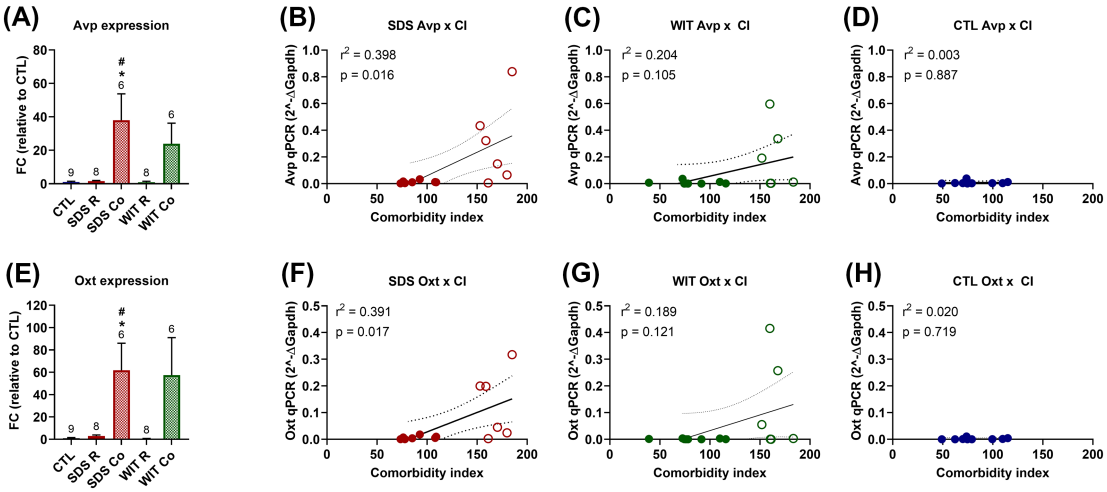

FIGURE 5 Avp and Oxt expression positively correlates with comorbidity index. Bar graphs indicating the mRNA levels of Avp A and Oxt E. Avp and Oxt expression are significantly upregulated in SDS co and show a trend to be upregulated for WIT co. Correlation between Avp expression and comorbidity index for SDS B, WIT C, and CTL D. Correlation between Oxt expression and comorbidity index for SDS F, WIT G, and CTL H. Avp and Oxt are positively correlated with comorbidity index in SDS animals and show a trend to be in WIT animals. Closed circles: resilient animals; open circles: comorbid animals; * compared to CTL; # compared to respective resilient group; *p <0.05. Co: comorbid; CI: comorbidity index; CTL: control; R: resilient; SDS: social defeat stress; WIT: witness

To determine whether prior alcohol exposure may affect *Avp* expression, we ran a correlational analysis between baseline alcohol self-administration and *Avp* expression. We did not find any correlation between *Avp* expression and baseline alcohol self-administration (Figure S3).

We then correlated the gene expression levels of *Avp* and *Oxt* with the comorbidity index of the rats. This demonstrated a positive correlation between the expression levels of both genes with the comorbidity index in SDS rats (*Avp*: r^2 = 0.40, p = 0.016; *Oxt*: r^2 = 0.39, p = 0.017; Figure 5B,F, respectively). A similar trend was observed in WIT rats for both genes (*Avp*: r^2 = 0.20, p = 0.1; *Oxt*: r^2 = 0.19, p = 0.1; Figure 5C,G, respectively).

4 | DISCUSSION

We found that social defeat and witness stress generated a range of individual responses resembling those observed in humans. These responses were stratified in three groups, the susceptible/comorbid group, which presented both increased alcohol self-administration and anxiety-like behavior; the intermediate group, which displayed either of the two behaviors; and the resilient group with no stress-induced changes in alcohol self-administration and anxiety-like behaviors. To investigate the neurobiological basis underlying resilience and susceptibility to stress, we conducted gene expression analysis in the resilient and comorbid groups. We found changes in amygdala gene expression that were specifically associated with these behavioral characteristics, pointing to candidate mechanisms for resilience and susceptibility to stress-induced comorbidity.

4.1 | Social defeat and witness stress induce persistent comorbid alcohol escalation and anxiety-like behavior in a subpopulation of rats

We found that exposure to SDS can lead to increased alcohol self-administration and anxiety-like behavior. Our results linking the effect of SDS with anxiety-like behavior are in line with the literature.[20,21] We found that SDS induced an increase in anxiety-like behavior in 47% of rats. Similar to our data, Bosh-Bouju et al. reported that 52% of defeated mice showed anxiety-like behaviors when measured 2 days after stress.[20] In the present study, anxiety-

like behavior was assessed 2 weeks after the last defeat episode, indicating a persistent effect of SDS on anxiety. In contrast, the effects of SDS on alcohol consumption are less clear in the literature. Not only temporal parameters but also individual variation in stress response may in part explain the discrepancies between studies. We found that SDS induced escalated alcohol self-administration in about 35% of our rats. Consistent with our work, clinical studies have shown that only a subset of people exposed to a traumatic event develop AUD, pointing to the importance of investigating individual susceptibility rather than group level effects. When examining anxiety-like behaviors and alcohol self-administration together, we show that, similar to humans,[45] SDS induces comorbid anxiety-like behaviors and alcohol escalation in a small proportion of animals (19%), supporting a translational relevance of the model. We further demonstrate that witnessing social defeat produces a psychological stress that is sufficient to induce comorbid alcohol escalation and anxiety-like behavior in 14% of rats. Previous studies also reported that witnessing another animal's distress leads to anxiety- and depression-like behaviors.[31] Our work extends these studies by demonstrating that both social defeat and witness stress lead to a range of maladaptive behaviors including comorbid anxiety-like behavior and escalation of alcohol self-administration.

Notably, our control rats were also subjected to a mild stressor, as they were single-housed to match with the housing condition of the SDS and WIT rats. Patki et al. suggested that social housing can impact the stress response, resulting in greater anxiety- and depression-like behaviors.[31] To exclude a possible social buffering, rats were therefore single-housed. Expectedly, a certain number of socially isolated control rats exhibited an escalation of alcohol self-administration and increased anxiety-like behavior. However, the proportion of control animals presenting a comorbid behavior was considerably lower (5%) compared to SDS and WIT rats (19% and 14%, respectively), indicating a robust effect of these stressors.

4.2 | The types of stress and resulting behavioral characteristics are associated with specific gene expression signatures

One aim of this study was to investigate whether comorbid behavioral characteristics induced by the two different stressors are driven by similar molecular mechanisms. To disentangle the psychological component from the combined physical and psychological stress of social defeat, a cage mate was made to witness the SDS. While this allows for a better understanding of defeat stress-induced behaviors, the SDS model cannot be applied to females, as it is difficult to use an aggression-based model in females. We therefore focused on males to allow a direct comparison between SDS and WIT rats.

SDS and WIT rats showed largely distinct gene expression profiles, suggesting that both stressors lead to escalated alcohol self-administration and anxiety-like behavior in part through different molecular mechanisms. However, focusing on the overlapping genes may help identify the mechanisms that mediate the effects of stress-induced comorbid AUD and ANX.[33]

We found that the transcripts coding for the neuropeptides oxytocin (*Oxt*) and vasopressin (*Avp*) were upregulated in the AMG of comorbid SDS and WIT rats. Both *Oxt* and *Avp* gene expression levels were positively correlated with the comorbidity index in SDS rats and a similar trend was observed in WIT rats. However, no association was found in the control group, suggesting a possible role of these two neuropeptides in stress-induced comorbidity. In line with our hypothesis, several studies have shown a role of *Avp* in the regulation of stress and anxiety.[46,47] *Avp* was found to be positively correlated with alcohol consumption in mice exposed to SDS.[48] A recent study also showed that AVP microinfusion into the central amygdala was sufficient to induce anxiety-like behavior, suggesting a functional role of AVP in anxiety.[49] Remarkably, *Oxt* mRNA levels were also increased in the AMG of comorbid SDS rats. In contradiction to our data, oxytocin has been shown to exert anxiolytic properties in rodents and humans[50] and has been proposed as a potential medication to enhance psychotherapy in PTSD patients.[51] Release of oxytocin in the AMG mainly comes from the hypothalamus, and no study has, to our knowledge, investigated the role of *Oxt* expression in AMG neurons. Given the chromosomal proximity of the *Oxt* and *Avp* genes, it is possible that the increased expression of *Oxt* may be driven by a common regulatory element and may therefore be a consequence of *Avp* upregulation rather than having a functional impact on anxiety and alcohol intake. Polymorphisms in the *Avp* promoter, leading to an increased expression of the gene, have previously been associated with increased anxiety-like behavior and acute response to stress in both Wistar rats[52] and humans.[53] This suggests that *Avp* may be a vulnerability factor underlying individual differences in susceptibility to stress. Additional experiments are needed to determine whether higher *Avp* expression observed in our comorbid rats reflects a pre-existing condition or whether it is induced from an interaction with the environment.

In our behavioral paradigm, rats are exposed to alcohol prior to stress exposure. It is therefore possible that the behavioral and molecular changes observed after stress exposure are influenced by an interaction between prior alcohol intake and stress. While determining the role of pre-exposure to moderate alcohol levels for stress-reactivity is important, it is outside the scope of this study. Our behavioral paradigm also mimics the human situation, in which most adults establish low-moderate levels of alcohol use, but only a minority of these users progresses into AUD.[54] Finally, the absence of a correlation between baseline alcohol self-administration, behaviors, and *Avp* expression makes it unlikely that these characteristics are influenced by prior exposure to baseline levels of alcohol self-administration.

In the present study, we demonstrate that social defeat and witness stress induce long-term comorbid anxiety and escalation of alcohol self-administration. Similar to the clinical situation, only a subpopulation of rats show susceptibility to the stress, supporting a translational validity of this model. Comorbid SDS and WIT rats present different gene expression profiles within the AMG, indicating

that the two stressors drive the comorbid behavioral changes in part through different mechanisms. However, our results also suggest a possible common mechanism, with a strong upregulation of *Avp* and *Oxt* in the AMG. This upregulation positively correlated with the magnitude of comorbidity in SDS rats and to a lesser extent with WIT rats. Together, these data provide novel insights into the neurobiological substrates underlying individual variation in susceptibility to comorbid AUD and ANX.

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CONFLICT OF INTEREST

The authors report no financial interests or potential conflict of interest.

AUTHORS CONTRIBUTION

RB: conceptualization, methodology, data curation, formal analysis, investigation, visualization, writing-original draft and review and editing, and project administration; KC: investigation, ED: investigation and writing-review and editing; FG: software, data curation, and visualization; AC: investigation; AA: investigation; ST: investigation; LH: investigation; GA: investigation; LX: investigation; EA: investigation; MH: visualization, writing-review and editing, and funding acquisition; EB: conceptualization, methodology, data curation, formal analysis, investigation, visualization, writing-original draft and review and editing, supervision, and project administration.

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