# Pheromone circuits and transcriptional cascades modulating transcriptional and chromatin states in the *Drosophila* central brain with social experience

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# Abstract

Social experience significantly influences the behavioral and physiological responses of animals, including humans. In many animals, social isolation increases aggression, courtship, locomotion, and feeding while disrupting sleep. This occurs when peripheral neurons detect social signals, such as pheromones, which activate decision-making circuits in the brain. However, the molecular and circuit mechanisms of how chronic social isolation or enrichment alter gene expression and affect neuronal function and behavior remain unclear. In this study, we examined how transcription patterns and chromatin marks in male *Drosophila* brains change in response to social experience, and the impact of pheromone circuits and transcription factors involved in social circuit function. We focused on pheromone receptors Or47b and Or67d, as well as transcription factors $Fru^M$ and $Dsx^M$. Our findings suggest that social experience impacts multiple genes in the central brain. Disrupting Or47b, Or67d, $Fru^M$, and $Dsx^M$ function normalized the transcriptional responses through antagonistic interactions. Specifically, Or47b circuits predominantly mediated transcriptional responses to social isolation through $Dsx^M$ function, while Or67d and $Fru^M$ regulated responses to group housing. Notably, mutants of $fru^M$ and $dsx^M$ exhibited more extensive transcriptional changes in the brain than Or mutants, especially for $Fru^M$/$Dsx^M$ target genes. While social experience did not lead to detectable alterations in the overall chromatin profile in the whole brain, mutants of the four genes resulted in significant changes in the enrichment of H3K4me3 and RNA polymerase II (RNAPolII) compared to wild type. Furthermore, mutants in $fru^M$ and $dsx^M$ generally eliminated social experience-dependent changes in sleep and locomotion behaviors, whereas Or mutants exhibited more modest disruptions. Overall, our results uncover the pheromone circuits and transcriptional cascades in regulating molecular and behavioral responses to social experience.

# Introduction

Social experience modulates many behavioral and physiological responses in animals, including humans [1-11]. During this process, social signals are detected by the peripheral neurons tuned to social cues like pheromones, which activate the pheromone and social circuits in the central brain, where information is integrated, and behavioral decisions are made (Fig. 1A). In the case of chronic social isolation or enrichment, the activity state of neuronal circuits likely alters gene expression at the transcriptional and chromatin levels, ultimately adaptively altering the neuronal circuit function in the brain and behavioral outputs [7, 12-22]. However, the molecular and circuit mechanisms underlying how social experience modulates behaviors remain largely unknown.

Social experience has been shown to influence gene regulation in the nervous systems, from insects to mammals [10, 13, 23-25]. *Drosophila melanogaster*, as an excellent behavioral model, has been used to study many social behaviors, as well as behaviors influenced by social experience [5, 10, 12, 13, 17, 26-31]. Previous research on the *Drosophila* peripheral olfactory system demonstrated that social experience and pheromone receptor signaling modify chromatin states and alter the expression of numerous neuromodulatory genes, in addition to changing neuronal responses [12, 13, 30]. These findings suggest a strong link between social signals, transcriptional and chromatin modulation, and neuronal responses within the peripheral nervous system. Although there is clear evidence that social experience can alter gene regulation in the peripheral nervous system, the changes in gene expression and chromatin states in the central brain in different social contexts and the circuits that mediate these changes remain unclear (Fig. 1A).

In *Drosophila*, two transcription factors (TFs), Fruitless (Fru) and Doublesex (Dsx), regulate the development and function of sex-specific social circuits and social behaviors [32-43]. The male isoform of Fru, $Fru^M$, encodes a BTB zinc finger transcription factor involved in the sexual differentiation of neural circuits that control male behaviors [32, 39, 44]. In addition, $fru^M$ is necessary for mediating social experience-dependent changes in male competitive copulation behaviors [30]. We also recently reported that changes in $fru^M$ expression and function mediate social experience-dependent changes in courtship behaviors [45]. Fru was also shown to form a complex with the transcriptional cofactor Bonus (Bon), which recruits the chromatin regulators Histone deacetylase 1 (HDAC1) or Heterochromatin protein 1a (HP1a) to regulate gene expression underlying neuronal sexual differentiation [46]. In contrast, Dsx belongs to the evolutionarily conserved DMRT (Doublesex and Mab-3 Related Transcription factor) gene family. Sex-specific isoforms of Dsx ($Dsx^M$ and $Dsx^F$) regulate sexual differentiation of both soma and the nervous system from invertebrates to vertebrates [47-49]. Perturbation of Dsx function changes courtship behavior in both males and females. Moreover, $Dsx^M$ is necessary and sufficient for males to acquire experience-dependent courtship behavior in the absence of $fru^M$. Both $Fru^M$ and $Dsx^M$ bind DNA to regulate the expression of overlapping sets of genes, although some genes are specifically regulated by either $Fru^M$ or Dsx [35, 50].

$Fru^M$ is expressed in interconnected neurons that label circuits for mediating social behaviors such as courtship and aggression [51-53]. $Fru^M$ positive neural circuits include peripheral neurons that detect social cues to neural centers for sensory processing, integration, and decision-making, as well as the descending motor pathways responsible for executing the behaviors [34, 35, 38, 39, 52, 54, 55]. In the olfactory system, two classes of pheromone-sensing olfactory receptor neurons (ORNs) that express pheromone receptors Or47b and Or67d are $fru^M$ positive [39, 52]. Or47b ORNs detect male and female pheromones and drive age and social experience-dependent male copulation advantage in a $fru^M$-

dependent manner [30, 56, 57]. Or67d ORNs detect male-specific pheromone 11-*cis*-vaccenyl acetate (*cis*-VA) and function to inhibit courtship behaviors in males [58, 59].

Previous studies demonstrated that social isolation alters many behavioral and physiological responses of *Drosophila,* increasing aggression, courtship, locomotion, and feeding, and disrupting sleep [5, 10, 12, 13, 17, 26-31]. In a related study, we demonstrated that changes in courtship behaviors, resulting from social experiences, are associated with alterations in the expression and function of $fru^M$, as well as specific circadian genes that are potential targets of $Fru^M$ and/or $Dsx^M$ [45]. Activating pheromone-sensing neurons through chronic exposure to social cues stimulates social circuits and engages the transcriptional functions of $Fru^M$ and $Dsx^M$ within these neurons.

Behavioral modulation in response to social experiences likely depends on detectors of social signals, such as pheromone receptors (like Or47b and Or67d), and regulators of social circuits like $Fru^M$ and $Dsx^M$. To test this hypothesis, we conducted a study exploring how transcriptional and chromatin patterns in the brain change when we functionally disrupt these four pathways using male mutants in *Or47b*, *Or67d*, $fru^M$, and $dsx^M$ raised in monosexual groups or in isolation (Fig. 1B). We additionally examined the impact of these mutants on social modulation of behaviors. Our results suggest that: 1) Social experience leads to changes in numerous genes involved in various processes, from metabolism to behavioral regulation, neural development, and immunity. Loss of *Or47b*, *Or67d*, $fru^M$, or $dsx^M$ normalized the transcriptional responses to social experience, with general antagonistic effects between Or47b and Or67d pheromone circuits, and between $Fru^M$ and $Dsx^M$ circuits. We found trends where Or47b pheromone circuits and $Dsx^M$ circuits function together in mediating transcriptional responses to social isolation, while Or67d neuronal circuits and $Fru^M$ circuits function together to drive transcriptional responses to group housing in the brain. 2) Under the same housing conditions, compared to Canton-S wild type (*CS*), $fru^M$ and $dsx^M$ mutant brains showed more extensive changes at the transcriptional level than those observed in *Or47b* or *Or67d* mutants. $Fru^M$ and $Dsx^M$ function as either transcriptional repressors or activators, influencing downstream target genes. 3) Despite transcriptional changes, at the chromatin level, social experience does not significantly alter the overall distribution of H3K4me3 or RNA polymerase II (RNAPolII) in the whole brain. However, compared to *CS*, the four mutants cause numerous changes to the enrichment of H3K4me3 or RNAPolII under the same social conditions. The transcriptional changes identified in different mutants are accompanied by modest concordant changes in H3K4me3 or RNAPolII enrichment. 4) Mutants in $fru^M$ and $dsx^M$ generally eliminate social experience-dependent changes in sleep and locomotion behaviors. In contrast, Or mutants exhibited more modest disruptions in social modulation of locomotion and courtship, with housing-specific defects observed in *Or47b* mutants. Our results identified pheromone circuits and transcriptional cascades that work with them in the modulation of molecular responses to social experience, which reprogram brain function and behaviors. These findings provide new insights into how social experiences affect molecular plasticity, laying the groundwork for future research on the molecular mechanisms linking social interactions to behavior and physiology.

# Results

## The contribution of pheromone receptors and social circuit regulators on transcriptional and chromatin responses to social experience in the brain

We recently reported differentially expressed genes (DEGs) between grouped and isolated *CS* male brains [45]. To explore the contribution of pheromone circuits and social circuit regulators in social modulation of gene expression, we analyzed transcriptional and chromatin profiles in *Or47b, Or67d,* $fru^M$, and $dsx^M$ mutant brains from grouped or isolated males (Fig. 1A-B). We performed bulk RNA sequencing of brain tissues from group-housed (GH) and single-housed (SH) male *CS* wild type, and $Or47b^{2/3}$, $Or67d^{GAL4/GAL4}$,

139 *fru*$^{LexA/4-40}$, and *dsx*$^{0683/1649}$ mutants (Fig. 1B). To explore potential chromatin-based mechanisms for social
140 experience-dependent gene regulation, we additionally conducted CUT&RUN (Cleavage Under Targets
141 and Release Using Nuclease) and sequencing in male brains using the same genotypes and social
142 conditions to assess the genome-wide enrichment of two active chromatin marks: H3K4me3 and
143 RNAPolII (Fig. 1B).

144 We first examined the quality of the sequencing results. From the PCA results of both RNA seq and
145 CUT&RUN, samples with the same genotypes are clustered together (Fig. 1C, D, and E). Both the
146 transcription profiling and chromatin landscape of *fru*$^{LexA/4-40}$ and *dsx*$^{0683/1649}$ mutants appeared more
147 distinct from the wild-type *CS*, while *Or47b*$^{2/3}$ and *Or67d*$^{GAL4/GAL4}$ mutants showed more modest changes
148 compared to the wild type (Fig. 1C, D, and E). Regarding the distribution of H3K4me3 and RNAPolII
149 peaks, over 50% of the peaks for both marks were found around the promoter regions throughout the
150 genome (Fig. 1F and G).

151 Normalized counts of RNA transcripts of several housekeeping genes or control genes (*RpS29*, *Hmt4-20*,
152 and *TSG101*) appeared consistent across all genotypes (Supplementary Fig. 1A-C). *fru*$^{LexA/4-40}$ mutants
153 significantly decreased the expression of *fru* (Supplementary Fig. 1E). Within the overall brain, *fru*
154 expression showed very little to no change with social experience in *CS* or mutants. Yet, similar to results
155 from the peripheral olfactory system [12], we found some *fru* exons to be differentially regulated in *CS*
156 with social experience (Fig. 1H frames). The same exons were also decreased in *Or67d*$^{GAL4/GAL4}$ mutants
157 and *dsx*$^{0683/1649}$ mutants (Fig. 1H frames). *fru*$^{LexA/4-40}$ males exhibited a reduction in H3K4me3 and
158 RNAPolII enrichment at the *fru* locus (Fig. 1H). This trend is also true for *dsx* expression, as well as
159 H3K4me3 and RNAPolII enrichment at the *dsx* locus in *dsx*$^{0683/1649}$ mutants (Fig. 1I; Supplementary Fig.
160 1F).

161 These results show that Fru$^{M}$ and Dsx$^{M}$ have a more dramatic impact on the transcriptional and chromatin
162 responses to social experience, compared to the effect of Or47b and Or67d pheromone circuits.

## 163 Or47b and Or67d pheromone circuits function antagonistically to influence transcriptional 164 responses to social experience in the central brain

165 Compared to the SH *CS*, we found approximately 130 genes that are transcriptionally up- or
166 downregulated in GH *CS* (Fig. 2A; Fig. 4A, B). These DEGs are involved in many biological processes,
167 especially immunity, metabolism, receptor signaling, and behaviors (Fig. 2B; Fig. 3B; Supplementary
168 Fig. 4A; Supplementary Table 1). We first examined how pheromone circuits drive transcription
169 responses to social experience. Both *Or47b*$^{2/3}$ and *Or67d*$^{GAL4/GAL4}$ mutants showed a significant reduction
170 in the number of DEGs between GH and SH (Fig. 4A-C). *Or47b*$^{2/3}$ mutants predominantly disrupted DEG
171 patterns in response to social isolation (Fig. 2A). In contrast, in *Or67d*$^{GAL4/GAL4}$ mutants, DEG patterns in
172 response to group housing were disrupted (Fig. 2A). We also compared the Gene Ontology (GO) terms of
173 DEGs in GH/SH comparisons of *CS*, *Or47b*$^{2/3}$ and *Or67d*$^{GAL4/GAL4}$ mutants to determine if the two
174 pheromone circuits modulate different biological processes and molecular functions in response to social
175 experience (Fig. 2B). For example, DEGs related to immune functions in *CS* male brains were no longer
176 DEGs in *Or67d*$^{GAL4/GAL4}$ mutant brains (Fig. 2A, B). This suggests that Or67d circuits may contribute to
177 the transcriptional differences in immune genes between grouped and isolated wild-type male brains. In
178 *Or47b*$^{2/3}$ mutants, on the other hand, most specific changes are observed in GO terms for locomotion and
179 hormone activity (Fig. 2A, B).

180 We next investigated how chromatin states changed in response to different social experiences. The
181 overall distribution of either H3K4me3 or RNAPolII did not show any significant change between the

182 GH/SH housing comparison in any genetic background (Fig. 6D), including *CS*, which had the most
183 DEGs. Interestingly, multiple papers over the past two decades indicate that this is a relatively common
184 observation [21, 60, 61]. This discrepancy, where DEGs are present without accompanying changes in
185 chromatin marks, may be due to technical limitations in detecting chromatin level changes that occur only
186 in specific cell populations within the brain. These localized changes might be masked when analyzing
187 chromatin at the global level across the entire brain. Furthermore, the hundreds to thousands of RNA
188 transcripts produced from a single gene in the genome—typically with only two copies in most cells—
189 could also explain the absence of a significant change in chromatin enrichment for H3K4me3 or
190 RNAPolII around DEGs under varying social conditions.

191 The results indicate that Or67d circuits primarily influence transcriptional changes in response to group
192 housing. In contrast, Or47b circuits mainly drive transcriptional responses to social isolation, although
193 their impact on the social modulation of transcriptional profiles in the brain is relatively weaker (Fig. 2L).
194 These data highlight the differences and opposing functions of the two pheromone circuits in
195 implementing the effects of social experience on gene expression throughout the brain.

## 196 The effect of Or47b and Or67d pheromone circuits on gene expression and chromatin state 197 in the brain

198 In addition to investigating the contribution of pheromone circuits in mediating social modulation of
199 transcription, we next examined how pheromone circuits affected overall transcription under the same
200 social condition. When compared to *CS* under both GH and SH conditions, the $Or47b^{2/3}$ mutant changed
201 the expression of many supramolecular and cytoskeletal-related genes (Fig. 2C-F; Supplementary Fig. 5).
202 $Or67d^{GAL4/GAL4}$ mutant, on the other hand, altered the expression of various metabolic processes and
203 defense responses under GH conditions (Fig. 2C-D; Supplementary Fig. 5). Under SH conditions, the
204 $Or67d^{GAL4/GAL4}$ mutant mainly altered the expression of genes in molecular binding and metabolic
205 processes (Fig. 2E-F; Supplementary Fig. 5). Overall, the effect of disrupting Or67d and Or47b function
206 on transcriptional profiles in both GH and SH brains was specific to each pheromone circuit, with little
207 overlap between the mutants (Fig. 2C-F, Fig. 4G and K). These results agree with specific and
208 overlapping roles for each pheromone circuit in mediating distinct cellular and transcriptional responses
209 in the central brain.

210 We next investigated how chromatin states change when different pheromone circuits are disrupted. We
211 did not find significant differentially enriched peaks (DEPs) for H3K4me3 or RNAPolII in male brains
212 when comparing GH/SH housing conditions in any of the genotypes (Fig. 6D). However, loss of *Or47b*
213 or *Or67d* exhibited DEPs for H3K4me3 and RNAPolII when compared to *CS* under either GH or SH
214 conditions (Fig. 2G-K). The effect of Or47b or Or67d circuits on H3K4me3 and RNAPolII enrichment
215 compared to wild type in both GH and SH brains was generally specific (Fig. 2G-J). A small portion of
216 DEPs were altered in the same direction in both mutants, yet to different extents (Fig. 2G-J). To
217 determine the genes with DEPs, we linked the DEPs to the nearest genes (Fig. 2G-K). Next, we
218 performed GO term analysis on the genes with DEPs in $Or47b^{2/3}$ and $Or67d^{GAL4/GAL4}$ mutants compared to
219 wild type in either social condition (Fig. 2K). We found that $Or47b^{2/3}$ mutants altered the RNAPolII
220 distribution around genes involved in channel activity-related processes under the SH condition but not in
221 the GH condition (Fig. 2K). Genes with RNAPolII or H3K4me3 DEPs in $Or67d^{GAL4/GAL4}$ mutants were
222 not enriched for any GO terms.

223 We next extended the analysis to determine if the changes in DEPs were concordant with the changes in
224 DEGs. We compared DEGs to genes with DEPs that changed in the same direction in Or mutants
225 (Supplementary Fig. 2). The number of genes with DEPs was much less than that of DEGs. We identified

226 a few genes exhibiting concordant changes in transcription and enrichment patterns for either active mark.
227 The genes with concordant changes in *Or47b*$^{2/3}$ mutants are involved in various metabolic, catabolic, and
228 intracellular transport processes, and while in *Or67d*$^{GAL4/GAL4}$ mutants, they are involved in enzyme
229 activity, DNA, and chromosome regulation (Supplementary Fig. 2E-F). These results suggest that Or47b
230 and Or67d ORN circuits reprogram chromatin states and transcription around unique sets of genes in the
231 brain, highlighting potential differences in cellular responses driven by different pheromone circuits.

## 232 Fru$^M$ and Dsx$^M$ function antagonistically to influence transcriptional responses to social 233 experience in the central brain

234 The effect of Fru$^M$ or Dsx$^M$ on gene regulation in the nervous system, or their modes of transcriptional
235 regulation, remains unclear. We performed a similar analysis on *fru*$^{LexA/4-40}$ and *dsx*$^{0683/1649}$ mutant brains.
236 First, we investigated whether the function of Fru$^M$ or Dsx$^M$ is required to drive transcriptional changes to
237 social experience observed in the wild type using *fru*$^{LexA/4-40}$ and *dsx*$^{0683/1649}$ mutant males that are either
238 grouped or isolated. The expression pattern of DEGs in GH/SH comparisons in *CS* brains showed that
239 Fru$^M$ and Dsx$^M$ have opposing roles in mediating gene expression with social experience. In *fru*$^{LexA/4-40}$ or
240 *dsx*$^{0683/1649}$ mutants, the transcriptional differences between GH and SH are largely eliminated, albeit in
241 different ways (Fig.3A; Fig.4A-D). Specifically, the transcriptional profile in both GH and SH *fru*$^{LexA/4-40}$
242 mutant brains appeared similar to that of SH *CS* brains (Fig.3A), suggesting that Fru$^M$ contributes to
243 mediating the transcriptional responses to GH. This pattern was opposite in *dsx*$^{0683/1649}$ mutant brains,
244 where, for many genes, transcriptional responses to isolation appeared more significantly disrupted
245 compared to GH (Fig.3A). This result indicates that, in contrast to Fru$^M$, Dsx$^M$ plays a significant role in
246 mediating the transcriptional responses to SH. Fru$^M$ or Dsx$^M$ were previously shown to bind to the
247 regulatory elements of many of the DEGs (Fig. 4D) [35, 50], suggesting that the transcriptional defects
248 observed are likely a direct downstream effect of the loss of Fru$^M$ and Dsx$^M$ function. The fact that some
249 of these DEGs were upregulated and others were downregulated in *fru*$^{LexA/4-40}$ and *dsx*$^{0683/1649}$ mutants also
250 reveals that both transcription factors (TFs) can function as transcriptional repressors or activators in a
251 gene-specific manner (Fig. 4D, H, L; Supplementary Fig. 6). Alternatively, Fru and Dsx proteins can
252 potentially form different homomeric or heteromeric complexes, which determine repressor or activator
253 functions on target genes as well as themselves, and mutations in either *fru* or *dsx* can alter the
254 stoichiometry of these complexes, determining their effects on transcription.

255 The total number of DEGs between GH and SH male brains is significantly lower in *fru*$^{LexA/4-40}$ or
256 *dsx*$^{0683/1649}$ mutants compared to that of wild-type male brains (Fig. 4A-C). A number of GO terms related
257 to metabolism and behavior from DEGs responding to social experience in *CS* were absent in *fru*$^{LexA/4-40}$
258 mutants (Fig.3A-B; Supplementary Table 1). Yet, the DEGs identified between GH and SH in *fru*$^{LexA/4-40}$
259 mutants are enriched in GO terms such as various receptor activities and binding processes (Fig. 3B;
260 Supplementary Fig. 4). The DEGs between grouped and isolated *dsx*$^{0683/1649}$ mutants were not enriched in
261 any GO terms but rather in defense response and cell communication Reactome or KEGG terms (Fig.3B;
262 Supplementary Fig. 4). In addition to the normalization of transcription in response to social experience in
263 *fru*$^{LexA/4-40}$ or *dsx*$^{0683/1649}$ mutants, the distribution of two active chromatin marks, H3K4me3 and
264 RNAPolII, did not show any significant changes between housing conditions in either mutant (Fig. 6D).
265 Overall, these data reveal opposing modes of regulation for Fru$^M$ and Dsx$^M$ in mediating transcriptional
266 responses to grouping and isolation in the brain, respectively (Fig. 3L).

## 267 The effect of Fru$^M$ and Dsx$^M$ function on gene expression and chromatin state in the brain

268 Next, we analyzed changes in transcription in *fru*$^{LexA/4-40}$ and *dsx*$^{0683/1649}$ mutant male brains compared to
269 the wild type under the same housing conditions (Fig. 3C-F), which can include genes directly regulated

by $Fru^M$ and $Dsx^M$. DEGs in GH conditions from $fru^{LexA/4-40}$ mutant/*CS* or $dsx^{0683/1649}$ mutant/*CS* comparison are involved in many metabolic pathways, oxidoreductase activity, structural processes, and RNA processing (Fig. 3C-D; Supplementary Fig. 5). DEGs in SH conditions in $fru^{LexA/4-40}$ mutants include genes involved in supramolecular and defense processes (Fig. 3E-F; Supplementary Fig. 5). In comparison, SH $dsx^{0683/1649}$ mutants changed the expression of genes regulating cell-cell junctions and communication, development of the nervous system, and various signaling pathways (Fig. 3E-F; Supplementary Fig. 5). Overall, transcriptional profiles in both GH and SH brains revealed some genes to be exclusively impacted in either $fru^{LexA/4-40}$ or $dsx^{0683/1649}$ mutants, while others were affected in both mutants. These results suggest that distinct and shared transcriptional responses in the central brain are mediated by $Fru^M$ and $Dsx^M$.

Under the same housing condition, H3K4me3 or RNAPolII displayed differential distribution in $fru^{LexA/4-40}$ or $dsx^{0683/1649}$ mutants compared to *CS* in male brains (Fig. 3G-K; Fig. 6). Genes linked with H3K4me3 or RNAPolII DEPs were enriched in diverse GO terms (Fig. 3K). The $dsx^{0683/1649}$ mutants exhibited varying levels of H3K4me3 enrichment near genes related to DNA catalytic activity compared to *CS* under the GH condition, and showed differential enrichment of RNAPolII around genes involved in mating and reproductive behaviors, developmental and metabolic processes (Fig. 3K). While $fru^{LexA/4-40}$ mutants showed differential enrichment of RNAPolII at genes involved in synapse assembly or organization in the GH condition (Fig. 3K). $fru^{LexA/4-40}$ and $dsx^{0683/1649}$ mutants show both specific and shared influences on chromatin states in each housing condition compared to wild type (Fig. 3G-J). Consistent with their unique and shared roles in transcriptional regulation, these data continue to support distinct housing-specific changes in chromatin profiles around each gene mediated by $Fru^M$ and $Dsx^M$.

The comparison of DEGs and genes with DEPs in $fru^{LexA/4-40}$ and $dsx^{0683/1649}$ mutants relative to *CS* revealed concordant changes at both transcription and chromatin levels, specifically regarding H3K4me3 or RNAPolII enrichment. Notably, a significant number of genes with H3K4me3 DEPs in $dsx^{0683/1649}$ mutants corresponded to changes in transcription levels in the same direction, including many genes related to behaviors and DNA processes (Supplementary Fig. 3). Interestingly, $fru^{LexA/4-40}$ and $dsx^{0683/1649}$ mutants changed both the transcript level and chromatin level for circadian and sleep-related genes, *per* and *sand* (Supplementary Fig. 3; Fig. 7). Strikingly, the *sand* gene displayed dramatically decreased H4K3me3 enrichment and transcript level in $fru^{LexA/4-40}$ and $dsx^{0683/1649}$ mutants, with the RNAPolII binding level remaining the same (Supplementary Fig. 9). This might suggest either transcriptional priming or RNAPolII pausing at the *sand* gene locus in $fru^{LexA/4-40}$ and $dsx^{0683/1649}$ mutants, possibly due to the lack of H3K4me3 enrichment.

## Or47b circuit functions through $Dsx^M$ in response to social isolation, whereas Or67d circuit functions through $Fru^M$ in response to group housing

Our analyses have shown that $Or47b^{2/3}$, $Or67d^{GAL4/GAL4}$, $fru^{LexA/4-40}$, and $dsx^{0683/1649}$ mutants all normalized the transcriptional differences between GH and SH comparison in unique ways (Fig. 2-4; Supplementary Fig. 7). Activation of pheromone-sensing neurons in the olfactory system activates ensembles of neurons within circuits driving social behaviors. This circuit activation can engage the functions of $Fru^M$ and $Dsx^M$ transcription factors expressed in a pheromone circuit-specific way to mediate the transcriptional and chromatin effects of social experience in the brain. To determine the neural and transcriptional cascades involved in this process and identify shared genes that are co-regulated by each pheromone circuit alongside $Fru^M$ and $Dsx^M$, we compared the DEGs and DEPs in each mutant (Fig. 4-6).

All mutants decreased the number of social experience-dependent DEGs in unique ways (Fig. 4A-D). In both $Or47b^{2/3}$ and $dsx^{0683/1649}$ mutants, the patterns of DEGs in response to social isolation are disrupted

314 (Fig. 4D). This suggests that the Or47b circuits and $Dsx^M$ are important for driving transcriptional
315 responses to isolation. Notably, *dsx*$^{0683/1649}$ mutants show a more significant defect in social experience-
316 induced transcriptional changes, both in terms of the number of genes affected and the level of
317 transcriptional change observed (Fig. 4A-D). In contrast, in *Or67d*$^{GAL4/GAL4}$ and *fru*$^{LexA/4-40}$ mutants,
318 transcriptional responses to group housing were largely disrupted (Fig. 4D), suggesting that Or67d
319 circuits and $Fru^M$ drive transcriptional responses to group housing. Again, *fru*$^{LexA/4-40}$ mutants exhibited a
320 stronger defect in transcription. Notably, *Dsk*, which encodes a neuropeptide involved in regulating
321 aggression and suppressing courtship behaviors [15, 16], is the only gene statistically unaltered between
322 grouped and isolated male brains across all wild-type and mutant genotypes (Fig. 4C; Supplementary Fig.
323 1D).

324 Comparing each mutant to *CS* under the GH condition revealed that *Or47b*$^{2/3}$ mutants showed the least
325 number of DEGs among other mutants (Fig. 4E-F). In contrast, under the SH condition, *Or67d*$^{GAL4/GAL4}$
326 mutants showed the least number of DEGs compared to *CS* (Fig. 4I-J). With respect to wild-type brains,
327 *fru*$^{LexA/4-40}$ mutants or *dsx*$^{0683/1649}$ mutants always had more dramatic effects on transcriptional profiles
328 compared to Or mutants (Fig. 4E-L), supporting the hypothesis that *fru*- or *dsx*-positive central circuits
329 likely integrate information from multiple pheromone circuits unexplored in this study.

330 We next aimed to determine whether we could identify functionally shared or segregated pathways
331 connecting different pheromone circuits to the $Fru^M$ and $Dsx^M$ transcriptional functions in the central
332 brain. We compared the DEGs in Or mutants to those in TF mutants versus *CS*. *Or47b*$^{2/3}$ mutants had a
333 large number of DEGs that uniquely overlapped (268) with *dsx*$^{0683/1649}$ mutants under the SH condition
334 (Fig. 4K). Meanwhile, a large number of DEGs in *Or67d*$^{GAL4/GAL4}$ and *fru*$^{LexA/4-40}$ mutant brains uniquely
335 overlapped (197) in the GH condition (Fig. 4G). Among all the DEGs from comparisons of any mutant
336 versus *CS*, a notable number showed altered expression in the same direction across all four mutants, or
337 exhibited unique directional changes in specific mutants (Fig. 5). A significant number of the remaining
338 DEGs showed interesting overlaps in expression patterns between Or (*Or47b* or *Or67d*) and TF (*fru*$^M$ or
339 *dsx*$^M$) mutants. We generally observed a trend indicating that, in both GH and SH conditions, the
340 transcriptional effects on DEGs seen in *Or47b*$^{2/3}$ mutants are largely similar to those in *dsx*$^{0683/1649}$
341 mutants, and occasionally to *fru*$^{LexA/4-40}$ mutants (Fig. 5 gray frames). We saw opposing trends with
342 *Or67d*$^{GAL4/GAL4}$ mutants, where the transcriptional effects generally aligned with *fru*$^{LexA/4-40}$ mutants, and
343 occasionally with *dsx*$^{0683/1649}$ mutants (Fig. 5 gray frames).

344 DEGs that changed in various combinations of directions were enriched in distinct GO terms (Fig. 5).
345 Though DEGs showed enrichment for specific biological processes, molecular functions or cellular
346 components in each mutant, under both housing conditions, DEGs in *Or47b*$^{2/3}$ and *dsx*$^{0683/1649}$ mutants
347 shared many of the top ranked GO and KEGG terms (Supplementary Fig. 5). In contrast, DEGs in
348 *Or67d*$^{GAL4/GAL4}$ and *fru*$^{LexA/4-40}$ mutants showed overlaps in GO and KEGG terms (Supplementary Fig. 5).
349 These results highlighted potential functional connections between Or47b and $Dsx^M$ neuronal circuits,
350 particularly in isolated males, and between Or67d and $Fru^M$ neuronal circuits especially in grouped males,
351 suggesting that Or47b circuits might communicate with *dsx*- positive neurons in response to social
352 isolation whereas Or67d circuits might function through *fru*-positive neural circuits in response to social
353 enrichment.

354 At the chromatin level, genes with H3K4me3 or RNAPolII DEPs were mostly unique to each mutant,
355 showing modest overlap with other genotypes (Fig. 6A-B). These genes were associated with distinct GO
356 terms that were more genotype-specific (Fig. 6C). Figure 7 summarizes genes exhibiting concordant
357 changes in both transcription and chromatin state (H3K4me3 or RNAPolII) in the same direction in each
358 genotype. These findings suggest that transcriptional changes in these genes may be driven, at least in

359 part, by broader chromatin remodeling events in the male brain induced by activation of different
360 pheromone circuits in response to social experience, as well as the functions of $Fru^M$ and $Dsx^M$.

## 361 Differential effects of pheromone circuits, $Fru^M$, and $Dsx^M$ on the social modulation of 362 $Fru^M/Dsx^M$ target gene expression

363 Our results suggest that prolonged activation of pheromone circuits impacts the transcriptional activity of
364 $Fru^M$ or $Dsx^M$-expressing neurons associated with sex-specific and/or social behaviors. This likely leads
365 to changes in the expression of $Fru^M$ and/or $Dsx^M$ downstream target genes, contributing to the social
366 modulation of behaviors. A comparison of $Fru^M$ and $Dsx^M$ binding sites in the genome revealed overlap
367 among the genes regulated by either $Fru^M$ or $Dsx^M$ (Fig. 4D, H, L; Supplementary Fig. 6A-B) [35, 50].
368 Our analysis identified genes that are exclusively regulated/co-regulated by $Fru^M$ and $Dsx^M$
369 (Supplementary Fig. 6), which have various functions, including neural development and function, as
370 well as modulation of circadian rhythms, metabolic processes, and signaling pathways [35, 50].

371 We first analyzed the effect of housing on $Fru^M$ and $Dsx^M$ target DEGs, and how they changed in $fru^{LexA/4-}$
372 $^{40}$ mutants and $dsx^{0683/1649}$ mutants. We found that many of the $Fru^M/Dsx^M$ target genes were misexpressed
373 primarily under one housing condition. Generally, $fru^{LexA/4-40}$ mutants exhibited defects in GH, while
374 $dsx^{0683/1649}$ mutants showed defects in SH (Supplementary Fig. 6A). These DEGs contain binding sites for
375 either $Fru^M$ or $Dsx^M$, or both, in their regulatory elements (Supplementary Fig. 6A). Under the same
376 housing conditions, some of the target DEGs were either up- or down-regulated in the same direction in
377 both mutants, albeit generally with one mutant having a more dominant effect (Supplementary Fig. 6B-
378 D). While many co-regulated DEGs showed changes in the same direction, others were affected in
379 opposite directions (Supplementary Fig. 6B-D). Some of the target DEGs were regulated exclusively by
380 either $Fru^M$ or $Dsx^M$ (Supplementary Fig. 6B-D). These data suggest that social experience-induced
381 changes in $Fru^M/Dsx^M$ target DEGs are mainly influenced by $Fru^M$ in group-housed conditions, while
382 $Dsx^M$ primarily drives responses to social isolation. Furthermore, they reveal both similarities and
383 differences in the mode of transcriptional regulation between $Fru^M$ and $Dsx^M$ on the expression of their
384 target genes, where both can function as activators or repressors of transcription.

385 To map the neural pathways from the olfactory pheromone-sensing neurons to the regulation of $Fru^M$,
386 $Dsx^M$ and their target DEGs in the central brain, we compared the log2 fold change of these DEGs in
387 $Or47b^{2/3}$ and $Or67d^{GAL4/GAL4}$ mutants as well. The effects of $Or47b^{2/3}$ and $Or67d^{GAL4/GAL4}$ mutants on the
388 $Fru^M/Dsx^M$ target DEGs were not as dramatic as $fru^{LexA/4-40}$ or $dsx^{0683/1649}$ mutants (Supplementary Fig. 6B-
389 D). We identified some DEGs that were either altered in the same direction in all 4 mutants, or in the
390 opposite direction only in a single mutant (Supplementary Fig. 6C-D). Similar to prior results, in both GH
391 and SH conditions, $Or47b^{2/3}$ mutants show transcriptional effects on target DEGs similar to $dsx^{0683/1649}$
392 mutants, and occasionally with $fru^{LexA/4-40}$ mutants (Supplementary Fig. 6C-D). In contrast, $Or67d^{GAL4/GAL4}$
393 mutants generally align with $fru^{LexA/4-40}$ mutants, and occasionally $dsx^{0683/1649}$ mutants (Supplementary Fig.
394 6C-D). These results are in agreement with the results above that suggest Or47b and Or67d circuits
395 predominantly work through $Fru^M$ and $Dsx^M$, respectively.

396 In an accompanying study, we discovered that social experience influences the expression of circadian-
397 related genes (i.e., *cry, Clk, Pdf,* and *Sik3*) in the central brain [45]. Interestingly, many of these circadian
398 genes are $Fru^M/Dsx^M$ target genes with $Fru^M$ and/or $Dsx^M$ binding in their upstream regulatory elements
399 [35, 50]. Due to the pleiotropic effects of the circadian system on behavior and physiology, social
400 experience-dependent alterations in $Fru^M/Dsx^M$ transcriptional cascades and the expression of
401 downstream target circadian genes may drive the impact of social experiences on behaviors. Indeed,
402 knockdown of $fru^M$ and mutants in various circadian genes either amplified or reduced the effects of

403 group housing-driven changes in courtship [35, 50]. These results indicate that changes in circadian gene
404 expression, influenced by social experience, may arise as a consequence of alterations in the levels or
405 functions of $Fru^M$ or $Dsx^M$. To test this hypothesis and more strictly temporally control transcriptional
406 changes in circadian genes, we sampled RNA at ZT7-8 from grouped and isolated male heads in different
407 mutants and performed qRT-PCR for *Clk, tim,* and *cry* with control genes (*TSG101*, *RpS29*, *Hmt4-20*, and
408 *Dsk*). We found that expression of *Clk* and *cry* trended towards an increase in response to isolation
409 (Supplementary Fig. 8A) [45]. Under both social conditions, *tim* and *cry* showed a decrease in $dsx^{0683/1649}$
410 mutants (Supplementary Fig. 8B). $Or67d^{GAL4/GAL4}$ mutants showed the strongest decrease in the expression
411 of *cry*, in the same direction as observed in $dsx^{0683/1649}$ mutants (Supplementary Fig. 8B). $dsx^{0683/1649}$
412 mutants also showed a slight increase in *Clk* expression in GH brains, which eliminated the difference in
413 *Clk* expression observed between GH and SH *CS* brains (Supplementary Fig. 8A-B). In contrast, in
414 $fru^{LexA/4-40}$ mutants, *cry* expression decreased in SH brains, while *tim* expression increased in GH brains
415 (Supplementary Fig. 8B). These results suggest that $Fru^M$ and $Dsx^M$ differentially regulate their target
416 genes, i.e., circadian genes, in the head, and social experience can modulate the expression of the
417 circadian genes in response to the differential activation of different pheromone circuits and
418 transcriptional function of $Fru^M$ and $Dsx^M$.

## 419 Differential effects of pheromone circuits, $Fru^M$, and $Dsx^M$ on social modulation of
## 420 behaviors

421 Considering the transcriptional changes in the brains of mutants of *Or67d*, *Or47b*, $fru^M$, and $dsx^M$, we
422 wanted to explore their effects on the social modulation of behaviors. Disrupting the function of Or47b or
423 Or67d affects the detection of diverse social cues, while loss of $Fru^M$ and $Dsx^M$ function disrupts the
424 processing of social signals in the central social circuits [38, 53, 56-59, 62]. Previous studies have shown
425 that social experience can reset the circadian clock in *Drosophila*, and grouped *CS* males show increased
426 sleep and decreased locomotion compared to isolated *CS* males [10, 31, 63]. Therefore, we assessed sleep
427 and locomotion activity in $Or47b^{2/3}$, $Or67d^{GAL4/GAL4}$, $fru^{LexA/4-40}$, and $dsx^{0683/1649}$ mutants to determine their
428 contribution to social experience-dependent changes in behaviors (Fig. 8; Supplementary Fig. 10). We
429 confirmed that GH *CS* males increased sleep and decreased locomotion compared to SH *CS* males (Fig.
430 8A-D). Both $fru^{LexA/4-40}$ and $dsx^{0683/1649}$ mutants diminished the differences in total sleep and locomotion
431 between GH and SH males (Fig. 8A-D), indicating that they both play a role in the social modulation of
432 these behaviors. In $dsx^{0683/1649}$ mutants, such elimination resulted from both a decreased GH sleep level
433 and an increased SH level (Supplementary Fig. 10A-D). In $fru^{LexA/4-40}$ mutants, the sleep pattern showed a
434 general decline in GH (Supplementary Fig. 10A-D). We also noted further differences in the transition of
435 sleep from nighttime to daytime in $fru^{LexA/4-40}$ mutants (Fig. 8A). In particular, the activity bout at the
436 transition from nighttime to daytime was not disrupted in $fru^{LexA/4-40}$ mutants as in other genotypes (Fig.
437 8A). This also agrees with their locomotion performance, in which $fru^{LexA/4-40}$ mutants showed an overall
438 decrease in activity peaks in the transition, and a less significant difference in locomotion between SH
439 and GH flies (Fig. 8C). $Or47b^{2/3}$ and $Or67d^{GAL4/GAL4}$ mutants did not dramatically alter sleep patterns or
440 trends between GH and SH (Fig. 8A-B). However, $Or47b^{2/3}$ mutants decreased locomotion in SH flies
441 (Fig. 8C-D; Supplementary Fig. 10E-H). This result aligns with transcriptional disruptions in genes
442 involved in locomotion of $Or47b^{2/3}$ mutants in SH brains (Fig. 2B). These findings suggest that $Fru^M$ and
443 $Dsx^M$ play essential but distinct roles in the social modulation of sleep and locomotion behaviors in
444 isolated and grouped flies, with a potential role for Or47b in mediating changes in locomotion in SH flies.

445 Since Or47b, Or67d, $Fru^M$, and $Dsx^M$ circuits are all related to courtship behaviors, we also performed
446 single-pair male-female courtship assays to determine their effect on social modulation of courtship.
447 Previous studies and our accompanying study showed that $fru^M$ and $dsx^M$ are necessary for normal

courtship behaviors in male flies, and *fru*$^{M}$ expression in the central brain neurons is necessary for mediating the social experience-induced changes in male courtship behaviors [45]. The role of *fru*$^{M}$ regulation with social experience is diverse: in some neurons, altered Fru$^{M}$ function enhances courtship behavior in SH males, while in other neurons it decreases courtship in response to GH [45]. Since social experience can influence gene regulation and behavior, and given that mutations in *Or47b* and *Or67d* affect the transcriptional profile and chromatin state of wild-type brains, we investigated whether either the Or47b or Or67d pheromone circuits play a role in how social experience modulates courtship behaviors. We quantified male courtship behaviors in *CS*, *Or47b*$^{2/3}$ and *Or67d*$^{GAL4/GAL4}$ mutants (Fig. 8E). Loss of *Or47b* function increased courtship in GH mutant males, though it did not completely eliminate the difference between GH and SH (Fig. 8E). The *Or67d*$^{GAL4/GAL4}$ mutants did not alter the courtship vigor in either social context (Fig. 8E). These results suggest that Or47b circuits contribute to courtship suppression in grouped males. Overall, our results suggest unique contributions of olfactory pheromone pathways and transcription factors critical for integrating social information on transcriptional and chromatin profiles are accompanied by equally unique effects on social modulation of various behaviors.

# Discussion

In this study, we investigated how transcription patterns and chromatin marks in the brains of male *Drosophila* change in response to social experience. To determine the neural and transcriptional pathways responding to social experience, we examined mutants in two pheromone receptors, Or47b and Or67d, along with two transcription factors, Fru$^{M}$ and Dsx$^{M}$, that influence sex-specific and social behaviors. The results show that *Or47b*$^{2/3}$, *Or67d*$^{GAL4/GAL4}$, *fru*$^{LexA/4-40}$, and *dsx*$^{0683/1649}$ mutants significantly reduced the transcriptional responses to social experience, suggesting that social modulation of gene expression involves all four genes and their associated circuits. *Or47b*$^{2/3}$ and *Or67d*$^{GAL4/GAL4}$ mutants exhibited opposing effects on DEGs that are altered by social experience in wild-type. Similar opposing functional effects were also observed in *fru*$^{LexA/4-40}$ and *dsx*$^{0683/1649}$ mutants. We found functionally segregated transcriptional cascades from individual pheromone circuits that engaged the function of either Fru$^{M}$ or Dsx$^{M}$ in the central brain. Mainly, we found that Or47b circuits generally function through Dsx$^{M}$ in response to isolation, while Or67d circuits work in group-housed males through Fru$^{M}$ to mediate social experience-dependent modulation of transcriptional responses. The RNA profiles from the brains of *fru*$^{LexA/4-40}$ and *dsx*$^{0683/1649}$ mutants also helped us identify both activation and repression functions for Fru$^{M}$ and Dsx$^{M}$, in addition to their shared and exclusive functions in mediating their effects on gene expression and behaviors in different housing conditions. And finally, we found that social experience-dependent changes in sleep and locomotion are generally eliminated in *fru*$^{LexA/4-40}$ and *dsx*$^{0683/1649}$ mutants, with moderate effects of pheromone receptors on some of the behaviors under certain social conditions. Given that many of the DEGs are direct targets of Fru$^{M}$ and/or Dsx$^{M}$, our study reveals how changes in social and pheromone circuit activity with social experience can alter transcriptional cascades reprogramming brains and behaviors.

One surprising finding from our study is that, although we identified DEGs between grouped and isolated male brains of wild-type flies, we did not observe dramatic changes in chromatin patterns of H3K4me3 and RNAPolII across any of the genotypes under different housing conditions. However, we detected numerous changes to the enrichment of H3K4me3 and RNAPolII in each mutant compared with wild-type brains under the same housing conditions. Some of these changes in chromatin were accompanied by concordant changes at the transcription level. Combinatorial function of multiple histone modifications together with RNAPolII is thought to be intimately connected to transcriptional states [64-66]. For instance, H3K4me3 and H3K27ac are considered marks for actively transcribed genes, while H3K27me3 is enriched around transcriptionally repressed genes [66-70]. However, many studies have also shown

discrepancies between the enrichment of specific chromatin marks and transcription responses [21, 60, 61]. For example, circadian analysis of transcriptional and chromatin states in mammalian cells throughout the day revealed that such correlations might represent an oversimplified view [71]. This study found that while both transcription and chromatin states displayed circadian patterns, numerous genes exhibited changes in chromatin patterns without affecting transcription levels and vice versa. Comparison of transcriptional changes and histone marks in dopaminergic neurons in flies raised in grouped versus isolated conditions also revealed only a small correlation in a limited number of genes [21]. They also discovered that many highly expressed genes showed unexpected increases in the enrichment of the repressive H3K27me3 mark, alongside decreases in active chromatin marks [21]. Similarly, chromatin accessibility and gene expression in *fru*+ versus *fru*- cells sorted from male and female fly brains show little overlap [60].

There are several explanations for the discrepancy we and others observe in transcript levels and chromatin mark enrichment. One possibility is cellular heterogeneity. Changes in chromatin states may occur in a small subset of cells in response to social experience. However, when analyzing the whole brain, these subtle changes can be obscured by other cells where such changes are not happening. Single-cell multiome ATAC and gene expression profiling techniques can simultaneously determine gene expression and chromatin landscape from the same cell, potentially increasing the resolution and confidence of molecular analysis [72]. An alternative explanation considers the transcriptional and chromatin states of a gene as it transitions through active, repressed, and poised states. Each of these states may be triggered by complex combinations of chromatin marks that accumulate around genes as cells respond to various signals and pathways. Transitioning between active and repressed transcriptional states may involve intermediate states as chromatin modifications build up around responsive genes. These conditions could lead to intermediate states where transcription decreases alongside an increase in repressive marks, while active marks remain unchanged. Conversely, transcription might increase due to the loss of a repressive mark, rather than the gain of an active mark. In this study, we only investigated two active marks, yet marks for repressed/closed chromatin are also crucial for regulating gene expression. Studies on mammalian cells demonstrated that the promoters of some transcriptionally repressed genes were marked with both active (H3K4me3) and repressive (H3K27me3) epigenetic marks [73]. These and numerous other studies indicate that chromatin states, as signaled by histone marks or accessibility, only offer a probabilistic prediction of transcription [74, 75]. Changes in gene expression result from the combined regulation of various factors, including chromatin accessibility, histone modifications, transcription factors, and post-transcriptional modifications [74, 75]. Comprehensive studies that analyze gene expression across all features will lead to a better understanding.

Despite the discrepancy in chromatin and transcriptional states, we did identify a small list of genes with concordant changes in transcript levels and active chromatin mark enrichment, like the *sand* gene. The *sand* gene, which encodes a KCNK18/TRESK homolog potassium channel, plays a crucial role in regulating various aspects of *Drosophila* behavior and physiology. It is involved in sleep regulation within the dorsal fan-shaped bodies [76] and cardiac function [77]. Additionally, it has been shown to operate in cortical glial cells to help control stress-induced seizures [78]. Considering the important role of the *sand* gene and the notable alterations in its transcriptional and chromatin state in $fru^{LexA/4\text{-}40}$ and $dsx^{0683/1649}$ mutants, it is possible that Sand may play a role in mediating some of the impaired neural, behavioral, and physiological processes observed in these mutants. Further studies are necessary to explore this hypothesis.

In a related paper, we demonstrated that the expression of $fru^{M}$ and core circadian genes, which are potentially regulated by $Fru^{M}$ and $Dsx^{M}$, is altered by social experience and essential for changes in

538 courtship behaviors [45]. These results suggest that social experience might exert its effect on behavioral
539 modulation through changes in circadian state. In this study, we further strengthen these findings by
540 highlighting the roles of both $Fru^M$ and $Dsx^M$ in mediating the effects of social experience on additional
541 behaviors known to be under circadian control, such as sleep and locomotion. We discovered that the
542 Or47b circuits influence the social modulation of locomotion and courtship behaviors in isolated and
543 grouped flies, respectively, correlating with changes in genes that mediate these behaviors in *Or47b*$^{2/3}$
544 mutant brains. Importantly, we identified the different effects of Or47b and Or67d pheromone circuits and
545 how they interact with the functions of $Fru^M$ and $Dsx^M$ to regulate their target genes under various social
546 conditions. Given that the regulation of circadian gene networks is complex and participates in the
547 modulation of various behaviors through changes in arousal states, understanding the diverse impacts of
548 $Fru^M$ and $Dsx^M$ on social modulation of target circadian gene expression in the brain is both important and
549 challenging. $Fru^M$, $Dsx^M$, and various circadian genes are expressed in different neuronal and non-
550 neuronal (glial and fat body) cell populations in the brain in different combinations. The cellular location
551 of social experience-dependent changes in $Fru^M$ and $Dsx^M$ function and target gene expression remains
552 unknown. Further studies are essential to identify the specific cells in which $Fru^M$ and $Dsx^M$ mediate
553 transcriptional differences in circadian genes in response to social experience. We anticipate that these
554 studies will provide a clearer understanding of how social experience modifies the complex gene
555 regulatory networks for circadian genes downstream of $Fru^M$ and $Dsx^M$ and how these modifications alter
556 behaviors.

557 Overall, our study uncovers how social experience alters transcription and chromatin states in the brain,
558 and provides insights into the roles played by pheromone circuits and transcription factors that influence
559 molecular and behavioral responses to social experience. In addition to modulating behavioral responses,
560 social experience and circadian pathways also influence many physiological responses. These are clearly
561 represented in our transcriptome dataset as social experience-dependent DEGs implicated in immune
562 responses, metabolism, signal transduction, homeostasis, and developmental pathways. The activity of
563 pheromone circuits, along with the functions of $Fru^M$ and $Dsx^M$, may also play a role in how social
564 experience influences physiological responses through changes in downstream target genes. Future
565 studies will reveal how social experience impacts additional behavioral and physiological responses, and
566 the effects of different pheromone circuits (from both olfactory and gustatory systems), as well as
567 transcriptional and circadian pathways, on these responses.

# 568 Methods

## 569 Drosophila strains and husbandry

570 Flies were raised on standard fly food from Archon Scientific Company at room temperature. Male flies
571 used in all experiments were 7-day-old. For male-female single-pair courtship behavior assays, unmated
572 males were used as the test fly, and 6-day-old $w^{1118}$ virgin females were used as the target fly. Specific
573 genotypes are listed here [79]: wild-type *CS* (*Canton-S*), *Or47b* mutant ($w^+/Y$; *Or47b*$^{2/3}$;+/+) [80], *Or67d*
574 mutant ($w^+/Y$; +/+; *Or67d*$^{GAL4/GAL4}$) [59], *fru* mutant ($w^+/Y$; +/+; *fru*$^{LexA/4-40}$) [43, 81], and *dsx* mutant ($w^-$
575 /Y; +/+; *dsx*$^{f00683\text{-}d07058/f01649\text{-}d09625}$) [82].

## 576 Social isolation and group housing setup

577 Dark pupae (80–100 hours old) were picked out and separated by sex. For the social isolation (single
578 housing, SH) condition, each pupa was placed into individual vials, allowed to eclose alone, and aged to 7
579 days. For the group housing (GH) condition, more than 30 pupae of the same sex were collected and
580 placed into food vials, and 30 newly eclosed and unmated flies with correct genotypes were collected and

581 put in a fresh new vial the next day of the collection day (day 0). All $w^{1118}$ virgin females used for the
582 courtship assay were raised under GH conditions.

## 583 Bulk tissue RNA sequencing

584 **Brain dissection and RNA extraction:** Fly brains were dissected in cold RNase-free 1X PBS and
585 transferred immediately to RNAlater (Sigma-Aldrich, R0901) on ice. Each genotype under the GH or SH
586 conditions has three biological replicates, and each biological replicate contains 30 male fly brains. After
587 the dissection, RNAlater with the sample was diluted 1:2 with 1X RNase-free PBS and centrifuged for 1
588 min at max speed, and the supernatant was removed. Brain samples were washed with 1 mL 1X RNase-
589 free PBS and centrifuged for 1 min at max speed. The supernatant was removed and replaced with 300 µL
590 of TRIzol reagent (Invitrogen, 15596026). To extract RNA, samples were ground sufficiently and
591 processed with QIAGEN Shredder kit (QIAGEN, 79654), and RNeasy Mini Kit (QIAGEN, 74104).
592 DNase I (TURBO DNA-free Kit, Invitrogen, Thermo Fisher Scientific AM1907) was applied to remove
593 genomic DNA after the RNA extraction.

594 **Library preparation and sequencing:** Around 500 ng total RNA in each sample was prepared for
595 library preparation. KAPA Stranded mRNA-Seq Kit was used to generate mRNA libraries. Libraries were
596 sequenced on the NextSeq 2000 sequencer, generating 75-bp or 100-bp paired-end reads.

597 **Bulk RNA sequencing data analysis:** Raw fastq files were processed with the standard nf-core/rnaseq
598 pipeline [83]. Briefly, reads were mapped to the *Drosophila melanogaster* BDGP6.46 genome with
599 STAR, and read counts were generated using the Salmon method. The following analysis was performed
600 in R [84, 85]. DEG analysis was performed with DESeq2 [86]. All control and mutant samples were
601 incorporated into a single DESeqDataSet (dds) object for differential expression analysis. The Wald
602 test was applied to the comparison between GH and SH of the same genotype, and the comparison
603 between mutant and *CS* under the same housing conditions. Gene ontology (GO), Reactome, and KEGG
604 analyses were performed using the clusterProfiler library [87].

## 605 CUT&RUN

606 **Brain dissociation and chromatin immunoprecipitation:** Fly brains were dissected in dissection buffer
607 (Schneider′s Insect Medium, Sigma-Aldrich S0146 with 1% BSA) and transferred into cold dissection
608 buffer in 1.5 ml tubes on ice. Each genotype under the GH or SH conditions has three biological
609 replicates. For H3K4me3 samples, fifteen male brains were used per sample, and for RNAPolII samples,
610 thirty male brains were used per sample. After completing the dissection, the old dissection buffer was
611 replaced with a fresh one. Collagenase Type I (Gibco, 17018029) stock solution was added directly into
612 samples for a final 2mg/mL concentration. Samples were incubated at 37 °C for 20 minutes. During the
613 incubation, samples were dissociated by gentle pipetting 20-30 times every 7 min with P200. The
614 collagenase solution was removed after centrifugation at 300 xg for 5 min. Cells were washed with wash
615 buffer supplied in CUTANA™ ChIC/CUT&RUN Kit (EpiCypher, 14-1048), and were centrifuged at 300
616 xg for 5 min. The old wash buffer was replaced with fresh wash buffer, and the cells were resuspended
617 and processed according to the instructions in the user manual. 1 ul of antibody (anti-H3K4me3
618 antibodies supplied by the CUT&RUN Kit, and anti-RNAPolII antibody from MilliporeSigma 05-623)
619 was added per sample.

620 **Library preparation and sequencing:** CUTANA™ CUT&RUN Library Prep Kit (EpiCypher, 14-1001,
621 14-1002) was used to prepare libraries. The quality of libraries was examined by the D5000 ScreenTape
622 station. Libraries were sequenced on the NextSeq 2000 sequencer, generating 75-bp paired-end reads.

**CUT&RUN data analysis:** Raw fastq files were processed using the standard nf-core/cutandrun pipeline [83]. Briefly, reads were trimmed by Trim Galore and aligned to the *Drosophila melanogaster* dmel-all-chromosome-r6.45 genome with Bowtie 2. Peak calling was performed by MACS2. The following analysis was performed in R [84, 85]. Counts of peaks were generated by csaw::regionCounts [88, 89]. DEP analysis was performed with DESeq2 [86]. All control and mutant samples were incorporated into a single DESeqDataSet (dds) object for DEP analysis. Wald test was applied to the comparison between GH and SH of the same genotype, and the comparison between mutant and *CS* under the same housing conditions. ChIPseeker was used to annotate DNA peaks with genes. Gene ontology (GO), Reactome, and KEGG analyses were performed using the clusterProfiler library [87].

## Quantitative reverse transcription PCR (RT-qPCR)

The RT-qPCR protocol was modified based on the previous protocol [90]. For each housing condition, three to four biological replicates were prepared separately, with each replicate containing 15-20 heads from male flies. Heads were dissected at ZT7-ZT8 on fly pads and immediately transferred into 300 ul TRIzol (Invitrogen, 15596026) on ice. RNA extraction and DNase treatment were as described above. cDNA was synthesized through reverse transcription reaction using approximately 200 ng of total RNA with the SuperScript IV First-Strand Synthesis Kit (Invitrogen, 18091050) and poly d(T) primers. qPCR was performed using the FastStart Essential DNA Green Master kit (Roche, 06924204001) on LightCycler® 96 instrument (Roche, 05815916001). Primers used were described in another study [45]. The expression level was calculated by the ΔCt method using *TSG101* as the standard gene. The calculation was performed in R. For each genotype, relative expression between GH and SH male flies was compared using two-sided independent t-tests. For each housing condition, differences among genotypes were assessed using one-way ANOVA followed by Tukey's HSD post-hoc test for pairwise comparisons. In both analyses, raw p-values were adjusted for multiple testing across all comparisons using the Benjamini–Hochberg procedure to control the false discovery rate. Adjusted p-values (*p.adj*) were used to determine statistical significance.

## Male-female single-pair courtship behavior assay setup and analysis

Courtship behavior assays were performed during ZT 7 – ZT 10 as described [45]. Briefly, one unmated male and one virgin $w^{1118}$ female were gently aspirated to each arena of an unused agarose chamber, separated by barriers before the start of the recording. When all flies were loaded, the chamber was placed under a camera with a stable, consistent light source. Barriers were removed to allow the two flies to meet each other, and video recording was started immediately. The trajectory of each fly was tracked by FlyTracker [91] on MATLAB. Fly identity and the start time of copulation were determined manually. JAABA was trained and used to automatically annotate other courtship behaviors [92]. For each courtship behavior video, only the first 10 minutes were used for the scoring of the courtship index (CI) (copulation time included):

CI = (the time a male fly spends on courtship)/(10 min)

## Sleep and locomotion behavior assay setup and analysis

Flies were grouped or isolated for 7 days, and then loaded individually into glass tubes containing food. The activity of each fly was monitored using the Drosophila Activity Monitor (DAM) system (TriKinetics, Waltham, MA) at 25 °C under 12-h light-dark (LD) cycles. The DAM system records activity counts $Ct(t)$ (the number of counts each fly passes the beam) every 30 seconds for two days after the loading to capture one full LD cycle.

Sleep is defined as 5 consecutive minutes or longer with no beam passing.

$$S(t) = \begin{cases} 1, if\ S(t-1) = 1\ and\ Ct(t) = 0 \\ 1, if \sum_{i=t}^{t+10} Ct(i) = 0 \\ 0, otherwise \end{cases}$$

Sleep traces (%) were generated by averaging the sleep data over a duration of 60 minutes.

$$Sc(t) = \frac{1}{120} \sum_{i=t-60}^{t+60} S(i) * 100$$

Locomotion traces (counts) were generated by averaging the activity counts over a duration of 60 minutes.

$$Lc(t) = \frac{1}{120} \sum_{i=t-60}^{t+60} Ct(i)$$


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# Author contributions

Conceptualization: CD and PCV. Investigation: CD, LJ, and SS with the help from SO and SR. Analysis/interpretation of data: CD, JESF, LJ, CDJ, and PCV. Manuscript—original draft: CD and PCV.

# Figures

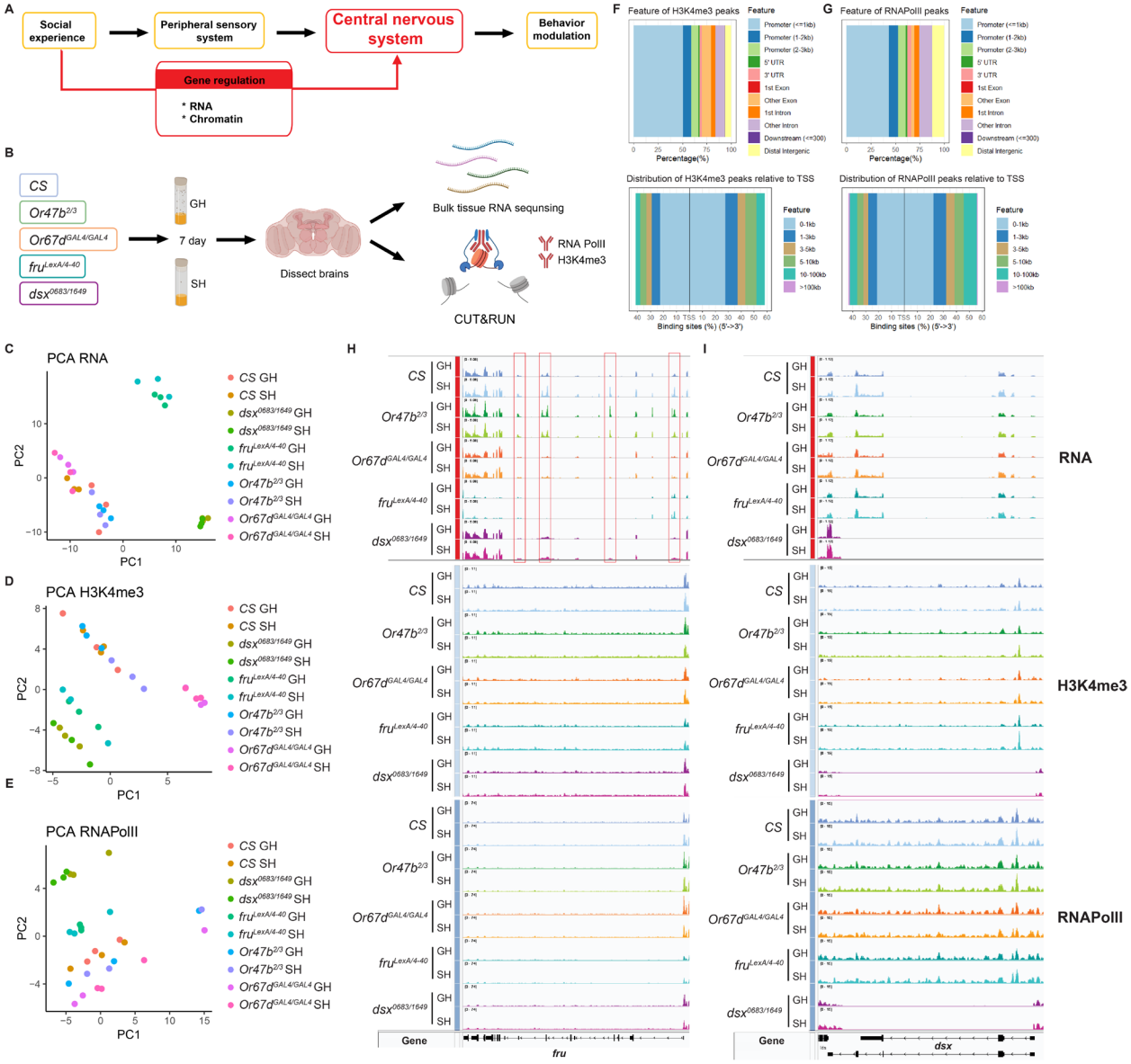
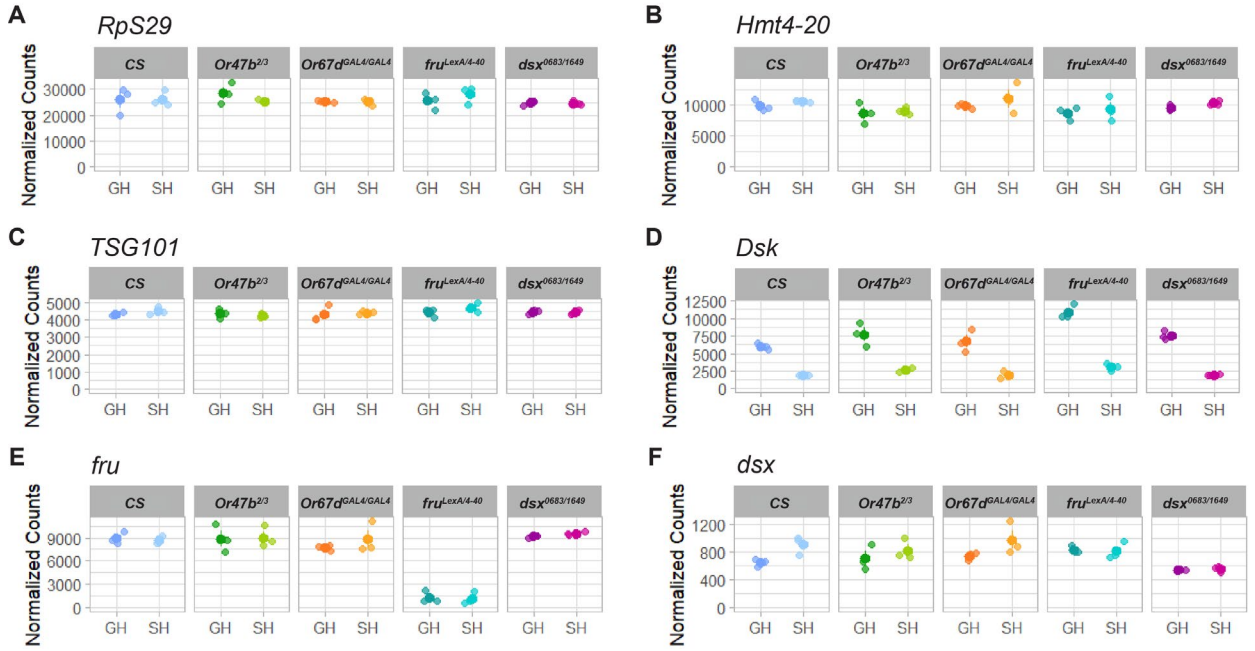



**Figure 1. The effect of *Or47b, Or67d, fru$^{M}$*, and *dsx$^{M}$* mutants on transcriptional and chromatin states in male brains of GH and SH *Drosophila*. (A)** Schematic for social signal transduction. **(B)** Brain RNA-seq and CUT&RUN workflow (Created with BioRender). **(C-E)** PCA analysis of transcriptional profiles **(C)**, and H3K4me3 **(D)** and RNAPolII enrichment chromatin profiles **(E)** from GH and SH wild-type *CS*, *Or47b$^{2/3}$*, *Or67d$^{GAL4/GAL4}$*, *fru$^{LexA/4–40}$* and *dsx$^{0683/1649}$* mutant male brains. **(F-G)** Feature distribution of H3K4me3 peaks **(F)** and RNAPolII peaks **(G)**. **(H-I)** IGV view of transcripts, H3K4me3 and RNAPolII distribution around *fru* **(H)** and *dsx* **(I)** locus among all genotypes (three biological replicates overlaid).




**Supplementary Figure 1: Normalized counts of genes of interest. (A-F)** Transcript levels for several representative housekeeping genes **(A-C)**, *Dsk* **(D)**, *fru* **(E),** and *dsx* **(F)** among all wild-type and mutant brains.

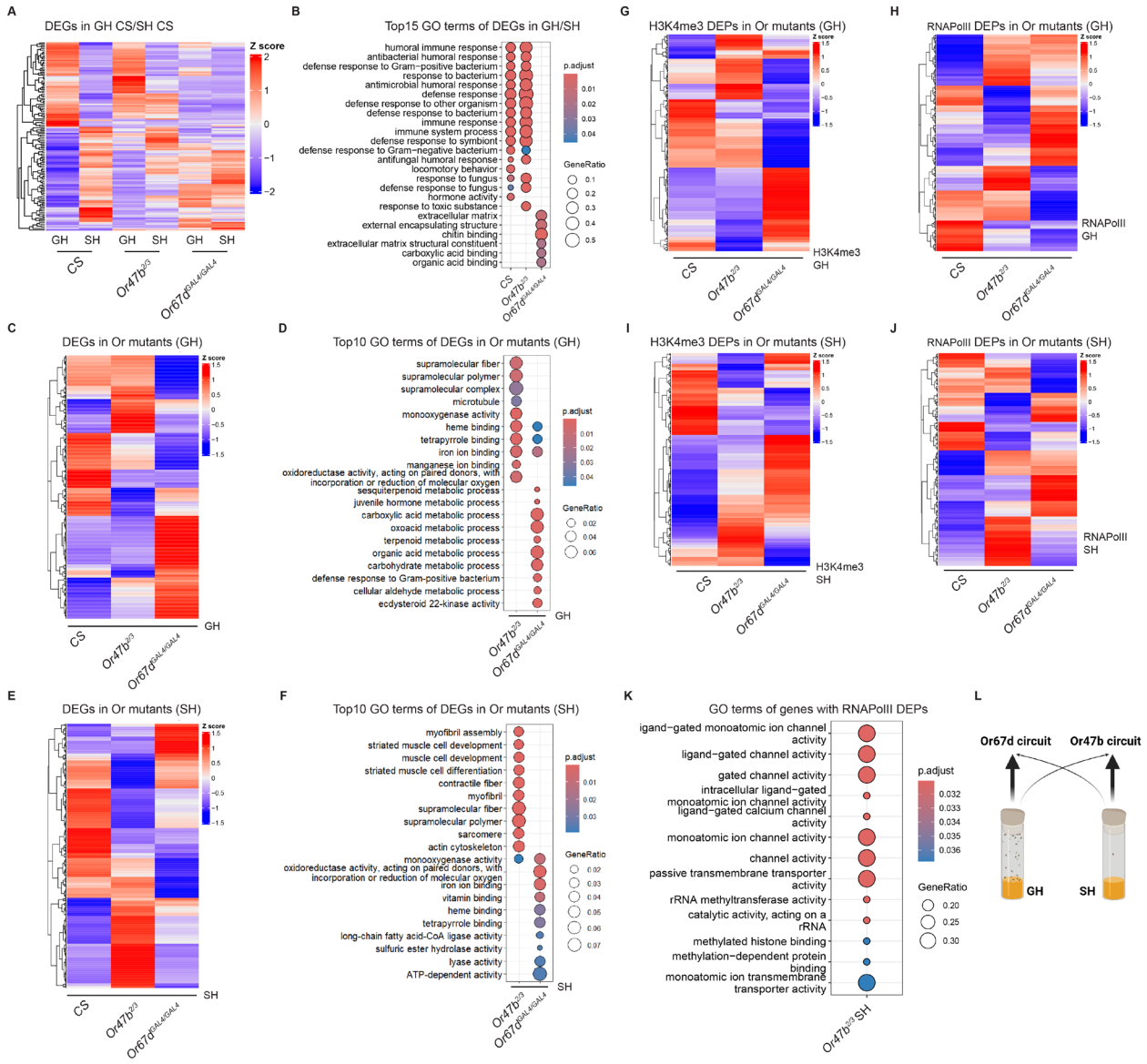
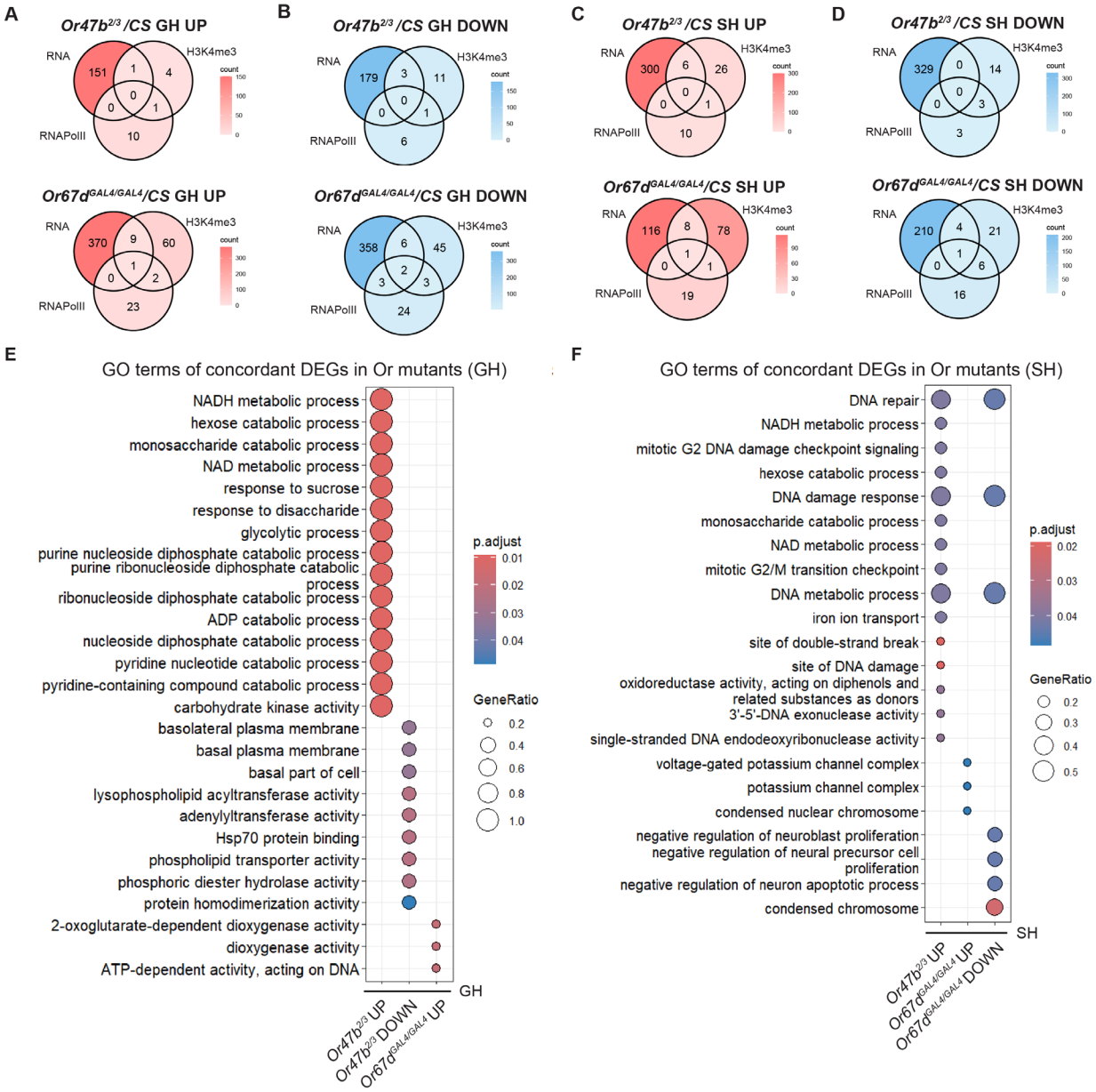



**Figure 2. The effect of Or47b and Or67d pheromone circuits on transcriptional and chromatin responses to social experience in the central brain. (A)** Heatmaps displaying Z-scores of the median of normalized transcript counts for DEGs identified in the male brains from the GH *CS*/SH *CS* comparison across *CS* and two Or mutants, *Or47b*$^{2/3}$ and *Or67d*$^{GAL4/GAL4}$ (adjusted p-value < 0.05, $|\log_2 FC| > 0.1$). **(B)** Top 15 most significantly enriched GO terms for DEGs of GH/SH comparisons of *CS* and two Or mutants, respectively (q-value < 0.05). **(C, E)** Heatmaps displaying Z-scores of the median of normalized transcript counts for DEGs identified from the Or mutants/*CS* comparison in GH **(C)** and SH **(E)** (adjusted P-value < 0.05, $|\log_2 FC| > 0.1$). **(D, F)** Top 10 most significantly enriched GO terms for DEGs of Or mutants/*CS* comparison in GH **(D)** and SH **(F)** (q-value < 0.05). **(G-H)** Heatmaps displaying Z-scores of the median of normalized enrichment levels of H3K4me3 **(G)** or RNAPolII **(H)** DEPs identified from Or mutants/*CS* comparison in GH brains (adjusted P-value < 0.01). **(I-J)** Heatmaps displaying Z-scores of the median of normalized enrichment levels of H3K4me3 **(I)** or RNAPolII **(J)** DEPs identified from Or mutants/*CS* comparison in SH brains (adjusted P-value < 0.01). **(K)** GO terms for genes with RNAPolII DEPs of *Or47b*$^{2/3}$ mutants/*CS* comparison in SH brains (q-value < 0.05). **(L)** A working model

721 depicting the roles of Or47b and Or67d pheromone circuits in mediating transcriptional response to social
722 experience in the central brain.




723

724 **Supplementary Figure 2: Comparisons of DEGs and DEPs in Or mutants. (A-D)** Venn diagram
725 comparing DEGs and genes with H3K4me3 or RNAPolII DEPs in the same direction from
726 *Or47b*$^{2/3}$or *Or67d*$^{GAL4/GAL4}$ mutants compared to GH **(A, B)** or SH *CS* **(C, D)**. **(E-F)** GO terms for genes
727 showing concordant changes between transcription and at least one active chromatin mark in Or
728 mutant/*CS* comparisons in GH **(E)** or SH **(F)** male brains (q-value < 0.05).

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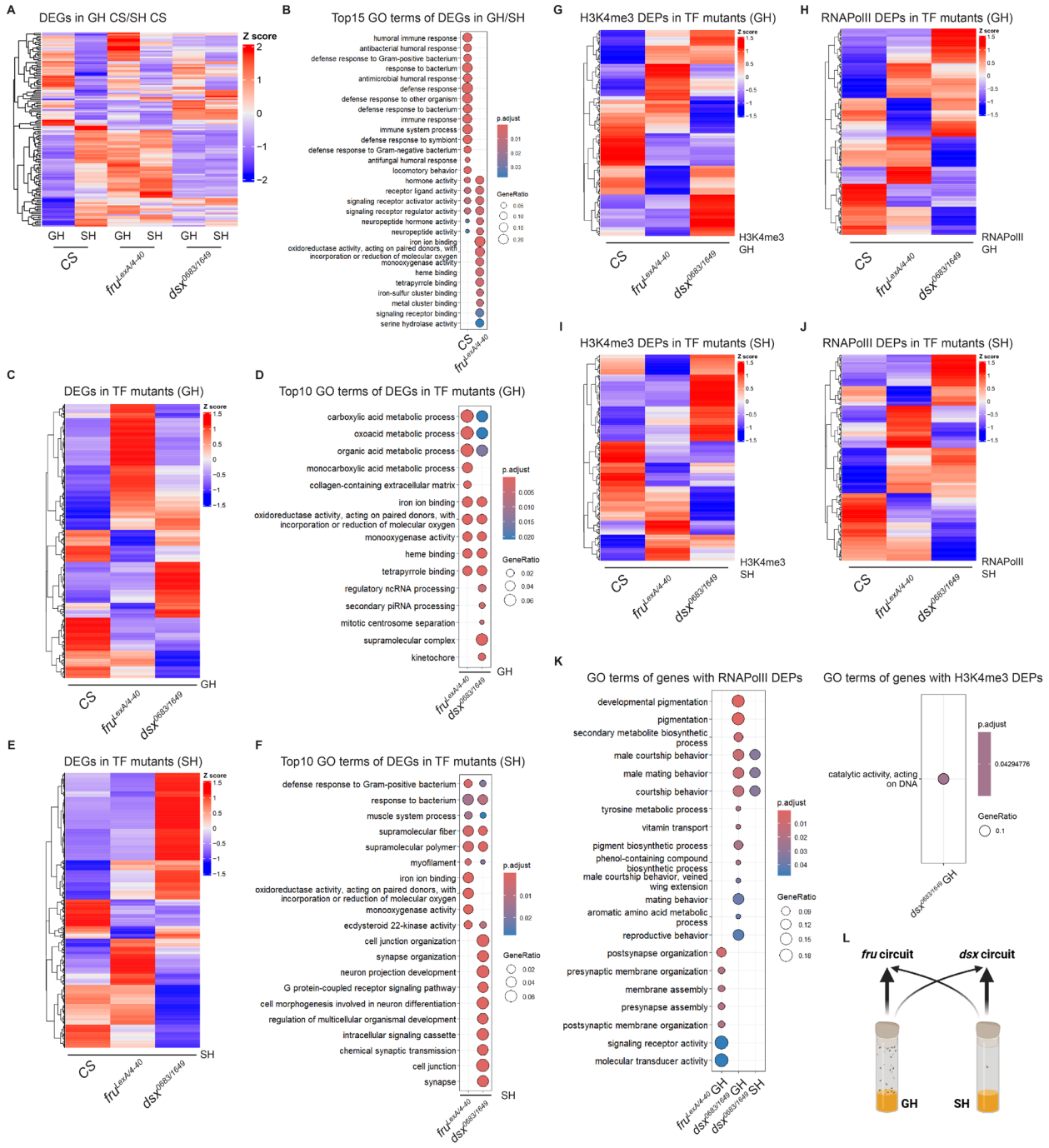


**Figure 3. The effect of transcription factors Fru$^{M}$ and Dsx$^{M}$ on transcriptional and chromatin responses to social experience in the central brain. (A)** Heatmaps displaying Z-scores of the median of normalized transcript counts for DEGs identified in the male brains from the GH *CS*/SH *CS* comparison across *CS,* and mutants in two transcription factors (TFs), *fru*$^{LexA/4-40}$, and *dsx*$^{0683/1649}$ (adjusted p-value < 0.05, $|\log_2 FC| > 0.1$). **(B)** Top 15 most significantly enriched GO terms for DEGs of GH/SH comparisons of *CS* and *fru*$^{LexA/4-40}$ mutant, respectively (q-value < 0.05). **(C, E)** Heatmaps displaying Z-scores of the median of normalized transcript counts for DEGs identified from TF mutants/*CS* comparison in GH **(C)**

739 and SH **(E)** (adjusted P-value < 0.05, |$\log_2$FC| > 0.1). **(D, F)** Top 10 most significantly enriched GO terms
740 for DEGs of TF mutants/*CS* comparison in GH **(D)** and SH **(F)** (q-value < 0.05). **(G-H)** Heatmaps
741 displaying Z-scores of the median of normalized enrichment levels of H3K4me3 **(G)** or RNAPolII **(H)**
742 DEPs identified from TF mutants/*CS* comparison in GH brains (adjusted P-value < 0.01). **(I-J)** Heatmaps
743 displaying Z-scores of the median of normalized enrichment levels of H3K4me3 **(I)** or RNAPolII **(J)**
744 DEPs identified from TF mutants/*CS* comparison in SH brains (adjusted P-value < 0.01). **(K)** GO terms
745 for genes with H3K4me3 or RNAPolII DEPs of TF mutants/*CS* comparison in either GH or SH condition
746 (q-value < 0.05). **(L)** A working model displaying the roles of $Fru^M$ and $Dsx^M$ circuits in mediating
747 transcriptional response to social experience in the central brain.

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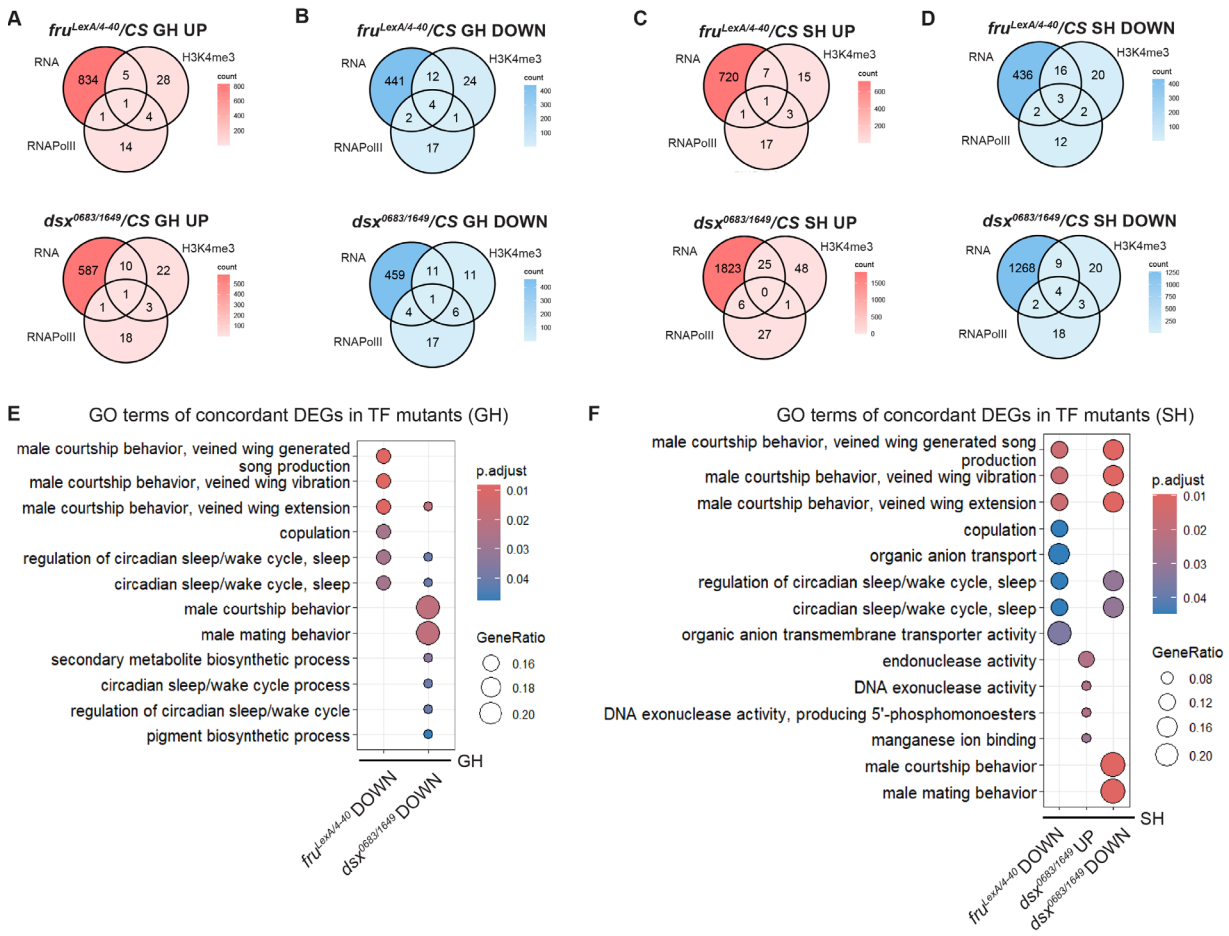


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750 **Supplementary Figure 3: Comparisons between DEGs and DEPs in TF mutants. (A-D)** Venn
751 diagram comparing DEGs and genes with H3K4me3 or RNAPolII DEPs in the same direction from
752 *fru*$^{LexA/4-40}$ or *dsx*$^{0683/1649}$ mutants compared to GH **(A, B)** or SH *CS* **(C, D)**. **(E-F)** GO terms for genes
753 showing concordant changes between transcription and at least one active chromatin mark in TF
754 mutant/*CS* comparisons in GH **(E)** or SH **(F)** male brains (q-value < 0.05).

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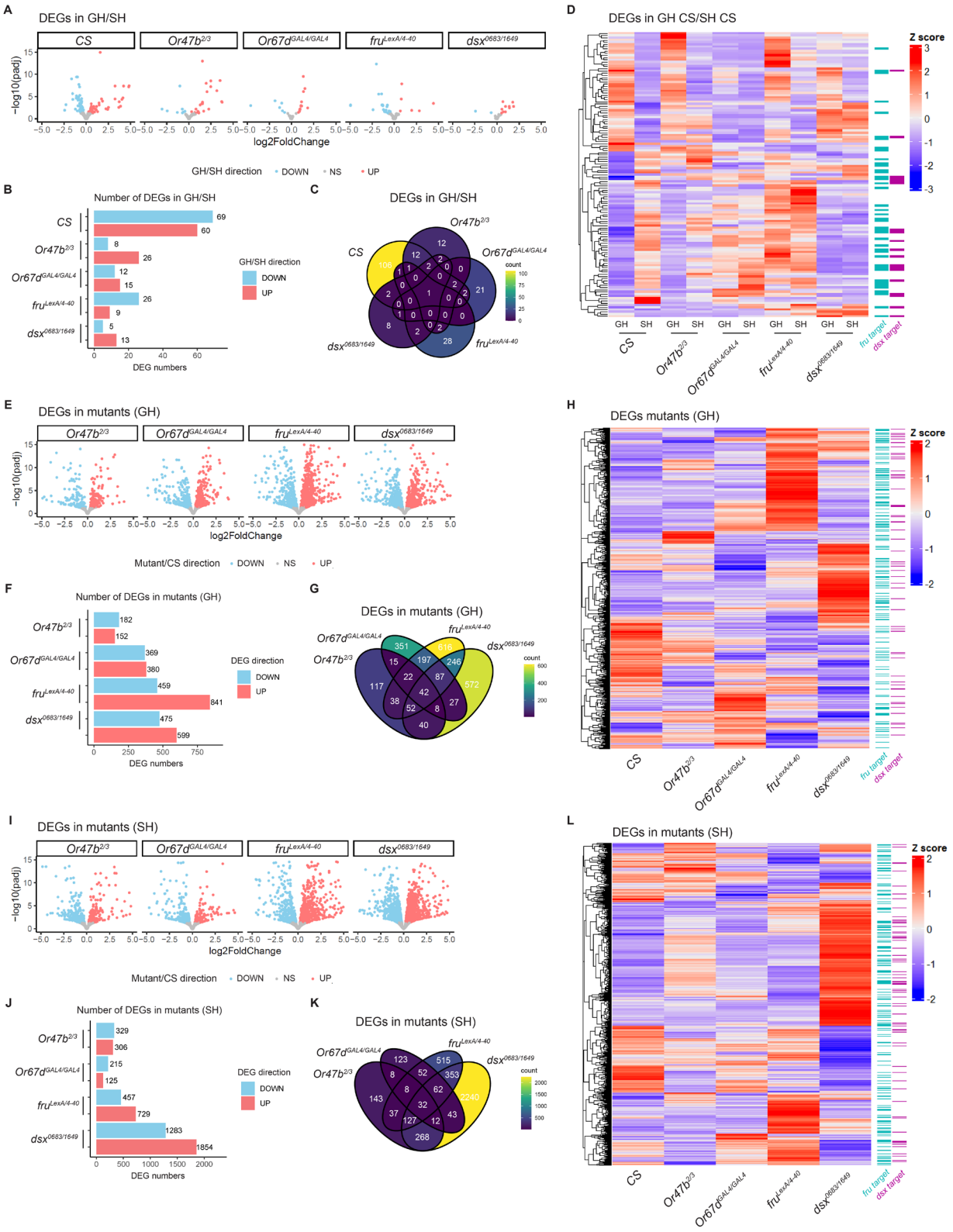
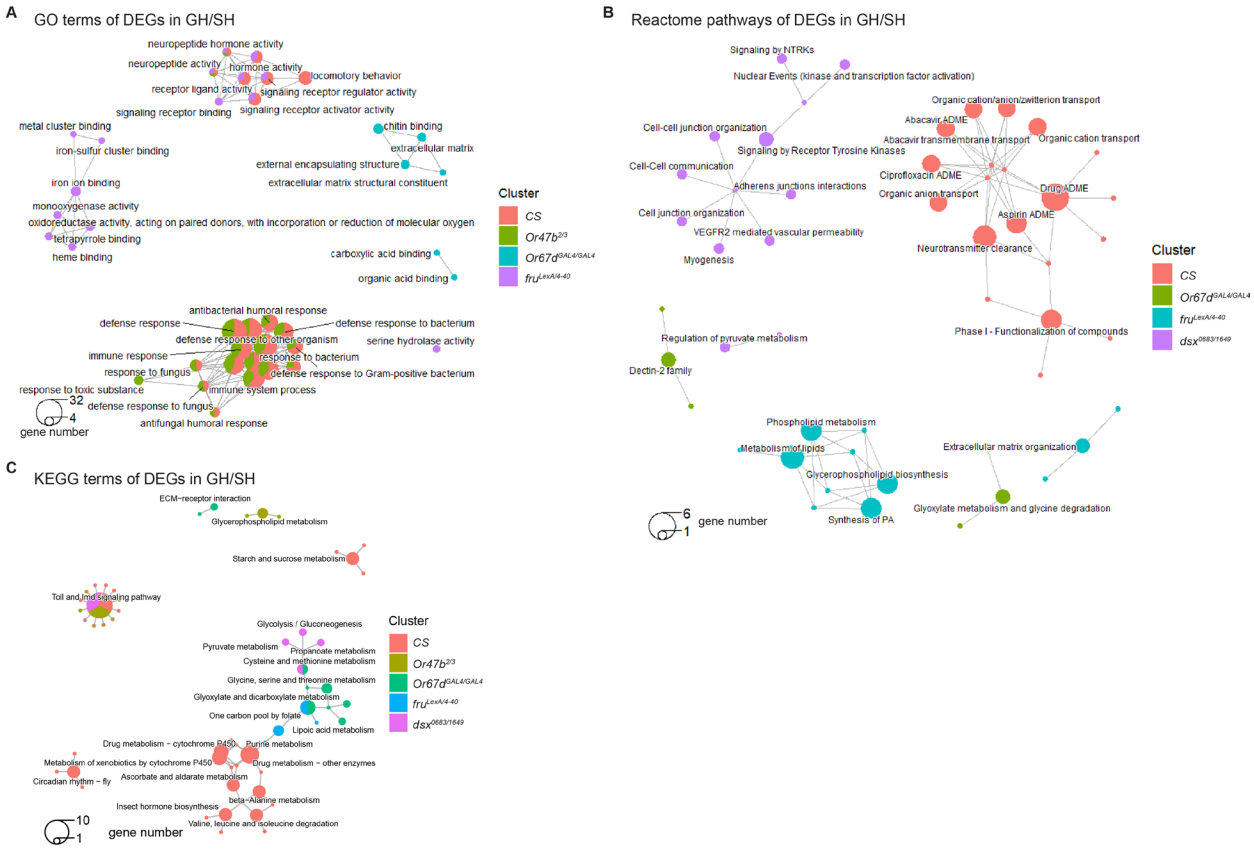
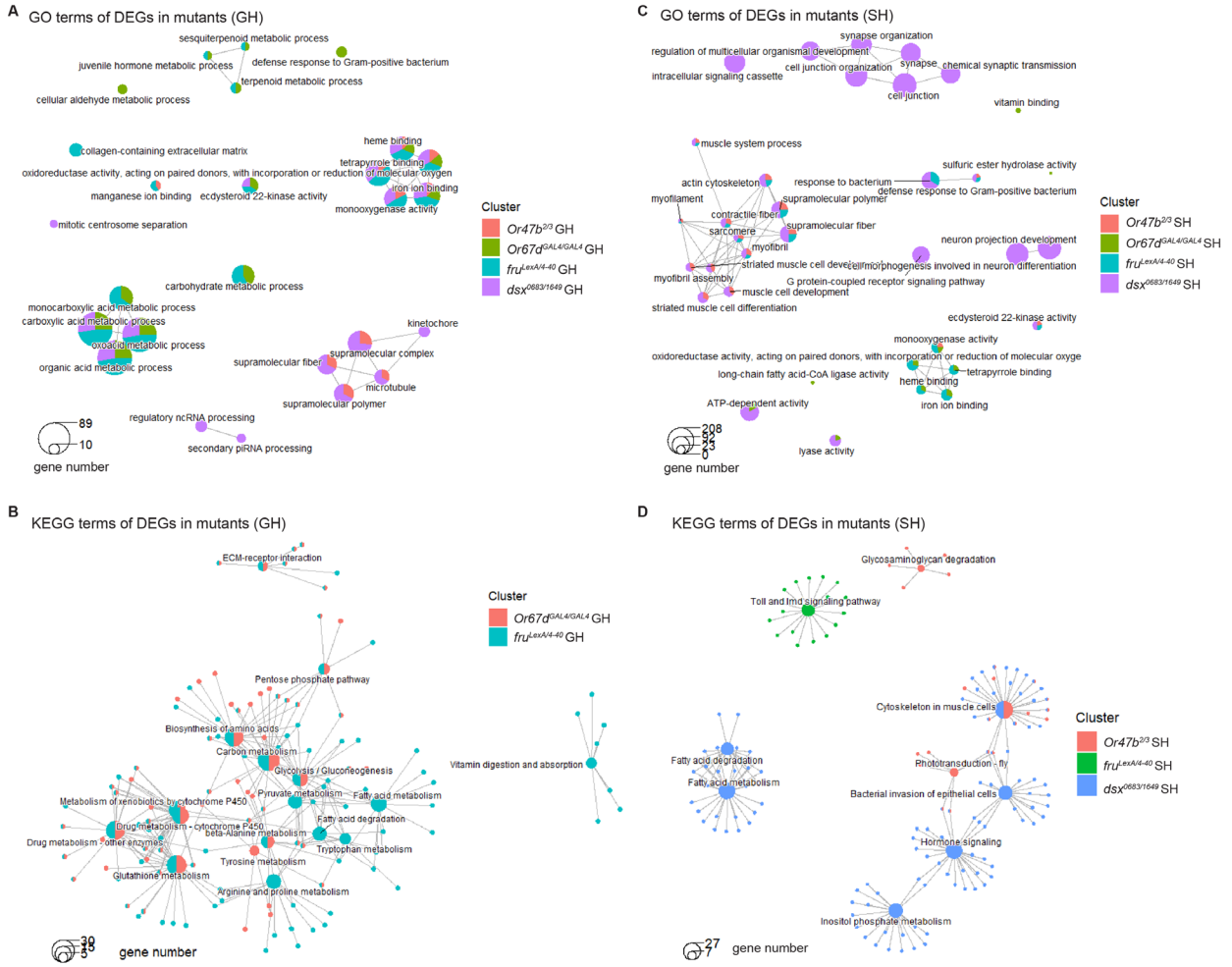



**Figure 4: Comparisons of the transcriptional effects of Or and TF mutants in male fly brains. (A)** Volcano plots displaying $\log_2$FoldChange (GH/SH) and -log10(p-adj) of genes in each genotype. DEGs

were labeled in red (higher in GH) or blue (lower in GH). **(B)** Numbers of DEGs in GH/SH comparisons for each genotype (adjusted p-value < 0.05, $|\log_2 FC| > 0.1$). **(C)** Venn diagram comparing DEGs from GH/SH comparisons for each genotype. **(D)** Heatmaps displaying Z-scores of the median of normalized transcript counts for DEGs from the GH *CS*/SH *CS* comparison across *CS*, Or mutants, and TF mutants (adjusted p-value < 0.05, $|\log_2 FC| > 0.1$). Putative $Fru^M$ or $Dsx^M$ target genes are labeled on the right. **(E, I)** Volcano plots displaying $\log_2$FoldChange (mutant/*CS*) and -log10(p-adj) of genes in each genotype under GH **(E)** or SH **(I)** condition. DEGs up- and down-regulated in mutants are labeled in red and blue, respectively. **(F, J)** Numbers of DEGs in mutant/*CS* comparisons for each genotype under GH **(F)** or SH **(J)** condition (adjusted p-value < 0.05, $|\log_2 FC| > 0.1$). **(G, K)** Venn diagram comparing DEGs from mutant/*CS* comparisons for each genotype under GH **(G)** or SH **(K)** conditions. **(H, L)** Heatmaps displaying Z-scores of the median of normalized transcript counts for all DEGs from any of the mutant/*CS* comparison under either GH **(H)** or SH **(L)** condition (adjusted p-value < 0.05, $|\log_2 FC| > 0.1$). Putative $Fru^M$ or $Dsx^M$ targets are labeled on the right.




**Supplementary Figure 4: GO, Reactome, and KEGG terms of social experience dependent DEGs in *CS*, Or mutants, and TF mutants. (A)** Connection plots of the top 15 most significantly enriched GO terms for social experience-dependent DEGs in *CS*, Or mutant, and TF mutant (q-value < 0.05) male brains. **(B)** Connection plots of the top 10 most significantly enriched Reactome pathways for DEGs from GH/SH comparisons of *CS*, Or mutants, and TF mutants (q-value < 0.05). **(C)** Connection plots of the top 10 most significantly enriched KEGG terms for social experience-dependent DEGs in *CS*, Or mutants, and TF mutants (q-value < 0.05).




**Supplementary Figure 5: GO and KEGG terms from DEGs in mutant/*CS* comparisons. (A, C)** Connection plots of the top 10 most significantly enriched GO terms for DEGs from mutant/*CS* comparisons under GH **(A)** or SH **(C)** conditions (q-value < 0.05). **(B, D)** Connection plots of the top 10 most significantly enriched KEGG terms for DEGs from mutant/*CS* comparisons under GH **(B)** or SH **(D)** conditions (q-value < 0.05).

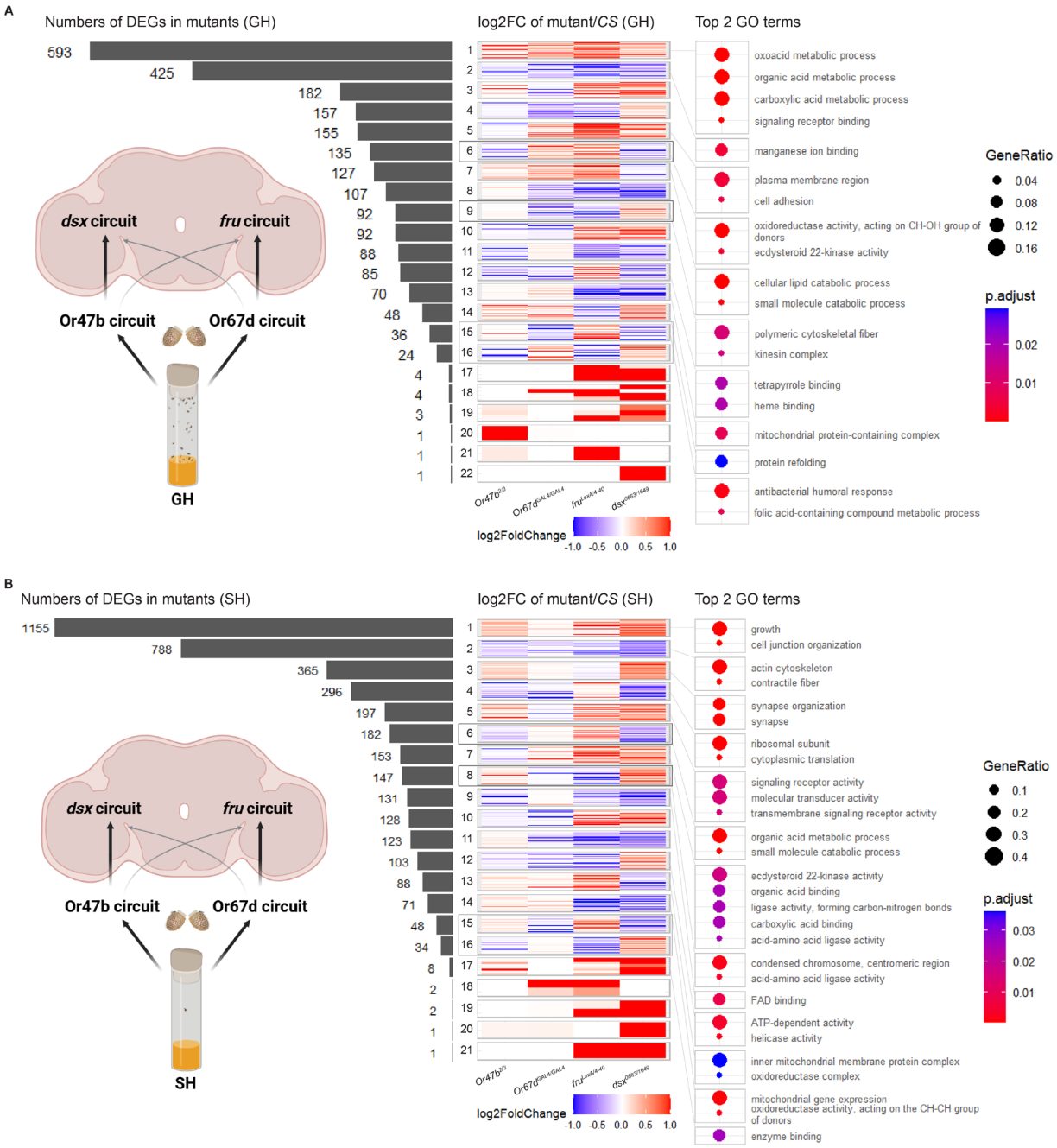
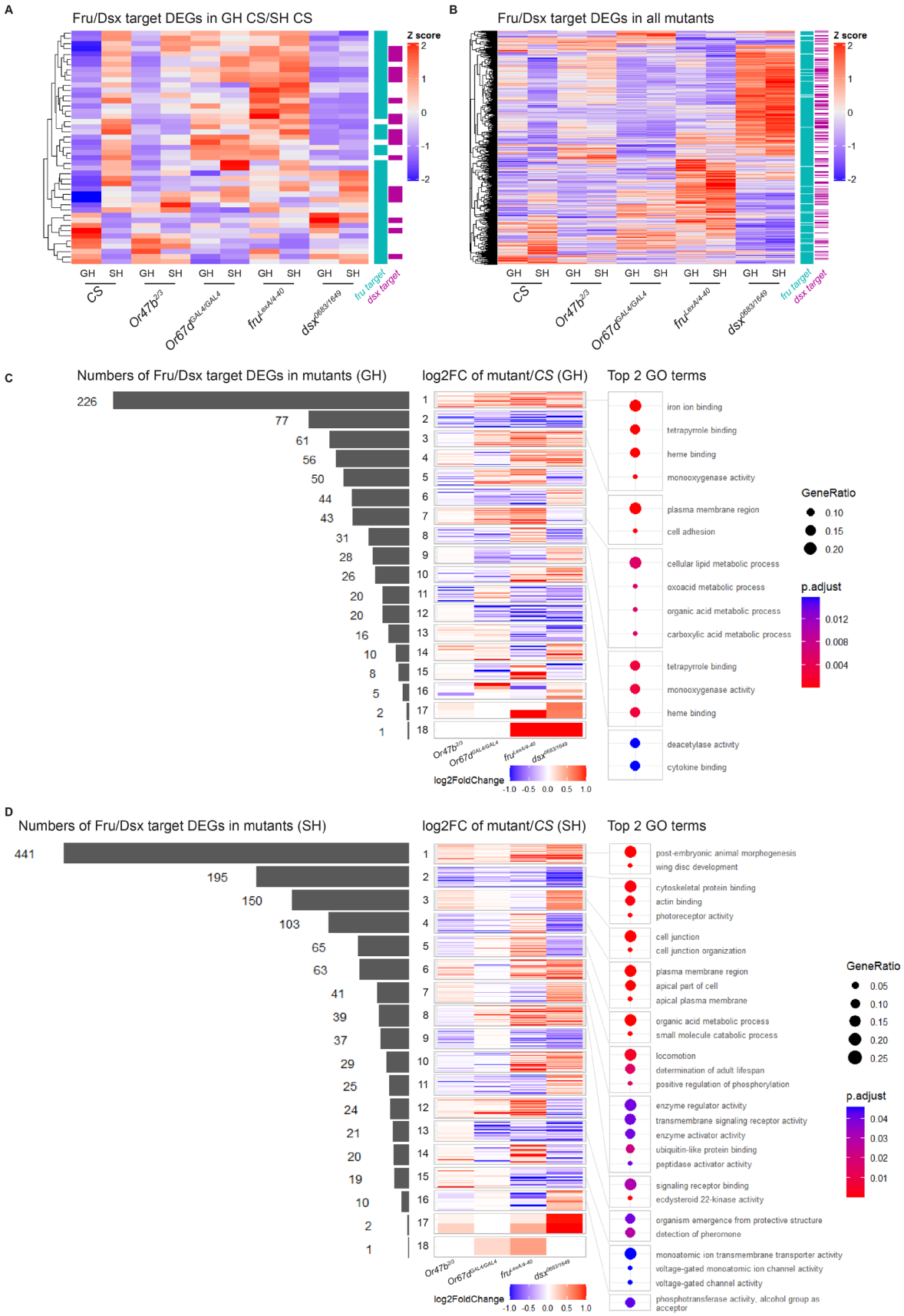



**Figure 5: Or47b and Or67d circuits contribute to social modulation of gene expression through the function of Dsx$^{M}$ and Fru$^{M}$, respectively. (A, B)** Bottom left: Models displaying pathways connecting Or47b or Or67d pheromone circuits to *fru* or *dsx* circuits in their transcriptional regulation on male fly brains under GH **(A)** or SH **(B)** condition. Left: Numbers of DEGs in mutant/*CS* comparisons across various combinations of log2FC in different directions. Middle: Heatmaps displaying log2FC of DEGs in mutant/*CS* comparisons across combinations of different directions. Dark gray frames indicate DEGs with log2FC in the same direction between *Or47b*$^{2/3}$and *dsx*$^{0683/1649}$ mutants, as well as between *Or67d*$^{GAL4/GAL4}$ and *fru*$^{LexA/4-40}$ mutants. Light gray frames indicate DEGs with log2FC in the same direction between *Or47b*$^{2/3}$and *fru*$^{LexA/4-40}$ mutants, and between *Or67d*$^{GAL4/GAL4}$ and *dsx*$^{0683/1649}$ mutants. Right: Top 2 GO

terms for DEGs in mutant/*CS* comparisons across combinations of log2FC in different directions in the middle.


A
Fru/Dsx target DEGs in GH CS/SH CS
Z score
GH SH GH SH GH SH GH SH GH SH
CS
Or47b2/3
Or67dGAL4/GAL4
fruLexA4-40
dsx9683/1649
fru target
dsx target
B
Fru/Dsx target DEGs in all mutants
C
Numbers of Fru/Dsx target DEGs in mutants (GH)
226
77
61
56
50
44
43
31
28
26
20
20
16
10
8
5
2
1
log2FC of mutant/CS (GH)
log2FoldChange
-1.0 -0.5 0.0 0.5 1.0
Top 2 GO terms
iron ion binding
tetrapyrrole binding
heme binding
monooxygenase activity
plasma membrane region
cell adhesion
cellular lipid metabolic process
oxoacid metabolic process
organic acid metabolic process
carboxylic acid metabolic process
tetrapyrrole binding
monooxygenase activity
heme binding
deacetylase activity
cytokine binding
GeneRatio
0.10
0.15
0.20
p.adjust
0.012
0.008
0.004
D
Numbers of Fru/Dsx target DEGs in mutants (SH)
441
195
150
103
65
63
41
39
37
29
25
24
21
20
19
10
2
1
log2FC of mutant/CS (SH)
log2FoldChange
-1.0 -0.5 0.0 0.5 1.0
Top 2 GO terms
post-embryonic animal morphogenesis
wing disc development
cytoskeletal protein binding
actin binding
photoreceptor activity
cell junction
cell junction organization
plasma membrane region
apical part of cell
apical plasma membrane
organic acid metabolic process
small molecule catabolic process
locomotion
determination of adult lifespan
positive regulation of phosphorylation
enzyme regulator activity
transmembrane signaling receptor activity
enzyme activator activity
ubiquitin-like protein binding
peptidase activator activity
signaling receptor binding
ecdysteroid 22-kinase activity
organism emergence from protective structure
detection of pheromone
monoatomic ion transmembrane transporter activity
voltage-gated monoatomic ion channel activity
voltage-gated channel activity
phosphotransferase activity, alcohol group as acceptor
GeneRatio
0.05
0.10
0.15
0.20
0.25
p.adjust
0.04
0.03
0.02
0.01

800

801 **Supplementary Figure 6: Differential effect of pheromone circuits, $Fru^M$, and $Dsx^M$ on social**
802 **modulation of $Fru^M$/$Dsx^M$ target gene expression. (A)** Heatmaps displaying Z-scores of the median of
803 normalized transcript counts for $Fru^M$ or $Dsx^M$ target DEGs identified from the GH *CS*/SH *CS*
804 comparison. **(B)** Heatmaps displaying Z-scores of the median of normalized transcript counts for $Fru^M$ or
805 $Dsx^M$ target DEGs identified from the mutant/*CS* comparison under either GH or SH conditions. **(C, D)**
806 Left: Numbers of DEGs in mutant/*CS* comparisons across various combinations of log2FC in different
807 directions under GH **(C)** or SH **(D)** conditions. Middle: Heatmaps displaying log2FC of DEGs in
808 mutant/*CS* comparisons across combinations of different directions. Right: Top 2 GO terms for DEGs in
809 mutant/*CS* comparisons across combinations of log2FC in different directions in the middle.

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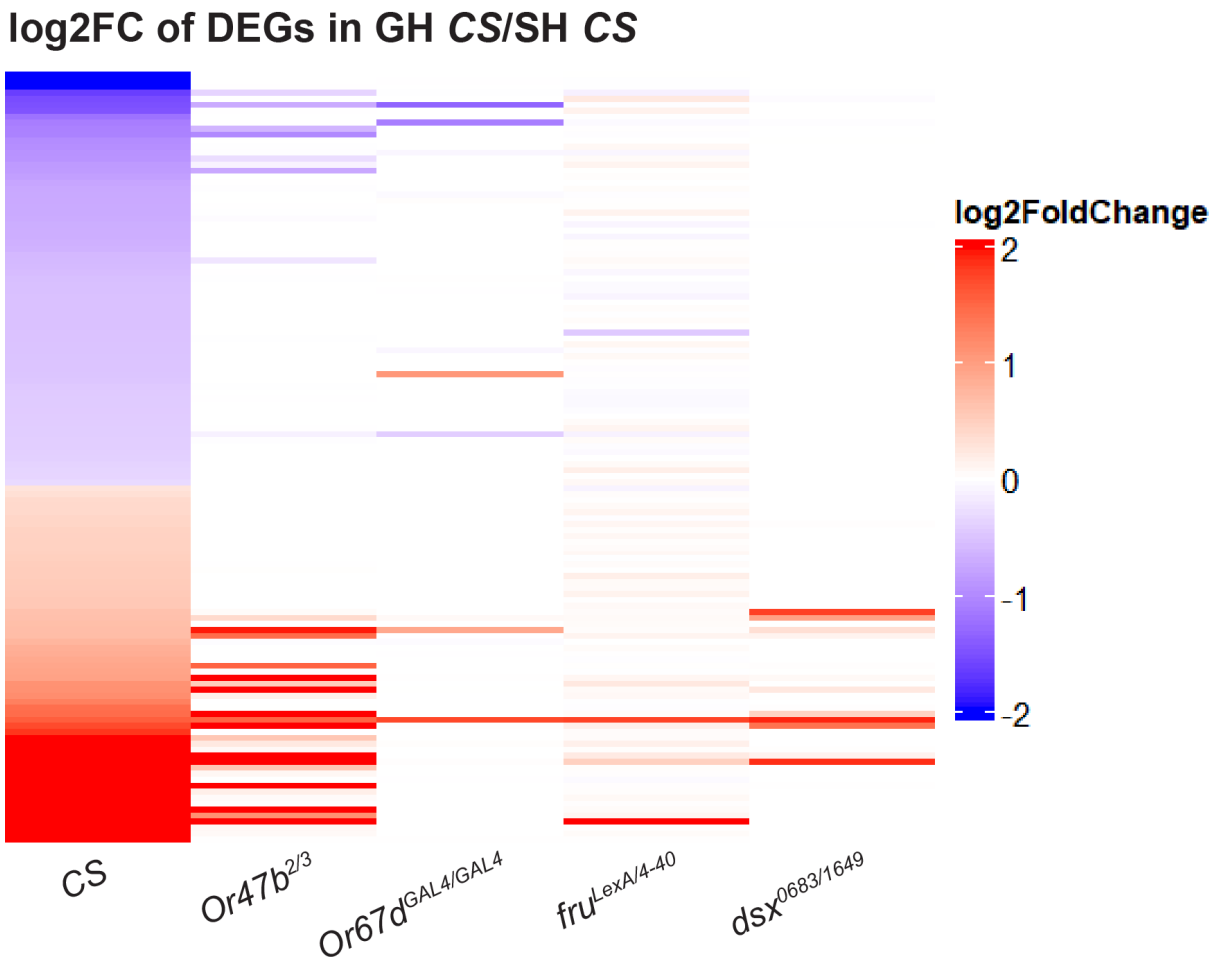


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812 **Supplementary Figure 7:** Heatmaps displaying log2FC of DEGs from GH *CS*/SH *CS* comparison in all
813 genotypes.

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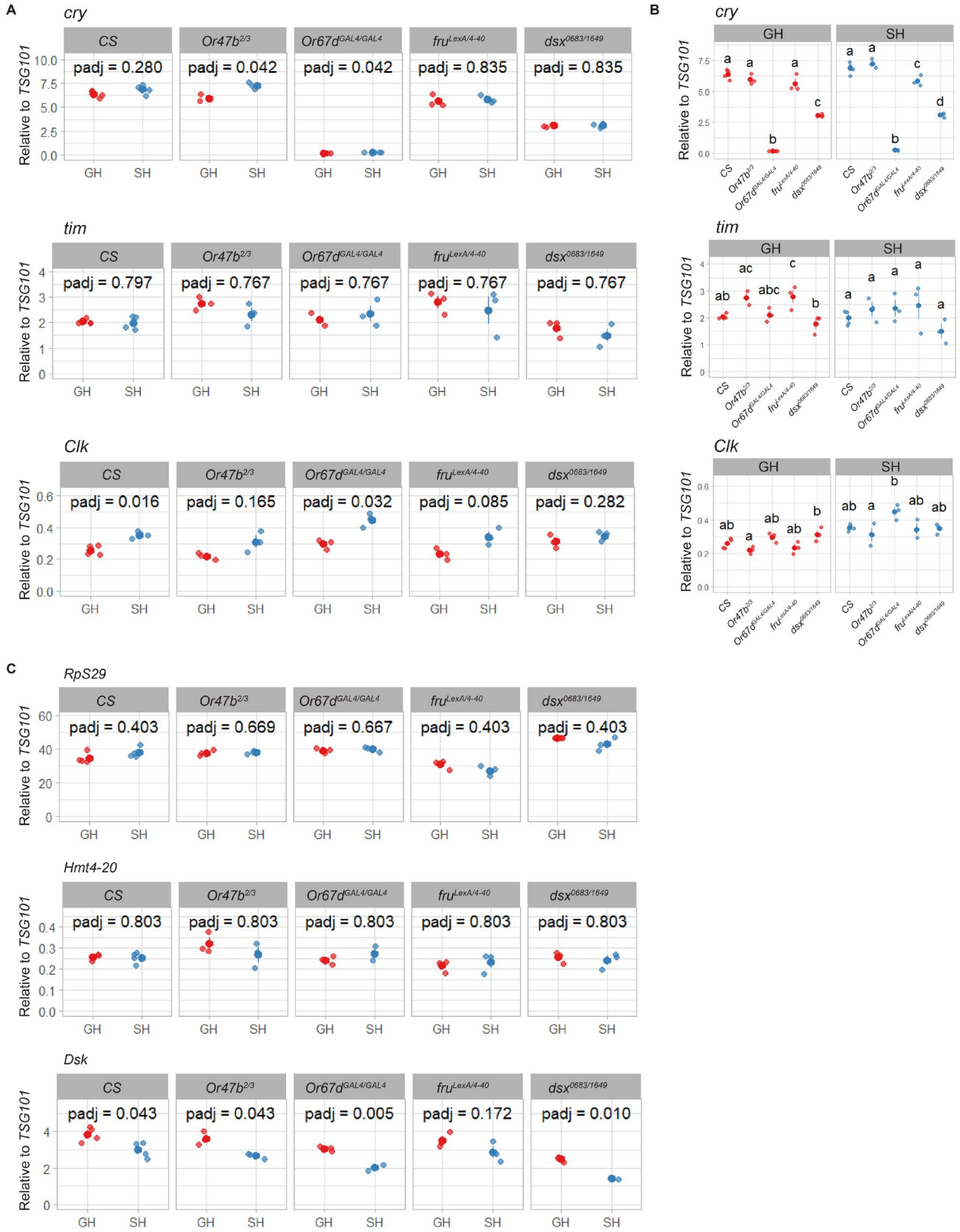
A
cry
CS
Or47b2/3
Or67dGAL4/GAL4
fruLexA/4-40
dsx0683/1649
padj = 0.280
padj = 0.042
padj = 0.042
padj = 0.835
padj = 0.835
tim
padj = 0.797
padj = 0.767
padj = 0.767
padj = 0.767
padj = 0.767
Clk
padj = 0.016
padj = 0.165
padj = 0.032
padj = 0.085
padj = 0.282
Relative to TSG101
GH
SH
B
C
RpS29
padj = 0.403
padj = 0.669
padj = 0.667
padj = 0.403
padj = 0.403
Hmt4-20
padj = 0.803
padj = 0.803
padj = 0.803
padj = 0.803
padj = 0.803
Dsk
padj = 0.043
padj = 0.043
padj = 0.005
padj = 0.172
padj = 0.010

816 **Supplementary Figure 8: Expression of circadian genes in male heads in response to social**
817 **experience. (A)** RT-qPCR analysis of *cry*, *tim*, and *Clk* transcript levels in male fly heads between GH
818 and SH across genotypes. Statistical comparisons were performed using two-sided t-tests. P-values were
819 adjusted for multiple comparisons across genotypes using the Benjamini–Hochberg method. **(B)**
820 Comparison of *cry*, *tim*, and *Clk* transcript levels in male fly heads among all genotypes under GH or SH
821 conditions. ANOVA analysis followed by Tukey's HSD test was applied to the statistics. **(C)** Comparison
822 of *RpS29*, *Hmt4-20*, and *Dsk* transcript levels in male fly heads between GH and SH across genotypes.
823 Statistical comparisons were performed using two-sided t-tests. P-values were adjusted for multiple
824 comparisons across genotypes using the Benjamini–Hochberg method.

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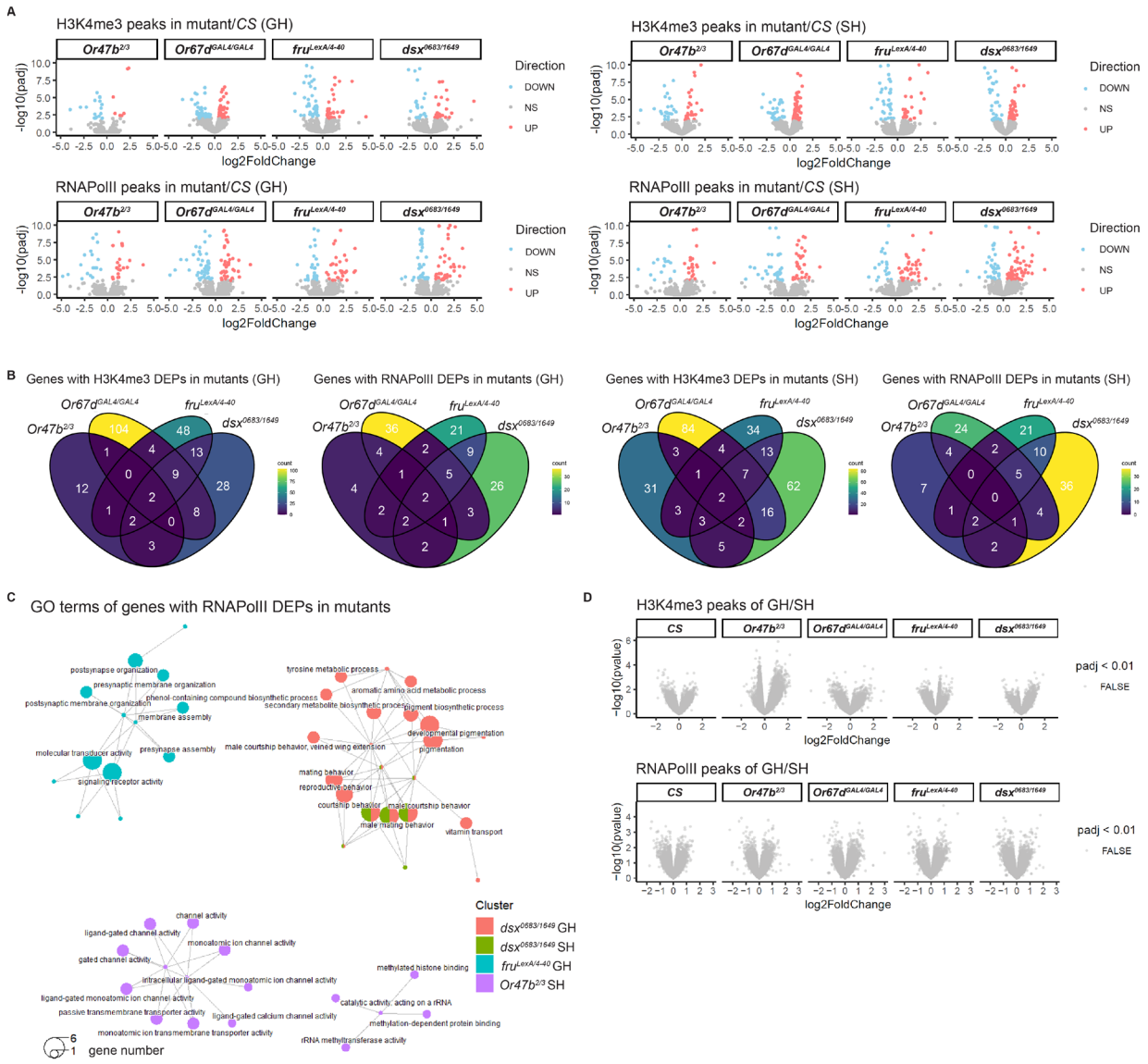



**Figure 6: Comparisons of DEPs across Or and TF mutants in male fly brains in different social contexts. (A)** Volcano plots showing log2FoldChange and –log10(padj) of mutant/*CS* comparisons for H3K4me3 or RNAPolII peaks under the same housing conditions. Peaks significantly enriched in mutants compared to *CS* are shown in red, while peaks significantly depleted are shown in blue (adjusted p-value < 0.01). **(B)** Venn diagram comparing genes with H3K4me3 or RNAPolII DEPs from mutant/*CS* comparisons under the same housing conditions. **(C)** Connection plots of GO terms for genes with RNAPolII DEPs from mutant/*CS* comparisons (q-value < 0.05). **(D)** Volcano plots showing log2FoldChange (GH/SH) and -log10(p-value) for H3K4me3 and RNAPolII peaks in each genotype. Gray labels peaks with adjusted p-value >= 0.01.

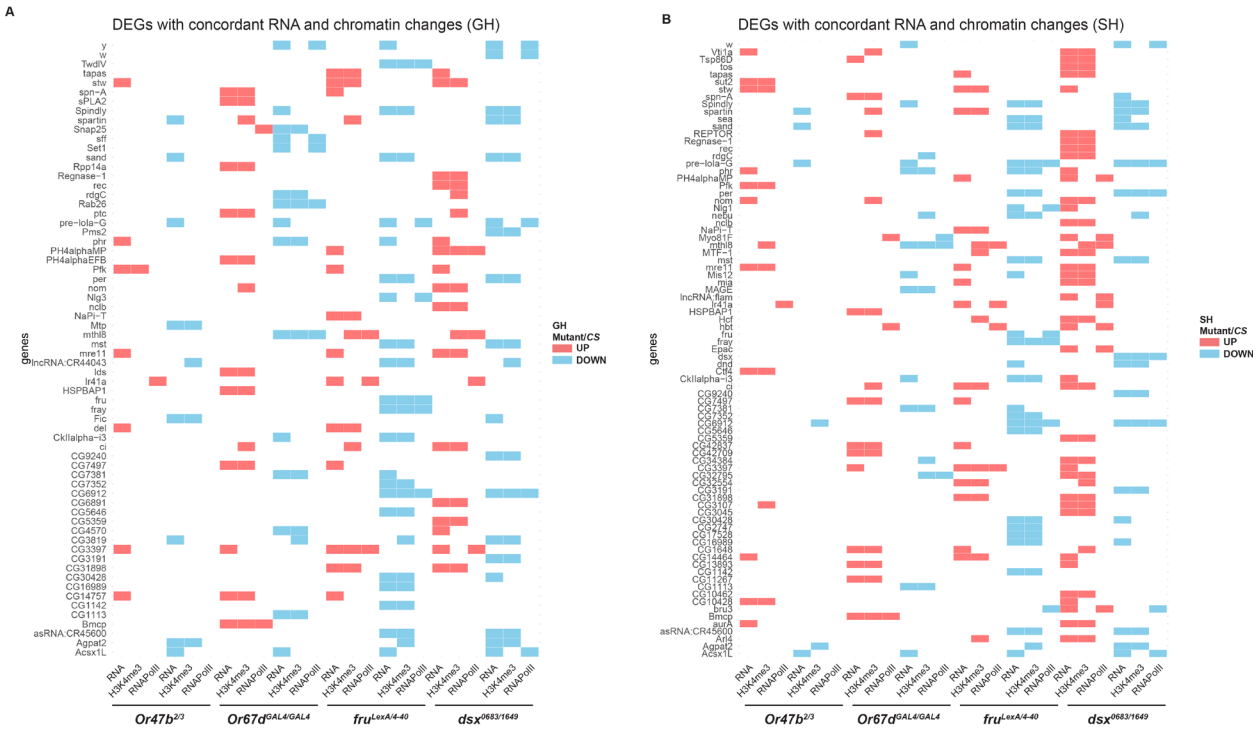
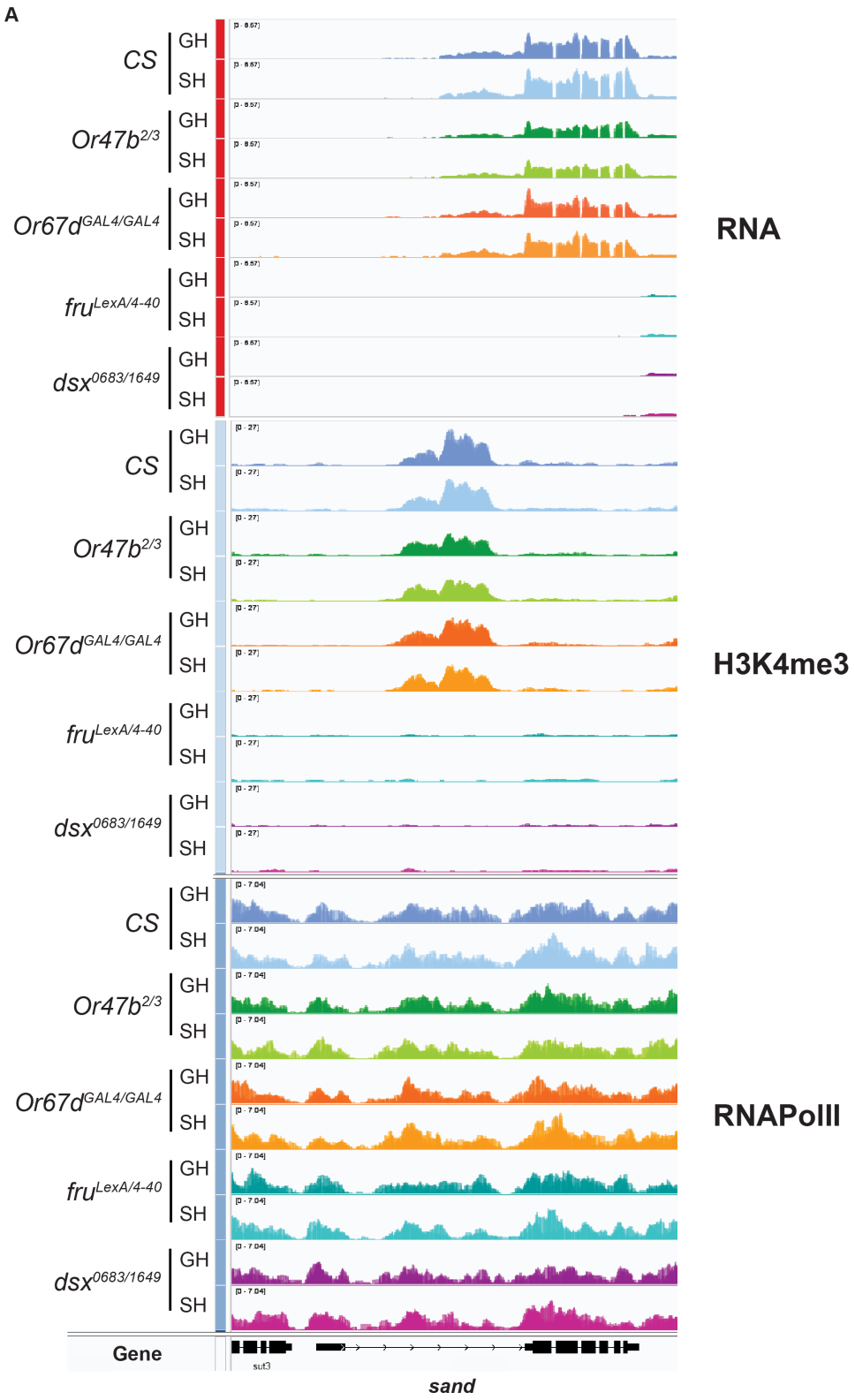



**Figure 7: Concordant changes in transcription and chromatin in response to grouping or isolation.** **(A, B)** Summary of genes exhibiting concordant changes in transcription and at least one active chromatin mark in each mutant/*CS* comparison under GH **(A)** or SH **(B)** conditions.

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846 **Supplementary Figure 9: Changes in transcript and chromatin marks at the *sand* locus in different**
847 **mutants and social conditions.** IGV tracks showing normalized transcript levels, H3K4me3, and
848 RNAPolII distributions at the *sand* locus across all genotypes (three biological replicates overlaid).

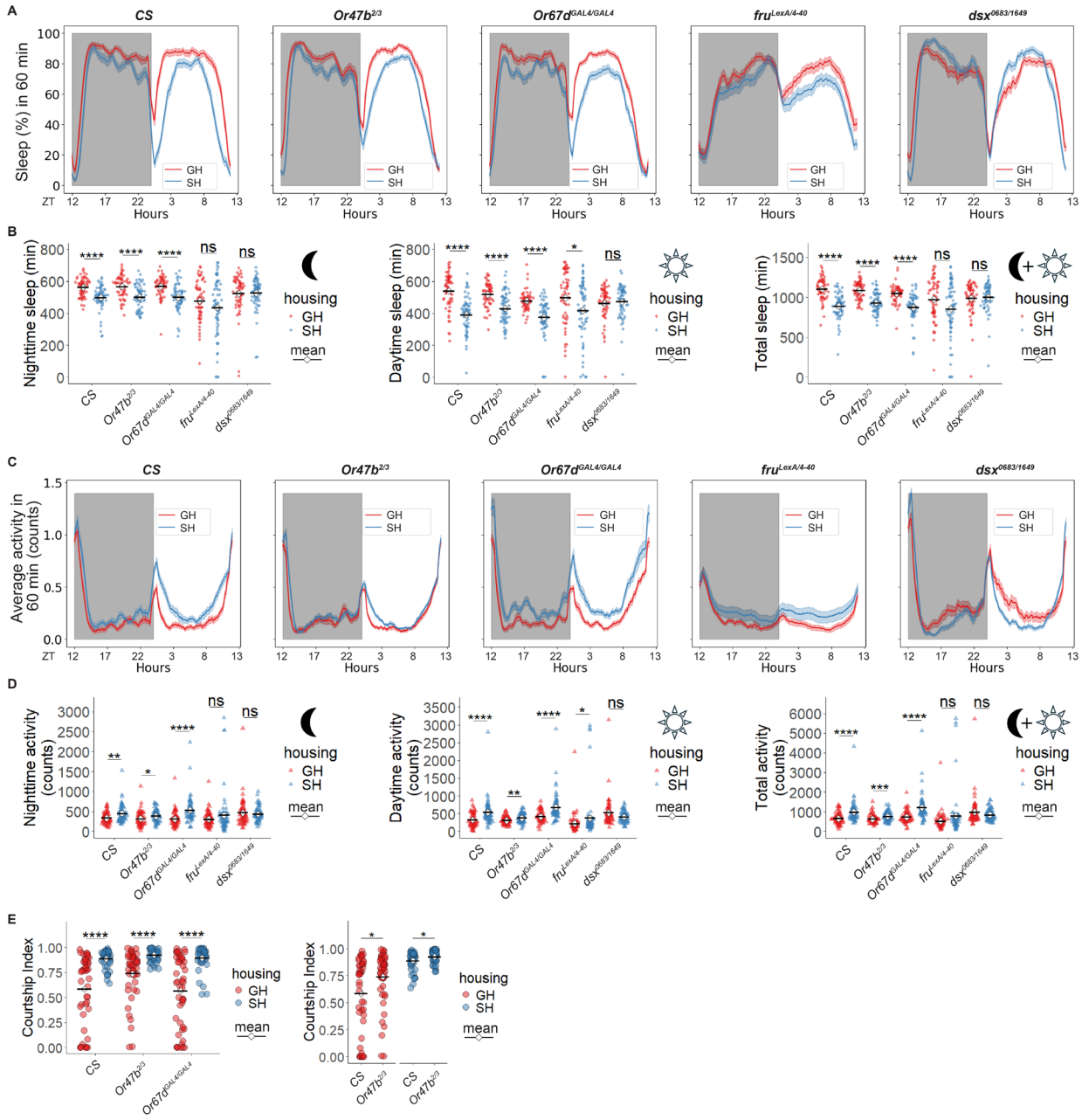


**Figure 8: Or47b and Or67d pheromone circuits, Fru$^{M}$, and Dsx$^{M}$ have differential effects on social modulation of behaviors. (A)** Sleep profiles of male flies from each genotype raised under GH or SH conditions, shown as the average proportion of sleep time per 60-minute interval during a 24-hour 12:12 dark-light cycle. Mean ±SEM. **(B)** Quantification of nighttime sleep, daytime sleep, and daily total sleep in **(A)**. Statistical comparisons were performed using the Wilcoxon test. P-values were adjusted for multiple comparisons across genotypes using the Benjamini–Hochberg method. **(C)** Locomotion activity profiles of male flies from each genotype raised under GH or SH conditions, shown as the average counts of beam crossings per 60-minute interval during a 24-hour 12:12 dark-light cycle. Mean ±SEM. **(D)** Quantification of nighttime activity, daytime activity and daily total activity in **(C)**. Statistical comparisons were performed using the Wilcoxon test. P-values were adjusted for multiple comparisons across genotypes using the Benjamini–Hochberg method. *P_adj<0.05, **P_adj<0.01, ***P_adj<0.001, ****P_adj<0.0001. (E) Male-female single pair courtship assay using *CS*, *Or47b$^{2/3}$*, and *Or67d$^{GAL4/GAL4}$*

862 males raised under GH and SH conditions. Statistical comparisons were performed using the Wilcoxon
863 test between GH and SH for each genotype (left) and the comparison between *Or47b*$^{2/3}$ and *CS* under the
864 same housing conditions (right). P-values were adjusted for multiple comparisons across genotypes (left)
865 or housing conditions (right) using the Benjamini–Hochberg method. Significance of adjusted P-value
866 (P_adj): *P_adj < 0.05, **P_adj < 0.01, ***P_adj < 0.001, ****P_adj < 0.0001.

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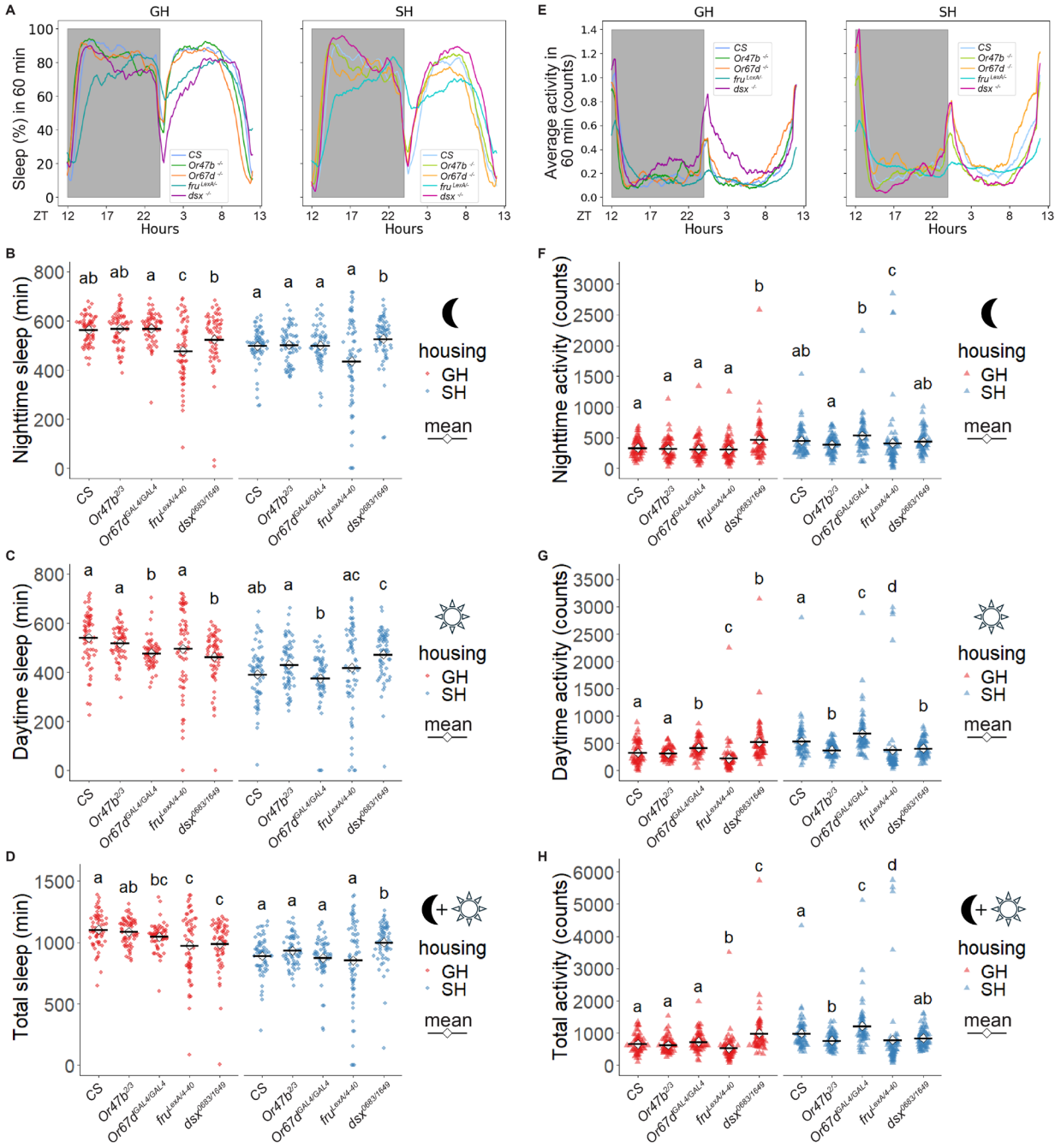


**Supplementary Figure 10: Differential effect of Or mutants and TF mutants on social modulation of behaviors. (A)** Sleep profiles of male flies from each genotype raised under GH or SH conditions, shown as the average proportion of sleep time per 60-minute interval during a 24-hour 12:12 dark-light cycle. **(B-D)** Quantification of nighttime sleep **(B)**, daytime sleep **(C),** and daily total sleep **(D)** in **(A)**. ANOVA analysis followed by Dunn's test was applied to the statistics. **(E)** Locomotion activity profiles of males from each genotype raised under GH or SH conditions, shown as the average counts of beam crossings per 60-minute interval during a 24-hour 12:12 dark-light cycle. **(F-H)** Quantification of nighttime activity **(F)**, daytime activity **(G),** and daily total activity **(H)** in **(E)**. ANOVA analysis followed

878 by Dunn's test was applied to the statistics. *P_adj<0.05, **P_adj<0.01, ***P_adj<0.001,
879 ****P_adj<0.0001.

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881 # References

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