Archival Report

Sex-Specific Transcriptional Changes in Response to Adolescent Social Stress in the Brain's Reward Circuitry

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ABSTRACT

BACKGROUND: Sex differences in addiction have been described in humans and animal models. A key factor that influences addiction in both males and females is adolescent experience. Adolescence is associated with higher vulnerability to substance use disorders, and male rodents subjected to adolescent social isolation (SI) stress form stronger preferences for drugs of abuse in adulthood. However, little is known about how females respond to SI, and few studies have investigated the transcriptional changes induced by SI in the brain's reward circuitry.

METHODS: We tested the hypothesis that SI alters the transcriptome in a persistent and sex-specific manner in prefrontal cortex, nucleus accumbens, and ventral tegmental area. Mice were isolated or group housed from postnatal day P22 to P42, then group housed until ~P90. Transcriptome-wide changes were investigated by RNA sequencing after acute or chronic cocaine or saline administration.

RESULTS: We found that SI disrupts sex-specific transcriptional responses to cocaine and reduces sex differences in gene expression across all three brain regions. Furthermore, SI induces gene expression profiles in males that more closely resemble group-housed females, suggesting that SI "feminizes" the male transcriptome. Coexpression analysis reveals that such disruption of sex differences in gene expression alters sex-specific gene networks and identifies potential sex-specific key drivers of these transcriptional changes.

CONCLUSIONS: Together, these data show that SI has region-specific effects on sex-specific transcriptional responses to cocaine and provide a better understanding of reward-associated transcription that differs in males and females.

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Sex differences in cocaine use disorder are known for every stage of addiction. Men are more likely to use drugs of abuse than women; however, when women do use drugs, they become addicted more quickly. Women also report greater craving during withdrawal, are more likely to relapse, and consume more drugs during relapse, which puts them at greater risk for overdoses (1). These effects are documented in rodents as well (2); however, the underlying molecular mechanisms remain poorly understood.

Adolescence is a crucial development period for the reward circuitry and for the progression to addiction in particular. Adolescence is associated with increased vulnerability to several psychiatric disorders, including substance use disorders (SUDs), and numerous studies show that adolescent rodents and humans are more sensitive to rewarding and stressful stimuli (3). The adolescent brain undergoes enormous structural and molecular changes, especially with regard to the mesocorticolimbic reward circuitry (4), suggesting that stimuli during this time may result in long-term behavioral consequences in adulthood. Indeed, adolescent social isolation (SI)

affects anxiety- and depression-related behaviors in the long term and enhances preference for numerous drugs of abuse, including cocaine, in male rodents. Likewise, in humans, social stress during adolescence influences susceptibility for SUDs (5–7). Adolescent SI in rodents has also been shown to disrupt dopamine signaling throughout the reward circuitry (8). However, few studies have investigated the transcriptional changes driven by adolescent SI in males, and no studies have investigated either phenomenon in females. An overarching goal of this study was to characterize the sex-specific response to cocaine under control conditions and determine whether those sex differences are disrupted by SI.

Recently, we showed that adolescent SI decreases certain sex differences in behaviors, but increases sex differences in cocaine place conditioning in mice (9). We also showed that these behavioral effects are reflected in transcriptional changes within the medial amygdala, a brain region traditionally thought to be outside the reward circuitry (9). Therefore, in this study, we tested the hypothesis that adolescent SI induces similar disruptions in transcriptional responses to cocaine in

three brain reward regions: prefrontal cortex (PFC), nucleus accumbens (NAc), and ventral tegmental area (VTA). Our findings offer new insight into the lasting interactions between adolescent stress and cocaine exposure in adulthood at the transcriptional level in both sexes.

METHODS AND MATERIALS

Detailed descriptions of experimental design and approaches are provided in Supplemental Methods in Supplement 1. All of the methods used have been published recently (9).

RESULTS

Adolescent SI Disrupts Sex-Specific Transcriptional Responses to Cocaine

To study the effect of adolescent SI on transcriptional responses to cocaine in an unbiased manner, we performed RNA sequencing on PFC, NAc, and VTA (Figure 1A, B). We analyzed transcriptome-wide gene expression changes in response to acute (1 hour after the first dose) (Figure 1C–H) or chronic (24 hours after the 10th dose) (Figure S1C–H in Supplement 1) experimenter-administered cocaine (7.5 mg/kg) (Table S1). This approach can model differences in drug exposure observed in the progression of SUD—that is, acute exposure models the sensitivity to first drug dose, and chronic exposure models longer-term alterations taking place over time (7). While there are sex differences in many aspects of addiction, the most well characterized are differences in acquisition of cocaine self-administration: females acquire the task more quickly (10–12). Therefore, we focused on acute effects of cocaine in the main figures.

To assess if SI disrupts sex-specific transcriptional responses to cocaine, we first focused on genes affected by cocaine in group-housed males (GHMs) and females (GHFs) after acute (Figure 1C–H) or chronic (Figure S1C–H in Supplement 1) exposure. Union heatmaps and Venn diagrams of genes regulated by cocaine in GHMs and GHFs reveal that in all three brain regions, GHMs regulate many more genes in response to acute (Figure 1C–H) but not chronic (Figure S1C–H in Supplement 1) cocaine exposure than GHFs, with very little overlap of those genes in the two sexes (Figure 1C–H; Figure S1C–H in Supplement 1). Importantly, SI disrupts this pattern in PFC and VTA, but not in NAc (Figure 1G; Figure S1G in Supplement 1). It is noteworthy that in the PFC, expression patterns in SI males (SIMs) mirror those of GHFs in response to both acute (Figure 1C) and chronic (Figure S1C in Supplement 1) cocaine exposure. In the NAc and VTA, there is very little overlap in expression when comparing SI mice with their GH counterparts after acute (Figure 1D, E) or chronic (Figure S1D, E in Supplement 1) drug exposure. Notably, in the VTA in response to acute cocaine exposure, SI females (SIFs) regulate similar genes as GHMs but in the opposite direction (Figure 1E). Another interesting pattern is the larger number of transcripts affected in the PFC and VTA of GHMs versus GHFs, a difference not seen in the NAc (Figure 1F–H). This pattern is reversed after SI, with many more transcripts regulated in SIM NAc. Together, these data suggest that SI disrupts sex-specific gene expression by cocaine in a brain region–

specific manner, with brain reward mechanisms likely being disrupted as a result.

We next used Ingenuity Pathway Analysis (Qiagen) to identify biological pathways that are affected by cocaine in GH and SI animals (activation z scores $\geq$ 2.0; $p <$ .05). We focused on pathways that are associated with neurotransmitters and intracellular signaling because molecules in the two pathways overlap significantly and largely reflect neuronal signaling. Numerous signaling pathways were identified in gene expression profiles after acute cocaine exposure in GHMs (Figure 1I), with minimal overlap in pathways predicted for GHFs, suggesting that pathways regulated in brain reward regions by the first cocaine dose differ greatly between males and females. By contrast, many of the same pathways were predicted to be regulated in SIMs and SIFs (Figure 1I), especially after chronic cocaine exposure (Figure S1I in Supplement 1). However, important region-specific differences in those pathways were observed. Together, these data suggest that adolescent SI reprograms both the acute and chronic response to cocaine in a sex- and region-specific manner.

Adolescent SI Causes Loss of Sex Differences in Gene Expression Throughout the Reward Circuitry

The finding that SI disrupts sex-specific transcriptional responses to cocaine suggests that baseline sex differences might be altered by SI. We tested this hypothesis by evaluating sex differences in gene expression in GHMs versus GHFs after acute or chronic saline (Figure 2A–G) or cocaine (Figure S2A–G in Supplement 1 and Table S2) exposure. Union heatmaps and Venn diagrams of sex differences in genes in GH animals under all conditions—acute/chronic saline (Figure 2) and cocaine (Figure S2 in Supplement 1) exposure—reveal that baseline sex differences in gene expression are dramatically attenuated after SI in all three brain regions. The signaling pathways that are associated with those sex differences in genes overlap with those regulated by cocaine (Figure 2H, I; Figure S2H, I in Supplement 1), suggesting that SI disrupts sex-specific biological pathways associated with neuronal signaling in a region-specific manner.

Pattern Analysis Indicates That Sex-Specific Gene Expression Changes Are Driven by a Loss and Gain of Baseline Sex Differences

We next used pattern analysis, a technique used previously (13), to account for the finding that SI disrupts both baseline sex-specific expression and transcriptional responses to cocaine. All differentially expressed gene data were compared with the same baseline (GHF chronic saline), and expression changes were categorized based on the patterns of expression across the four groups (GHM, GHF, SIM, and SIF). This dimensional reduction technique identified many patterns (Table S2). We focused on two, based on the finding that sex differences in gene expression were lost (different between GHMs and GHFs but not after SI) or gained (not different between GHMs and GHFs but different after SI) (Figure 3A). For both patterns, genes were first categorized by their differential expression between GHMs and GHFs and then characterized by how they changed after SI. For example, if the loss of sex difference after SI was driven by the fact that SIM expression
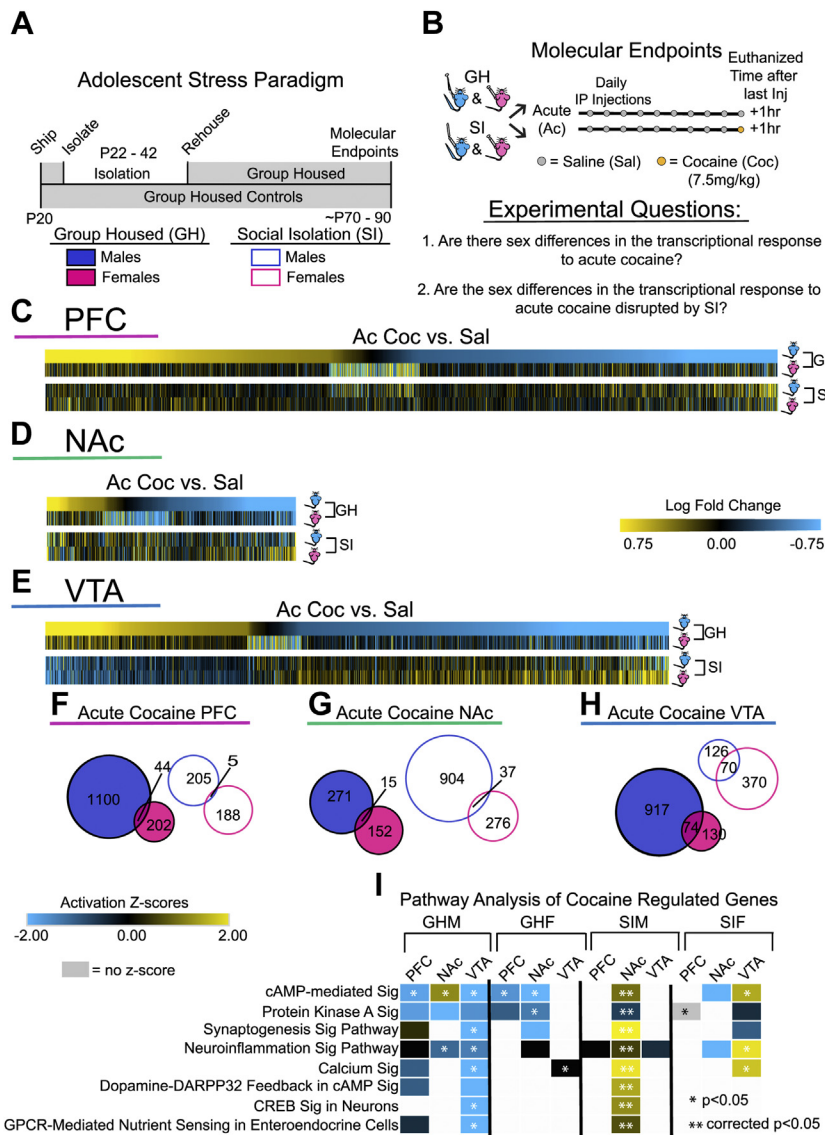
Figure 1. Sex-specific transcriptional responses to acute cocaine exposure are disrupted by SI throughout the reward circuitry. **(A, B)** Schematic of experimental design. Heatmaps show differentially expressed genes with at least a nominal $p \leq .05$ and a fold change ≥ 1.3 in response to acute cocaine exposure for the **(C)** PFC, **(D)** NAc, and **(E)** VTA (yellow = upregulated; blue = downregulated). Heatmaps are seeded by GHMs. All comparisons are made with the acute saline exposure control for the group. Venn diagrams of differentially expressed genes in GH (closed circles) and SI (open circles) M/F after acute cocaine exposure for the **(F)** PFC, **(G)** NAc, and **(H)** VTA. There was very little overlap between males and females across all three brain regions. **(I)** Ingenuity Pathway Analysis shows intracellular signaling, neurotransmitter, and other nervous system signaling pathways that are regulated by acute cocaine exposure (activation z scores ≥ 2.0; $p \leq .05$; yellow = predicted activated; blue = predicted inhibited; black = no predicted activation/inhibition). *$p < .05$; **corrected $p < .05$. Ac, acute; F, female; GH, group housed; IP, intraperitoneal; M, male; NAc, nucleus accumbens; PFC, prefrontal cortex; SI, social isolation; VTA, ventral tegmental area.

looked like GHF expression, it was categorized as "feminized" (Figure 3A). Pattern analysis revealed that the loss of sex differences in expression in all three brain regions and under all treatment conditions was driven by feminization of expression in SIMs: approximately 40% of sex differences in genes were feminized by SI. Masculinization of sex differences in genes did not make up a large proportion of the patterns observed under baseline condition (chronic saline). However, after SIFs were exposed to another stimulus, male-typical expression patterns were observed across all three brain regions (Figure 3B, E, H). Similar effects were observed in the patterns representing a gain of sex differences (Figure 3D, G, J). Genes only differentially expressed in SIMs made up a large proportion of the genes categorized as a "gain of sex difference" under all treatment conditions and all brain regions. The one exception to this was in the NAc, where under baseline conditions

(chronic saline), genes are disproportionally altered in SIFs (Figure 3G). These data suggest that many of the sex-specific transcriptional changes observed after SI are driven by changes in SIMs and point to a more responsive transcriptional state in SIMs throughout the reward circuitry. Finally, this analysis revealed that animals regulate many more transcripts in response to acute stimuli (saline or cocaine) in a sex-specific manner. This occurs even after several injections meant to induce habituation, suggesting that habituation is a transcriptionally active event and differs between the sexes. Indeed, we showed previously that stress resilience is associated with greater changes in transcription than stress susceptibility in males (14). The present data suggest a similar phenomenon regulating habituation to handling and injection stress.

We next focused on pathways predicted to be regulated by cocaine (Figure 1) or sex-specific expression (Figure 2) to
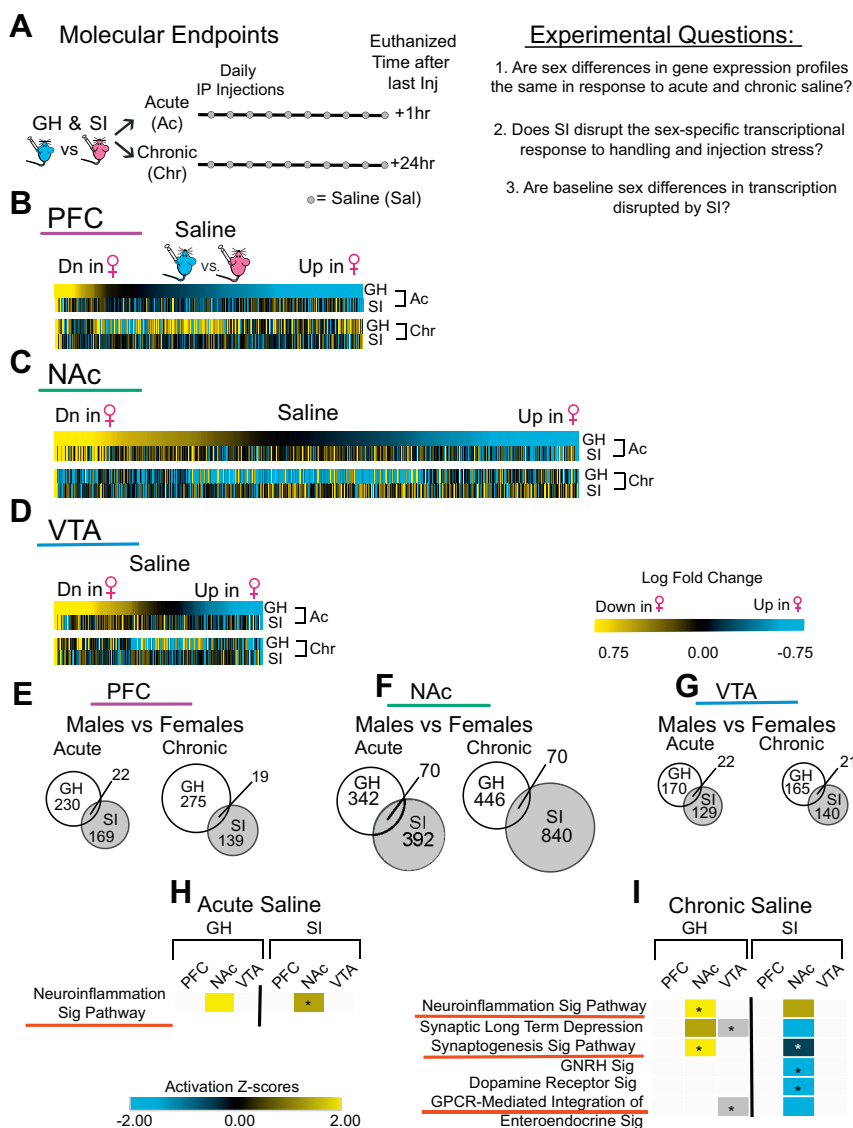
Figure 2. Adolescent SI disrupts expression of baseline sex differences in the reward circuitry. **(A)** Schematic of experimental design. **(B–D)** Union heatmaps of sex differences in differentially expressed genes in GH males vs. GH females after acute or chronic saline exposure. Blue represents genes upregulated in females, and yellow represents genes downregulated in females. **(E–G)** Venn diagrams of sex differences in genes in GH and SI animals. Sex differences in all three brain regions are lost after SI. Ingenuity Pathway Analysis shows pathways composed of sex differences in genes overlap with pathways affected by cocaine after experiencing **(H)** acute and **(I)** chronic saline exposure (activation z-scores ≥ 2.0; $p \leq .05$). *$p < .05$. Ac, acute; Chr, chronic; Dn, down; GH, group housed; IP, intraperitoneal; NAc, nucleus accumbens; PFC, prefrontal cortex; SI, social isolation; VTA, ventral tegmental area.

determine if the pathways reflect a loss or gain of sex-specific expression. Pathways that were predicted to be feminized under saline conditions were predicted to be regulated by cocaine only in SIMs in the NAc and PFC (Figure 3C, F). This was not the case for pathways predicted to be regulated in SIFs, where most were predicted to be regulated under saline conditions (both acute and chronic). These data suggest that neuronal signaling pathways are disrupted by SI more in males than in females.

Coexpression Analysis Reveals That Sex-Specific Gene Networks Are Disrupted by SI

We next asked if sex-specific disruption of transcription altered transcriptomic structure using multiscale embedded gene coexpression network analysis. This approach is unique in that unlike other coexpression analyses, multiscale embedded gene coexpression network analysis identifies

modules of genes at different compactness scales, leading to a hierarchical module structure (15). We hypothesized that disruption of sex-specific transcription disrupts coexpression modules on a global scale, providing insight into hubs that might be important for regulating sex-specific transcription in the reward circuitry. Each brain region was analyzed separately. Because we hypothesized that SI disrupts sex-specific transcriptional regulation under various conditions, modules were identified for each of the four groups (GHM/F, SIM/F) by collapsing data from all four treatment paradigms (acute/chronic cocaine and saline). We first focused on sex-specific coexpression networks between GHMs and GHFs and then determined how SI disrupted sex-specific coexpression on a global scale in each brain region (Figures 4–6). By comparing the number of parent modules, we identified disruptions to transcriptomic structure across the groups (Figures 4E, 5E, 6E). Because parent modules are the largest groups of
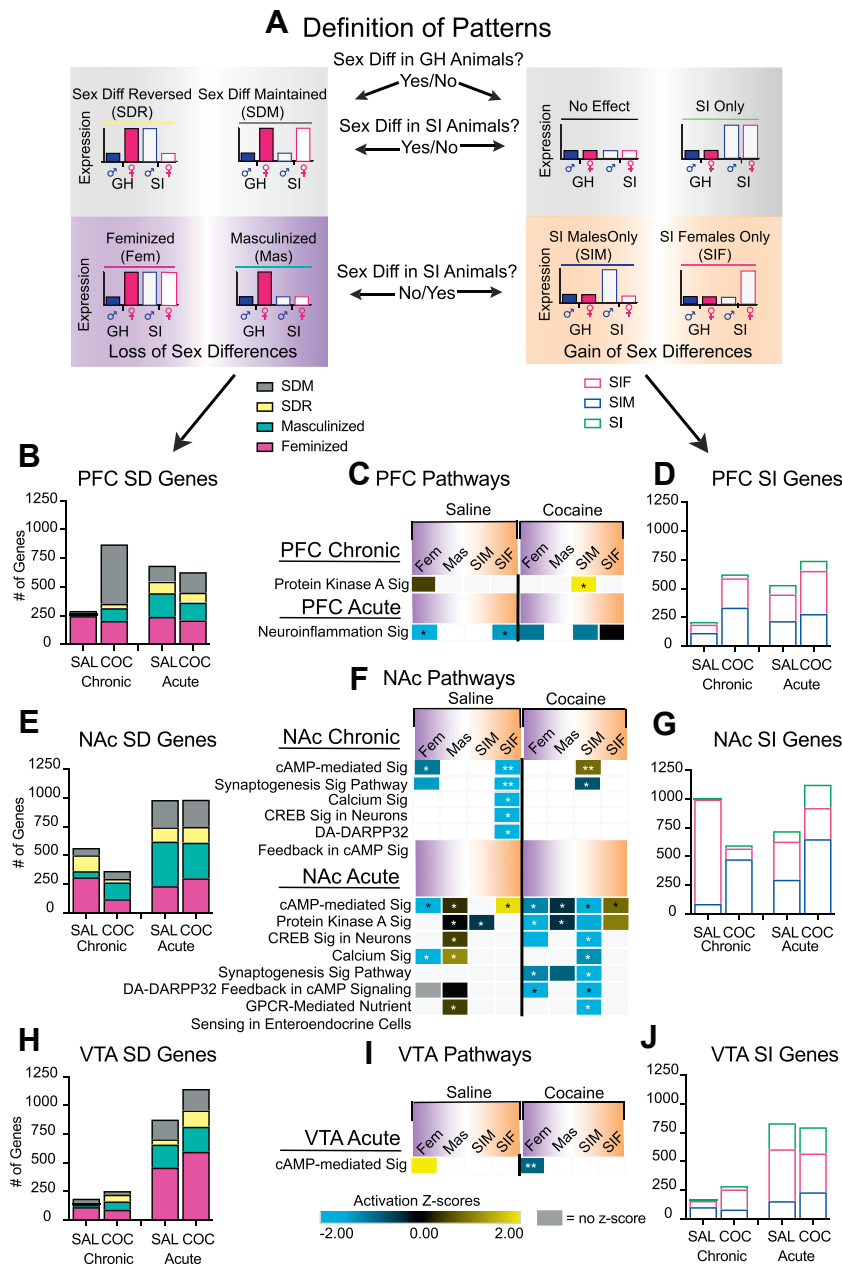
Figure 3. Pattern analysis reveals sex differences that are lost or gained after SI. **(A)** Schematic of decision-tree for categorizing genes in pattern analysis. Pattern analysis of **(B)** PFC, **(E)** NAc, and **(H)** VTA sex differences in genes in all four conditions. Gray = SDM, yellow = SDR, blue = masculinized, and pink = feminized. Ingenuity Pathway Analysis of the **(C)** PFC, **(F)** NAc, and **(I)** VTA reveals same pathways that are affected by cocaine and sex-specific gene expression. Only those pathways that overlap with those regulated by cocaine are included **(C, F, I)**. See Figure S3 in Supplement 1 for the complete list of the predicted pathways associated with the genes identified through pattern analysis (activation z-scores ≥ 2.0; $p \leq .05$; yellow = predicted activated; blue = predicted inhibited; black = no predicted activation/inhibition). Pattern analysis of SI effects after all four treatment conditions in the **(D)** PFC, **(G)** NAc, and **(J)** VTA. Translucent pink = SIF effects only, translucent blue = SIM effects only, and translucent green = SI effects. *$p <$ .05; **corrected $p <$ .05. COC, cocaine; F, female; Fem, feminized; GH, group housed; M, male; Mas, masculinized; NAc, nucleus accumbens; PFC, prefrontal cortex; SAL, saline; SDM, sex difference maintained; SDR, sex difference reversed; SI, social isolation; VTA, ventral tegmental area.

coexpressed genes identified within the root module, a transcriptome with fewer parent modules has more "structure."

Using module differential connectivity analysis, we identified parent modules that were differentially connected between GHMs and GHFs, with the predicted sex differences in module differential connectivity disrupted after SI (Figures 4F, 5F, 6F). We next used Fisher's exact tests to find parent modules composed of the same genes in GHMs and GHFs (Figures 4G, 5G, 6G; Figure S5B in Supplement 1) and then focused on parent modules enriched in genes categorized by our pattern analysis. This approach identified conserved modules that might be important for driving the region- and

sex-specific transcriptomic effects observed across the three brain regions (Figures 4H and I, 5H and I, 6H and I). Within those parent modules, we highlighted "children" and "grandchildren" submodules with conserved hubs across all groups but displaying sex-specific connectivity (Figures 4H, 5H, 6H) and expression patterns that fall into our categories identified by pattern analysis (Figures 4I, 5I, 6I). We focused on key drivers that met these criteria because previous work from our laboratory showed that manipulation of a key driver that is conserved across all groups results in predicted behavioral effects, whereas manipulation of a key driver that is not conserved across all four groups has no impact on
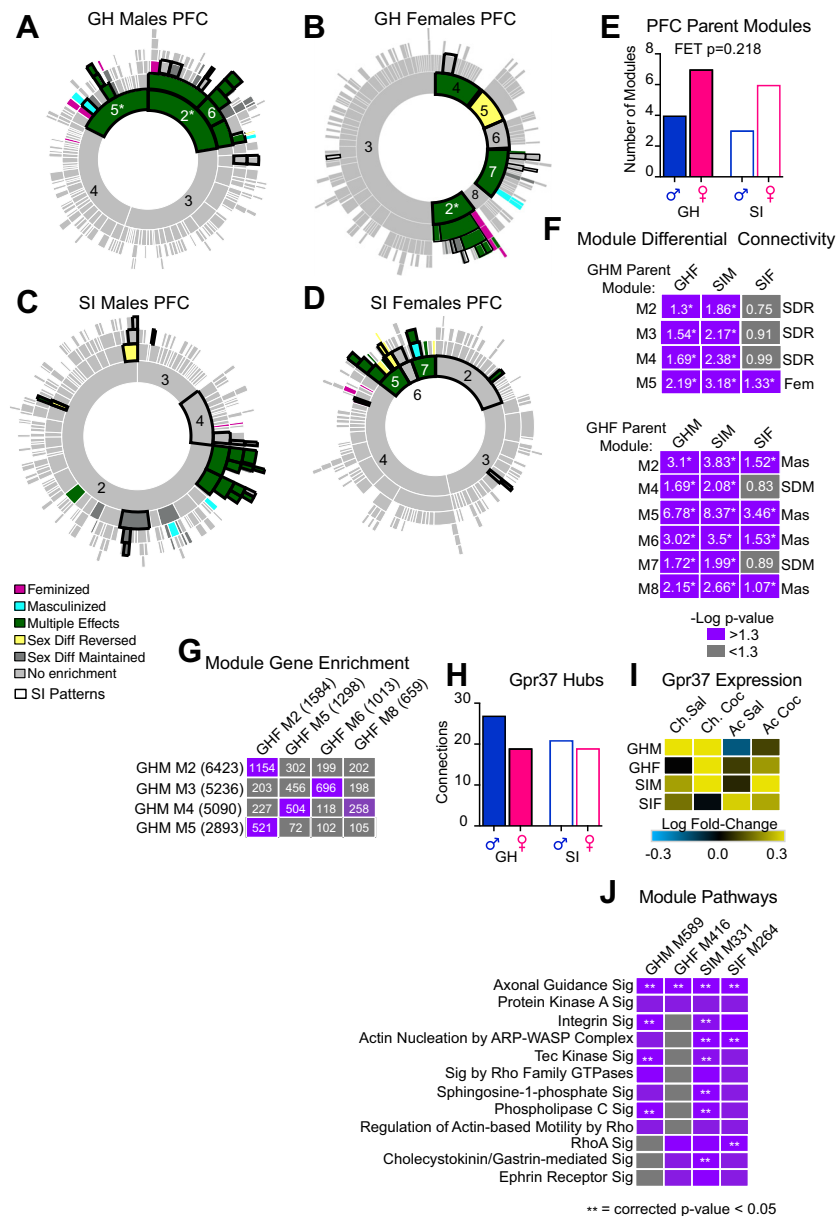
Figure 4. Sex-specific coexpression networks are disrupted by SI in PFC. **(A–D)** Sunburst plots in the PFC of **(A)** GHM, **(B)** GHF, **(C)** SIM, and **(D)** SIF. Plots are organized with parent modules on the innermost rings, followed by children, grandchildren modules, etc. Modules with asterisks are of interest because of conservation across all four groups and a loss or gain of sex difference. A variety of colors indicate enrichment of pattern genes; gray = no enrichment. **(E)** Bar graph of number of parent modules shows transcriptomic structure is conserved. **(F)** Module differential connectivity plots show comparison of module connectivity across parent modules. Numbers = module differential connectivity value for comparison; purple = differentially connected, and gray = not differentially connected. **(G)** Enrichment plot of conserved hub genes across differentially connected parent modules. Significant enrichment is indicated by purple, number of genes in each module is in parentheses, and number of overlapping genes is in each box. **(H)** Bar graph of the number of connections of the hub gene *Gpr37* in GH and SI males and females. **(I)** Heatmap showing the relative expression of the hub gene *Gpr37* in response to acute and chronic saline and cocaine exposure. **(J)** Pathway analysis of the genes in each module reveals sex-specific pathways associate with coexpression modules is disrupted by SI; purple = enrichment of genes associated with pathways; gray = no enrichment. *$p < .05$; **corrected $p < .05$. Ac, acute; Ch, chronic; Coc, cocaine; F, female; Fem, feminized; FET, Fisher's exact test; GH, group housed; M, male; Mas, masculinized; PFC, prefrontal cortex; Sal, saline; SDM, sex difference maintained; SDR, sex difference reversed; SI, social isolation.

behavior ([9]). This approach yielded more than one possible key driver for follow-up investigation in each brain region ([Table S4](#)); we highlight one key driver per brain region as an illustration ([Figures 4–6](#); [Figure S4–6](#) in [Supplement 1](#)).

Coexpression Analysis Reveals That SI Disrupts Differential Connectivity in Brain Reward Regions

We observed that SI disrupted transcriptome structure in the NAc and VTA but not in the PFC ([Figures 4–6A, B](#)). In PFC, we identified 4 parent modules in GHMs versus 7 in GHFs, numbers that were similar to those observed in SIMs and SIFs (Fisher's exact test $p = .219$) ([Figure 4E](#)). Even though the

number of modules was not disrupted by SI, we hypothesized that the parent modules would be differentially connected in GHMs than in GHFs and that SI would disrupt this sex-specific connectivity ([Figure 4F](#)). Modules were constructed in all four groups such that the same genes did not always comprise the same modules across comparisons. Therefore, module differential connectivity analysis was conducted by comparing the connectivity of the genes within a module constructed in one group (e.g., GHMs or GHFs) with the connectivity of the same genes in the other groups. Interestingly, every parent module identified in GH animals was differentially connected when compared with connectivity of genes in the GH animal of the
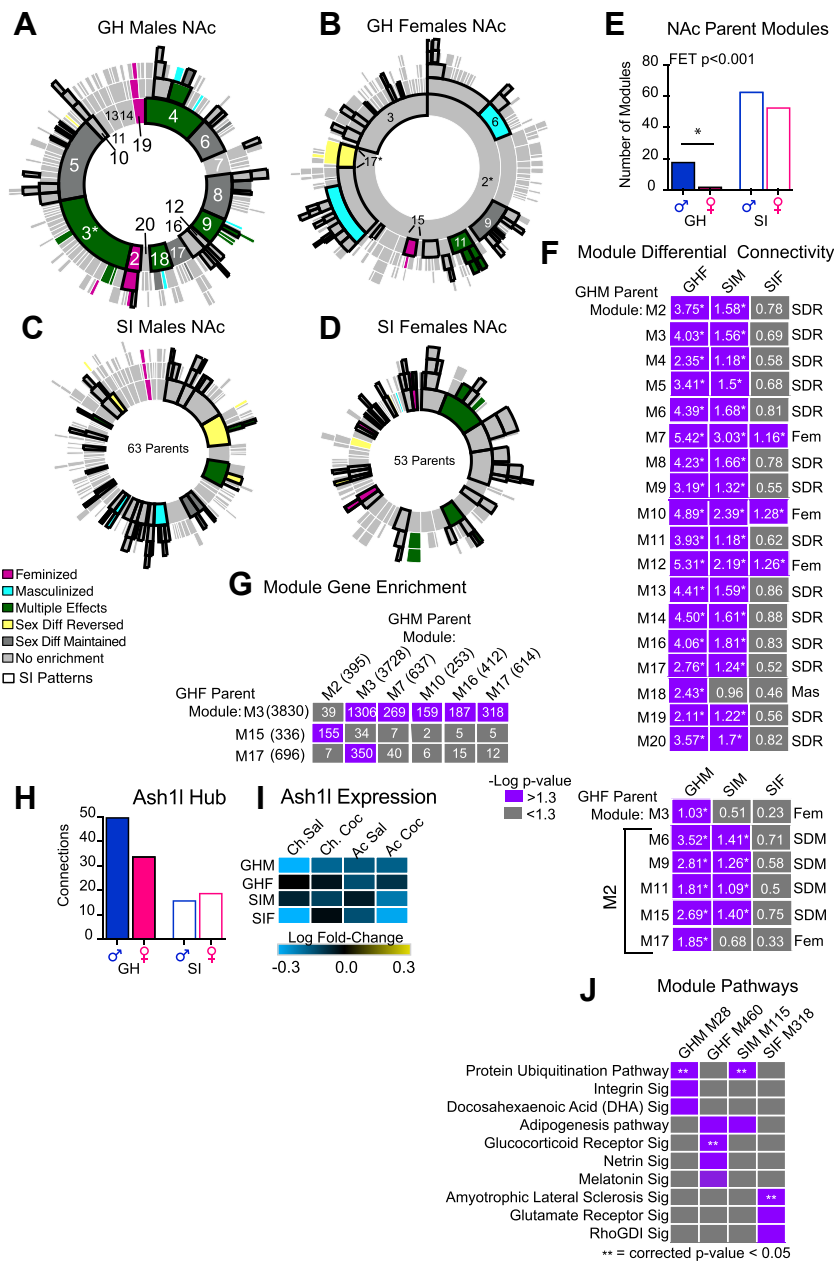
Figure 5. Sex-specific coexpression networks are disrupted by SI in NAc. **(A–D)** Sunburst plots in the NAc of **(A)** GHM, **(B)** GHF, **(C)** SIM, and **(D)** SIF. Plots are organized with parent and submodules as in Figure 4, where asterisks and colors are also defined. **(E)** Bar graph of number of parent module shows loss of transcriptomic structure. **(F)** Module differential connectivity plots show comparison of module connectivity across parent modules. Numbers and colors are defined in Figure 4. **(G)** Enrichment plot of conserved hub genes across differentially connected parent modules. Numbers and colors are defined in Figure 4. **(H)** Bar graph of the number of connections of the hub gene *Ash1l* in GH and SI males and females. **(I)** Heatmap showing the relative expression of the hub gene *Ash1l* in response to acute and chronic saline and cocaine exposure. **(J)** Comparison analysis of pathways associated with the genes modules reveals sex- and SI-specific pathways associate with coexpression modules. *$p < .05$; **corrected $p < .05$. Ac, acute; Ch, chronic; Coc, cocaine; F, female; Fem, feminized; FET, Fisher's exact test; GH, group housed; M, male; Mas, masculinized; NAc, nucleus accumbens; Sal, saline; SDM, sex difference maintained; SDR, sex difference reversed; SI, social isolation.

opposite sex (e.g., GHM modules vs. GHF gene coexpression). However, SI disrupted module connectivity in a sex-specific manner, especially modules constructed in GHMs (Figure 4F). This suggests that SI disrupts sex-specific coexpression of a majority of the parent modules representing the global PFC transcriptome.

Unlike the PFC, NAc transcriptomic structure is disrupted by SI. A large sex difference was observed in the number of parent modules in GH (GHM = 18; GHF = 2; Fisher's exact test $p < .001$; χ^2 between GHM vs. GHF $p < .001$) but not SI (SIM = 63 vs. SIF = 53) (Figure 5A–E). Notably, we only identified two parent modules in GHFs, and one of those modules contains

approximately 80% of the genes in the transcriptome. Modules this large generally offer limited insight. Therefore, we focused on the second layer of modules that compose module 2 for GHFs. Again, all parent modules identified in GHMs are differentially connected when compared with coexpression of those genes in GHFs, which was disrupted after SI. This effect does not translate to GHF modules where the vast majority of the modules that make up parent module 2 are differentially connected when compared with GHMs and SIMs but not SIFs (Figure S5A in Supplement 1). This suggests that the SI effects in the NAc may be driven by changes in SIMs to a greater extent than in SIFs.
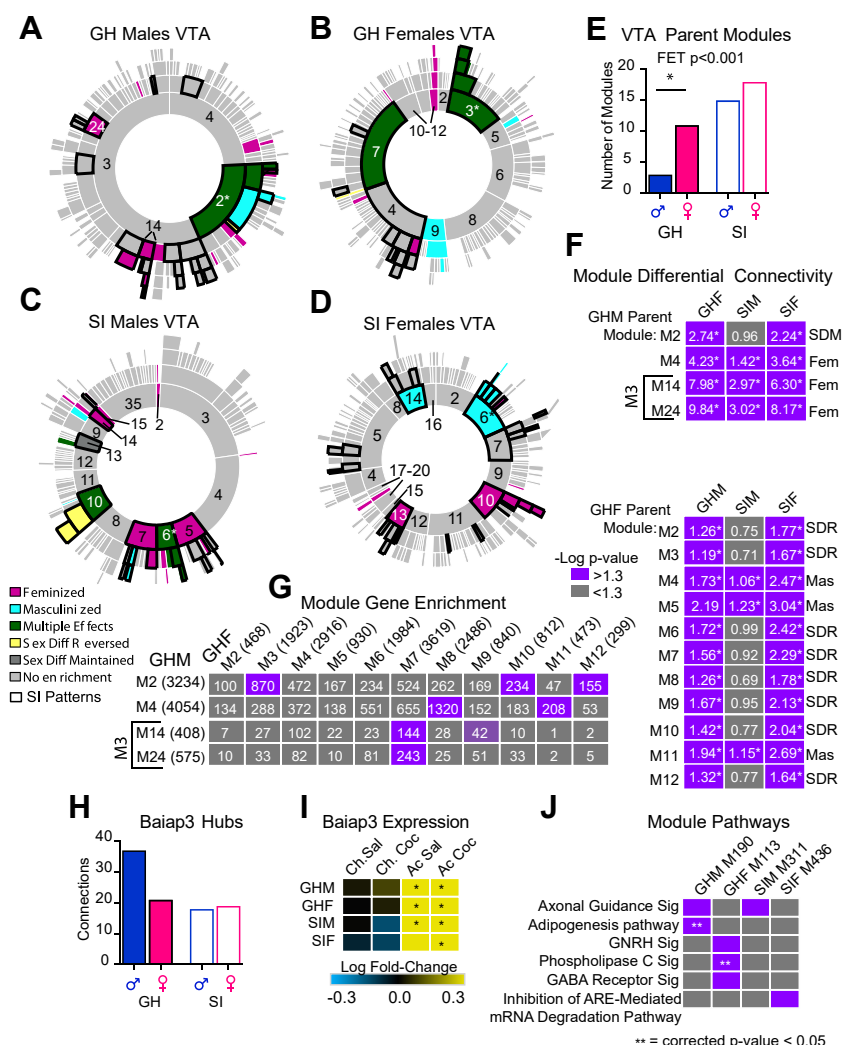
Figure 6. Sex-specific coexpression networks are disrupted by SI in the VTA. **(A–D)** Sunburst plots in the VTA of **(A)** GHM, **(B)** GHF, **(C)** SIM, and **(D)** SIF. Plots are organized with parent and submodules as in Figure 4, where asterisks and colors are also defined. **(E)** Bar graph of number of parent module shows loss of transcriptomic structure in SI animals. **(F)** Module differential connectivity plots show comparison of module connectivity across parent modules. Numbers and colors are defined in Figure 4. **(G)** Enrichment plot of conserved hub genes across differentially connected parent modules. Numbers and colors are defined in Figure 4. **(H)** Bar graph of the number of connections of the hub gene *Baiap3* in GH and SI males and females. **(I)** Heatmap showing the relative expression of the hub gene *Baiap3* in response to acute and chronic saline and cocaine exposure. **(J)** Comparison analysis of pathway enrichment of gene modules in each condition show no overlap between males and females independent of SI. *p < .05; **corrected p < .05. Ac, acute; Ch, chronic; Coc, cocaine; F, female; Fem, feminized; FET, Fisher's exact test; GH, group housed; M, male; Mas, masculinized; Sal, saline; SDM, sex difference maintained; SDR, sex difference reversed; SI, social isolation; VTA, ventral tegmental area.

In the VTA, GHFs have more modules, and thus less coexpression structure, than GHMs (Fisher's exact test $p < .001$; χ^2 GHM vs. GHF $p < .001$) (Figure 6A, B, E), which is disrupted by SI. Both SIM and SIF transcriptomes are composed of more modules suggesting disruption of global coexpression (Figure 6C–E). Analogous to the PFC and NAc, parent modules are differentially connected between GHMs and GHFs and disrupted by SI (Figure 6F). Together, these data suggest that global transcriptomic structure and connectivity are different in GHMs versus GHFs across all three brain regions and that this sex difference is disrupted by SI in a region-specific manner.

Identification of Sex-Specific Hub Genes in Brain Reward Regions

We used the criteria discussed above to identify key hub or driver genes in each brain region that might be important for regulating sex-specific gene expression patterns. In the PFC, this approach identified 9 conserved hubs, 3 of which displayed sex-specific connectivity ±30% connections between GHMs and GHFs within the modules of interest. Two key drivers lost the sex difference in connectivity after SI: *Cnn3* and *Gpr37* (Figure 4H). Analysis of their expression profiles revealed that only *Gpr37* is also a pattern gene under multiple conditions in the PFC (Figure 4I). *Gpr37* encodes an orphan GPCR (G protein–coupled receptor) that is associated with Parkinson's disease and has been proposed to interact with dopamine receptor 2 (16), as knockout of *Gpr37* alters dopamine dynamics (17). In humans, *GPR37* has been identified as a sex-biased gene across development and throughout the brain (18). Mice lacking *Gpr37* fail to form a preference for cocaine and amphetamine (19) and display altered cocaine-induced corticostriatal long-term depression (20). In our dataset, *Gpr37* is different between the sexes in the PFC of GH animals (male > female) and feminized by SI after chronic saline exposure. A gain of sex difference in cocaine responses is induced in that *Gpr37* is upregulated by acute and chronic cocaine exposure in SIM only.

Thus, behavioral and transcriptional effects of SI are reflected in expression profiles of *Gpr37* and give credence to this molecule as an important regulator of the sex-specific transcriptional effects of cocaine in the PFC. Pathway analysis of PFC modules containing *Gpr37* as a hub displays a similar pattern of regulation, with minimal overlap of predicted pathways observed between GHM and GHF modules. Interestingly, the neuronal signaling pathways associated with the modules are overrepresented in the pathways associated with sex differences in genes in GH and SI animals after chronic exposure to cocaine (Figure S2I in Supplement 1).

In NAc, there are very few shared hubs across the four groups (GHM/F and SIM/F) (Table S4). However, we identified 2 conserved hubs in parent modules GHM M3 and GHF M17 or their children and grandchildren submodules. Only one of those key drivers, Ash1-like histone lysine methyltransferase (*Ash1l*), displayed sex-specific connectivity within the GH modules (Figure 5H). *Ash1l* encodes an activity-dependent histone methyltransferase that is important for maintenance of H3K36me2 at intergenic regions (21) and has been associated with neuronal development (22). *Ash1l* deletion abolished activity-dependent repression of neurexin1a, a cell adhesion molecule important for synaptic plasticity (23). Finally, *Ash1l* is associated with autism in human males (24). *Ash1l* was not captured in our original pattern analysis approach because its expression did not change >30% (Table S4). However, because only one hub met our criteria, we characterized its expression changes using our pattern analysis criteria (Figure 3). Sex differences in *Ash1l* expression are reversed by SI ($p < .05$), and only SIFs decrease expression in response to acute cocaine exposure ($p < .05$) (Figure 5I). Pathway analysis of NAc modules that contain *Ash1l* as a hub (Figure 5L) reveals little overlap of neuronal signaling pathways across the modules. However, integrin signaling and RhoGDI signaling pathways are overrepresented in sex differences in gene expression after chronic exposure to cocaine in SI animals (Figure S5I in Supplement 1), and genes associated with RhoGDI signaling are enriched in SIF and SIM pattern genes after chronic saline and cocaine exposure, respectively (Figure S3C in Supplement 1).

Finally, we identified brain-specific angiogenesis inhibitor I–associated protein 3 (*Baiap3*) as a sex-specific hub in VTA. *Baiap3* encodes a transmembrane protein that is important for recycling secretory vesicles: its knockout causes vesicle accumulation (25–27). *Baiap3* is also associated with dopaminergic (14) and GABAergic (gamma-aminobutyric acidergic) (26) signaling. Inactivation of this gene induces sex-specific effects on anxiety and reward in humans and mice. Females lacking *BAIAP3* display increased anxiety, whereas males display increased benzodiazepine dependence (28). Pathways associated with the modules containing *Baiap3* do not overlap between GHMs and GHFs (Figure 6I) but do overlap with pathways associated with sex-specific expression after acute cocaine exposure in SI animals (Figure S2H in Supplement 1).

Together, these data suggest that integrating all levels of our bioinformatic analyses identified several sex-specific hub genes previously implicated in key neurobiological actions associated with stress and addiction and support our interpretation that coherence of gene expression patterns throughout the reward circuitry is disrupted by adolescent SI.

Threshold-Free Analyses Reveal That Sex-Specific Expression Is Maintained Across Brain Regions in GH Animals

Because the three brain regions studied are interconnected, we investigated if disruption of transcriptomic structure by SI is observed across brain regions. We used rank-rank hypergeometric overlay, which compares two large datasets in a threshold-free manner, to determine whether sex-specific expression across the transcriptome is conserved across brain regions in GH animals and whether this is disrupted by SI. We found strong coregulation of genes in the same direction in response to acute cocaine exposure between all brain regions (Figure S7A–C in Supplement 1), indicating that sex differences align across these regions in GHMs and GHFs. SI disrupts this pattern in VTA, with opposite regulation observed for many of the same genes (Figure S7D–F in Supplement 1). Similar patterns of sex-specific expression are seen across all brain regions after acute saline (Figure S8A–C in Supplement 1), chronic saline (Figure S8D–F in Supplement 1), and chronic cocaine (Figure S8G–I in Supplement 1) exposure. These findings suggest that SI profoundly disrupts sex-specific expression in the VTA, which may impact signaling between its connected regions.

DISCUSSION

We utilized adolescent SI, which has been used for decades to induce susceptibility to addiction-related behaviors in males, to understand how SI alters transcriptional responses to cocaine. Including females enabled us to determine that adolescent SI disrupts sex-specific behaviors and gene expression in several brain reward regions. By use of multiple bioinformatics techniques, we identified potential regulators of sex-specific transcriptional responses to cocaine in these regions and highlighted biological pathways that appear critical for sex differences in SUDs. Together, these data serve as an important resource in the study of SUDs because, to the best of our knowledge, this is the first unbiased study of the transcriptional effects of acute and chronic cocaine exposure in both males and females in the brain's reward circuitry.

Sex-Specific Transcriptional Responses to Cocaine Are Disrupted by Adolescent SI

A prominent finding of this study is that transcriptional responses to cocaine are vastly different between males and females. This is in line with recent findings for other psychiatric disorders, both in humans (29,30) and rodent models (31,32). We found very little overlap between the sexes in the specific transcripts regulated by cocaine (Figure 1C–H; Figure S1C–H in Supplement 1), the biological pathways associated with those transcripts (Figure 1I; Figure S1I in Supplement 1), or transcriptome-wide coexpression patterns (Figures 4–6) across the reward circuitry. This finding is surprising, partly because we observed a similar preference for cocaine in males and females (6), suggesting that very different sex-specific

transcriptional profiles yield similar behavioral phenotypes. This too is similar to the effects observed for other psychiatric disorders, including depression and anxiety (29–32). We hypothesize that this reflects the fact that sex differences in reward are necessary for normal reproductive behaviors and strategies (33) such that different molecular and cellular mechanisms are utilized by the two sexes for adaptation. Our data are thus important for understanding how these mechanisms are hijacked differently by drugs of abuse in males and females. For instance, our pathway analysis sheds light on sex-specific processes and cell types that influence behaviors related to stress and cocaine in males and females. We identify, for example, pathways associated with dopamine signaling in males, but not females, exposed to cocaine. Others have shown that psychostimulant-induced dopamine release is reportedly lower in females than in males (34), suggesting that cocaine-induced dopamine signaling may be sex specific.

We next leveraged the sex-specific disruption induced by adolescent SI to better understand sex-specific transcription in brain. We found that adolescence SI reduces sex differences in the transcriptome at baseline and increases sex differences in response to cocaine in the PFC, NAc, and VTA (Figure 2; Figure S2 in Supplement 1). These effects are reflected in results from our pattern analysis, which indicates that both the loss and gain of sex differences are driven overwhelmingly by changes in SIMs rather than in SIFs. This effect was observed across all three brain regions and under almost all treatment groups (Figure 3).

Coexpression Analysis Reveals That Sex-Specific Disruptions Are Transcriptome-wide

We used coexpression analysis to determine how transcriptomic structure is altered by adolescent SI. We observed in the NAc and VTA, but not in the PFC, a loss of transcriptomic structure after adolescent SI. This finding is crucial for understanding how sex-specific disruptions might affect the entire transcriptome and suggests that altered expression of a small number of sex-specific genes leads to important changes in the entire transcriptomic network (Figures 4–6A–E). In addition to transcriptomic structure, we found that parent modules are differentially connected between GHMs and GHFs across all three brain regions, which is also disrupted by SI. In many cases, SI resulted in genes with connectivity that is similar to the GH module of the opposite sex comparison rather than their same-sex counterpart (i.e., genes in SIMs were not differentially connected when compared with GHF modules but were when compared with GHM modules). While, to the best of our knowledge, our study is the first to investigate how cocaine influences sex-specific module differential connectivity, others have found similar effects in response to stress (31). Importantly, these effects were reflected in our rank-rank hypergeometric overlay analyses (Figures S7 and S8 in Supplement 1) and especially in the disruption of the sex-specific alignment of expression after acute cocaine exposure that is strikingly disrupted in the VTA and to a lesser extent in the NAc (Figure S7 in Supplement 1). Together, our findings suggest that disruption of sex-specific gene expression in the reward circuitry substantially impacts organization of the transcriptome and alignment of sex differences in multiple brain regions.

Coexpression Analysis Identified Conserved Hubs That Are Associated With Sex-Specific Expression and Dopamine Regulation

Coexpression analysis identifies key driver genes associated with behavioral phenotypes (29,35). We identified 3 hubs that are conserved in each of the three brain regions studied across all four groups of animals. While the functions of these molecules are quite different, they have all been identified as sex-specific genes in rodents (28) or humans (18,24). Additionally, even though the network analysis identified hubs based on gene coexpression and not function, it is worth noting that all 3 of these key driver genes are implicated in dopamine function (14,16,17,36).

Overall, this work provides a genome-wide map of transcriptomic effects of stress × cocaine interactions that occur in three brain reward regions and highlights dramatic sex differences. While it is beyond the scope of this study to determine the mechanisms by which adolescent stress reprograms the transcriptome, other studies have found that early-life experience influences serum hormone concentrations and motivated behaviors (37) and epigenetic mechanisms (38). These, along with cell-type–specific analyses, will be important factors to investigate in the future. Our study serves as a guide to better understand the influence of adolescent stress on SUDs and to develop sex-specific precision treatments for these conditions.

ARTICLE INFORMATION

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