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The *Drosophila* NPY-like system protects against chronic stress–induced learning deficit by preventing the disruption of autophagic flux

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Chronic stress may induce learning and memory deficits that are associated with a depression-like state in *Drosophila melanogaster*. The molecular and neural mechanisms underlying the etiology of chronic stress–induced learning deficit (CSLD) remain elusive. Here, we show that the autophagy-lysosomal pathway, a conserved cellular signaling mechanism, is associated with chronic stress in *Drosophila*, as indicated by time-series transcriptome profiling. Our findings demonstrate that chronic stress induces the disruption of autophagic flux, and chronic disruption of autophagic flux could lead to a learning deficit. Remarkably, preventing the disruption of autophagic flux by up-regulating the basal autophagy level is sufficient to protect against CSLD. Consistent with the essential role of the dopaminergic system in modulating susceptibility to CSLD, dopamine neuronal activity is also indispensable for chronic stress to induce the disruption of autophagic flux. By screening knockout mutants, we found that neuropeptide F, the *Drosophila* homolog of neuropeptide Y, is necessary for normal autophagic flux and promotes resilience to CSLD. Moreover, neuropeptide F signaling during chronic stress treatment promotes resilience to CSLD by preventing the disruption of autophagic flux. Importantly, neuropeptide F receptor activity in dopamine neurons also promotes resilience to CSLD. Together, our data elucidate a mechanism by which stress-induced excessive dopaminergic activity precipitates the disruption of autophagic flux, and chronic disruption of autophagic flux leads to CSLD, while inhibitory neuropeptide F signaling to dopamine neurons promotes resilience to CSLD by preventing the disruption of autophagic flux.

Drosophila melanogaster | chronic stress | learning and memory | autophagy | neuropeptide F

Significance

Cognitive impairment is a core symptom of depression and affects millions of people worldwide. The present study investigates the underlying molecular and neural mechanisms using a *Drosophila* model in which chronic stress treatments were employed to induce learning and memory deficits associated with a depression-like state. We found that chronic stress induces chronic disruption of autophagic flux which leads to a learning deficit and that neuropeptide F (the *Drosophila* homolog of neuropeptide Y) promotes resilience to chronic stress–induced learning deficit (CSLD). Furthermore, our data suggest that neuropeptide F signaling protects against CSLD by inhibiting stress-induced dopaminergic activity and maintaining autophagic flux. These findings provide important mechanistic insights into the etiology of depression-associated cognitive disorders and suggest potential therapeutic targets.

While acute stress can have beneficial effects on brain function, chronic stress often leads to a range of mental disorders, such as depression, schizophrenia, mania, anxiety, and Alzheimer's disease (1–3). Although extensively studied, the molecular and cellular mechanisms underlying susceptibility and resilience to chronic stress remain poorly understood, partly due to complicated neural circuits and cellular signaling pathways involved (4–9). To cope with this complexity, chronic stress paradigms have been developed in the relatively simple *Drosophila melanogaster*. Chronic stress can induce depression-like states in *Drosophila*, characterized by a variety of behavioral aberrations that indicate lack of motivation, anhedonia, proneness to despair, and cognitive impairment. Importantly, feeding antidepressants could relieve some of the depression-like behavioral symptoms (10–12). Mechanistically, reduced serotonin activity in the mushroom body (MB), a *Drosophila* brain center for controlling adaptive behaviors, has been linked to the lack of gap-climbing motivation (10, 13), while dopaminergic (DAergic) activity mediates chronic stress signals to induce abnormal neural activities in MBs that lead to chronic stress–induced learning deficit (CSLD) (12). However, the molecular and cellular mechanisms underlying the etiology of *Drosophila* depression-like state still lack study. Here, we first used transcriptome profiling to probe the signaling pathways associated with the development of the chronic stress–induced depression-like state. We then demonstrated that chronic stress–induced disruption of autophagic flux (CSDA) promotes the development of CSLD. Moreover, we found that neuropeptide F (NPF), the *Drosophila* homolog of neuropeptide Y (NPY), is necessary for maintaining normal autophagic flux, and NPFergic activity promotes resilience to CSLD by preventing the disruption of autophagic flux. Our data indicated that the protective effect of NPF on CSLD is mediated by the inhibition of DAergic activity.

Results

Transcriptome Profiling Revealed That the Autophagy-Lysosome Pathway Is Associated with a Chronic Stress–Induced Depression-Like State. In our previous study, we established a chronic stress paradigm that 4 d of chronic stress treatments (CST) could induce a

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significant learning deficit that is associated with a depression-like state (Fig. 1*A*) (12). In our chronic stress treatment, in addition to daily mechanical shock, flies are maintained under crowding condition, which in itself is a stressful condition. We sampled fly heads at five time points (0, 3, 24, 27, and 96-h) during the process of CST for RNA-sequencing (RNA-seq) analyses (Fig. 1*A*). By 96-h, the flies showed a strong learning deficit, whereas learning performance appeared normal at earlier time points (*SI Appendix*, Fig. S1) (12). We compared gene expression between the initial time point (0-h) and the subsequent time points. Global gene-expression profiles changed drastically while *Drosophila* transitioned from the normal state (0-h) to the depression-like state (96-h). In the first 3 h, the expression of 1,880 genes was changed, and these differentially expressed genes (DEGs) were predominantly up-regulated (*SI Appendix*, Fig. S2*D*). Similar patterns of gene expression changes were observed across the other time points. A total of 2,594 DEGs were identified after combining the DEGs between each time point and 0-h (*SI Appendix*, Fig. S2*D*) (Dataset S2). Enrichment analyses suggested that these DEGs are mainly enriched in the regulation of carbon metabolism, oxidative phosphorylation, glycolysis, biosynthesis of amino acids, pentose phosphate, proteasome, mitochondria, and peptidase (*SI Appendix*, Fig. S2 *E* and *F*). With Venn analysis, we found that 1,580 out of 2,594 DEGs were shared across all time points, suggesting that a subset of genes may be persistently active. Moreover, there are 26 unique DEGs at the 3-h time point, 215 unique DEGs at 24-h, 22 unique DEGs at 27-h, and 233 unique DEGs at 96-h. These findings suggest that gene expression patterns undergo dynamic changes during the course of CST, indicating different states of the flies at each respective time point (Fig. 1*B*). To identify the regulators of the etiology of chronic stress–induced depression-like state, we performed time-series cluster analyses with the 2,594 DEGs, which resulted in five gene clusters each exhibiting distinct temporal dynamic patterns (Fig. 1*C*) (Dataset S3). Clusters 1 and 5 drew our attention because the majority of the 233 endpoint DEGs distribute in these clusters (Fig. 1*D*) (Dataset S4). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses showed that autophagy and lysosome are enriched in clusters 1 and 5, respectively (Fig. 1*E*), suggesting that the autophagy-lysosome pathway might be associated with the development of chronic stress–induced depression-like state. Consistent with this idea, we identified a total of 58 autophagy-lysosomal genes from the 2,594 DEGs (*SI Appendix*, Fig. S2*G*) (Dataset S5). We randomly selected some of these genes and verified the expression pattern at the 96-h time point by qRT-PCR (*SI Appendix*, Fig. S2*H*). The autophagy-lysosome pathway is an evolutionarily conserved stress response pathway that degrades abnormal proteins and damaged organelles to maintain cellular homeostasis and has been linked to many neural disorders including depression (14–17). Thus, our transcriptome analyses suggested that the autophagy-lysosome pathway might have important roles in the etiology of chronic stress–induced depression-like state.

Chronic Stress Induces the Disruption of Autophagic Flux. There are three main subtypes of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is the major form of autophagic degradation that responds to a variety of environmental and cellular stresses (18). We thus focus here only on macroautophagy, which is referred to as autophagy in this study. Because changes in RNA levels could be interpreted as either disease-causing or compensatory, we proceeded to investigate further the effects of CST on the autophagy-lysosome pathway with other techniques. We assessed autophagy level by western blot (WB) to measure Atg8a (the *Drosophila* homolog of Atg8/LC3) and Ref(2)p (the *Drosophila* homolog of SQSTM1/p62) (19). Similar to LC3, Atg8a can be detected in two forms, Atg8a-I (cytosolic) and Atg8a-II (conjugated to phosphatidylethanolamine and present in the inner and outer membranes of autophagosomes and autolysosomes). An increase in the Atg8a-II/Atg8a-I ratio in WB indicates autophagy activation or degradation block (20, 21). Ref(2)p/p62 is a well-studied marker for autophagic flux, increased levels of Ref(2)p signals autophagy inhibition or degradation block (22). Compared with no-treatment controls, both the Atg8a-II/Atg8a-I ratio and Ref(2)p level were significantly up-regulated after CST (Fig. 1 *F*–*H*). The increases were not attributed to changes at the transcription level, as Ref(2)p and Atg8a mRNA levels did not increase after CST (Fig. 1 *I* and *J*). These data suggest that CST might induce the disruption of autophagic flux since protein degradation is blocked after CST. We verified this idea by feeding flies with chloroquine (CQ), a widely used autophagic flux blocker (23). CQ feeding mimicked the effects of CST on the Atg8a-II/Atg8a-I ratio and Ref(2)p level, and importantly did not further enhance the Atg8a-II/Atg8a-I ratio and Ref(2)p level of the CST group (Fig. 1 *K*–*M*). Consistently, ubiquitin-conjugated proteins, a marker for incomplete autophagic flux (24), were accumulated in the CST group (Fig. 1 *N* and *O*). Since the disruption of autophagic flux is often associated with lysosome abnormalities (19), we examined lysosomes by staining fly brains with LysoTracker Red DND-99. The CST group showed stronger LysoTracker signals in the central brain compared with the no-treatment control. Both the puncta counts and the total puncta area increased significantly after CST (Fig. 1 *P*–*R*). These lysosome abnormalities indicate that protein degradation insufficiency might be caused by chronic stress–induced lysosome dysfunction. Consistent with this hypothesis, the active or mature form of cathepsin L, m-CTSL, was significantly down-regulated by CST (Fig. 1 *S* and *T*). Together, the above data demonstrate that CST induces lysosome dysfunction, which in turn leads to the disruption of autophagic flux. Interestingly, the level of phosphorylated S6-Kinase (pS6K-T398), a readout of mTOR activity, was diminished after CST (Fig. 1 *U* and *V*). This is in line with the idea that chronic stress induces lysosome dysfunction, as a downregulation of mTOR activity could be detected after the suppression of lysosome function (25).

Chronic Disruption of Autophagic Flux Results in a Learning Deficit. Since chronic stress treatment could lead to the disruption of autophagic flux (Fig. 1) as well as learning deficits (Fig. 2*A*) (12), we wondered whether the disruption of autophagic flux might be responsible for CSLD. We tested this idea by feeding flies with the autophagic flux blocker, CQ. Although 3 h of CQ feeding resulted in normal olfactory learning (Fig. 2*B*), 3 d of CQ treatment was sufficient to induce a significant learning deficit (Fig. 2*C*), suggesting that chronic but not acute disruption of autophagic flux leads to a learning deficit. Remarkably, feeding flies with CQ during the 4-d CST did not exacerbate the learning deficit induced by chronic stress (Fig. 2*D*). Importantly, neither CST nor CQ feeding diminished shock reactivity and odor acuity (*SI Appendix*, Fig. S3). These data support our hypothesis that chronic disruption of autophagic flux accounts for CSLD.

Preventing the Disruption of Autophagy Is Sufficient to Protect against CSLD. We reasoned that if chronic disruption of autophagic flux is the underlying cause of CSLD, preventing the disruption of autophagy should protect against CSLD. Since moderately elevating basal autophagy levels could have beneficial effects on aging and neurodegeneration by better maintaining cellular homeostasis (24, 26, 27), we speculated that upregulation of basal

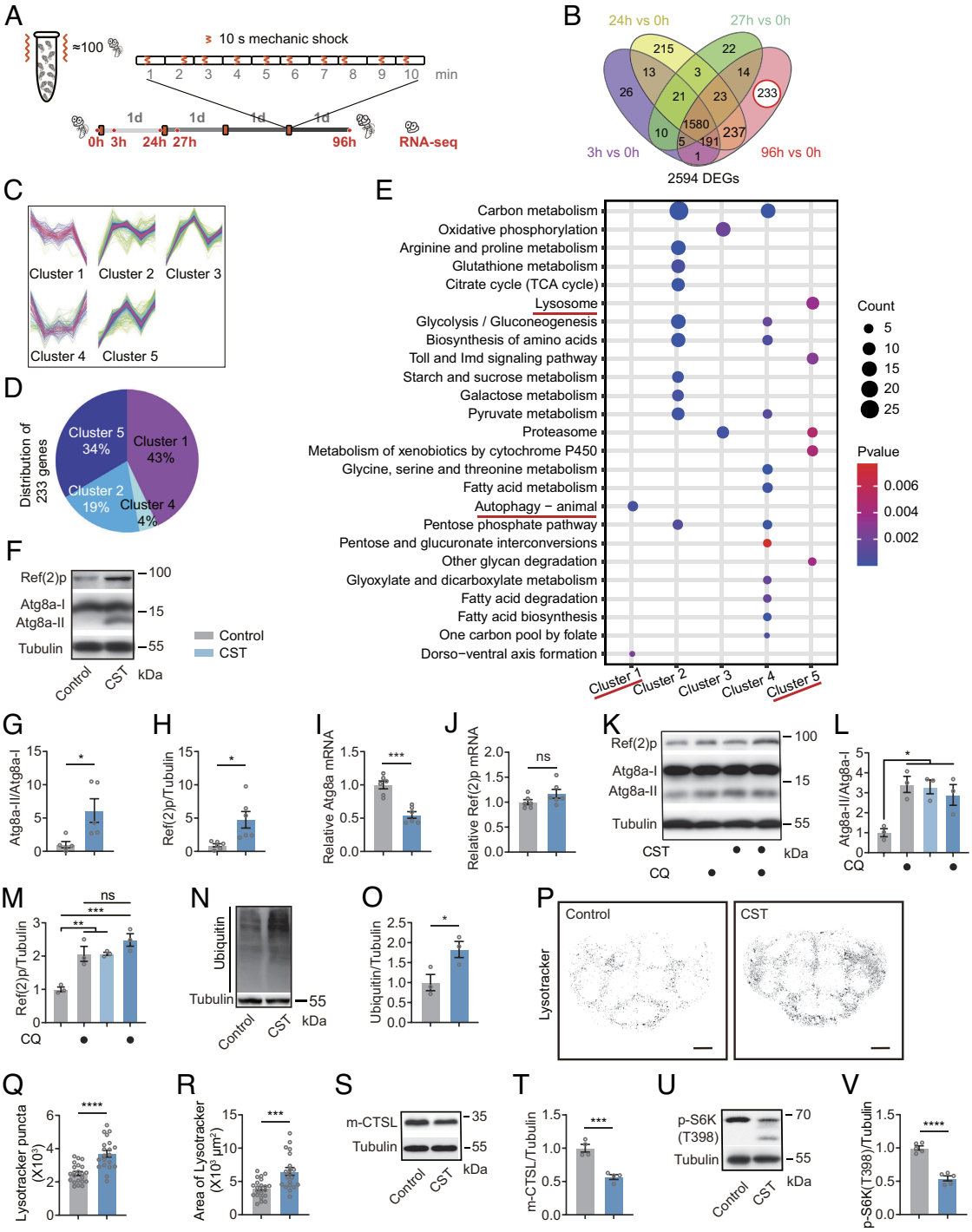

Fig. 1. CST induces the disruption of autophagic flux. (*A*) Schematic of the standard CST procedure and sampling time for RNA-Seq. Red spots indicate the time points (0, 3, 24, 27, and 96-h) of fly head sample collection. (*B*) A total of 2,594 differentially expressed genes between each time point and 0-h were presented with a Venn chart. Note that among the 2,594 DEGs, 233 genes were unique for 96-h vs. 0-h. (*C*) Time-series cluster analyses with all of the 2,594 DEGs resulted in the five gene clusters. (*D*) The distribution of the 233 endpoint DEGs in five gene clusters. Note that the majority of these genes distribute in cluster I and cluster V. (*E*) KEGG analyses of the five gene Clusters. (*F–H*) Western blot images and quantification of Atg8a-II/Atg8a-I ratio and Ref(2)p protein levels in adult head extracts to examine the impact of CST on autophagy. Atg8a-II/Atg8a-I ratio was significantly higher in CST than in control (Welch's corrected *t* test, $^{*}P < 0.05$, n = 5) (*G*); Ref(2)p level was significantly increased after CST as well (Welch's corrected *t* test, $^{*}P < 0.05$, n = 6) (*H*). (*I* and *J*) qRT-PCR of Atg8a and Ref(2)p to examine their mRNA levels in the adult head. Chronic stress down-regulated Atg8a mRNA level (*t* test, $^{***}P < 0.001$; n = 6) (*I*), while the mRNA level of Ref(2)p was not affected by CST (*t* test, $P > 0.05$; n = 6) (*J*). (*K–M*) Western blot images and quantification of Atg8a-II/Atg8a-I ratio and Ref(2)p. CQ feeding significantly increased the Atg8a-II/Atg8a-I ratio in control (one-way ANOVA, $^{**}P < 0.01$; Tukey's test, $^{**}P < 0.01$, n = 3) (*L*), but not CST (Tukey's test, $P > 0.05$, n = 3) (*L*). Similarly, CQ feeding also significantly increased Ref(2)p level in control (one-way ANOVA, $^{***}P < 0.001$; Tukey's test, $^{**}P < 0.01$, n = 3) (*M*), but not CST (Tukey's test, $P > 0.05$, n = 3) (*M*). (*N* and *O*) Western blot images and quantification of ubiquitinated proteins in adult head extracts. The amount of ubiquitin-conjugated proteins was significantly increased after CST (*t* test, $^{*}P < 0.05$; n = 3). (*P–R*) Images and quantification of LysoTracker staining. Representative images of the central brain region of adult flies were presented. (Scale bars represent 50 μm.) Chronic stress significantly increased both puncta number (Welch's corrected *t* test, $^{****}P < 0.0001$, n ≥ 20) (*Q*) and area (Welch's corrected *t* test, $^{***}P < 0.001$, n ≥ 20) (*R*). (*S* and *T*) Western blot images and quantification of mature CTSL. m-CTSL was significantly down-regulated after CST (*t* test, $^{***}P < 0.001$, n = 4). (*U* and *V*) Western blot images and quantification of pS6K-T398. pS6K-T398 level was significantly down-regulated after CST (*t* test, $^{****}P < 0.0001$; n = 5). Tubulin was the internal control in all western blot experiments. Data are represented as mean ± SEM. The stars indicate significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$); ns indicates not significant ($P > 0.05$). See also *SI Appendix*, Figs. S1 and S2.

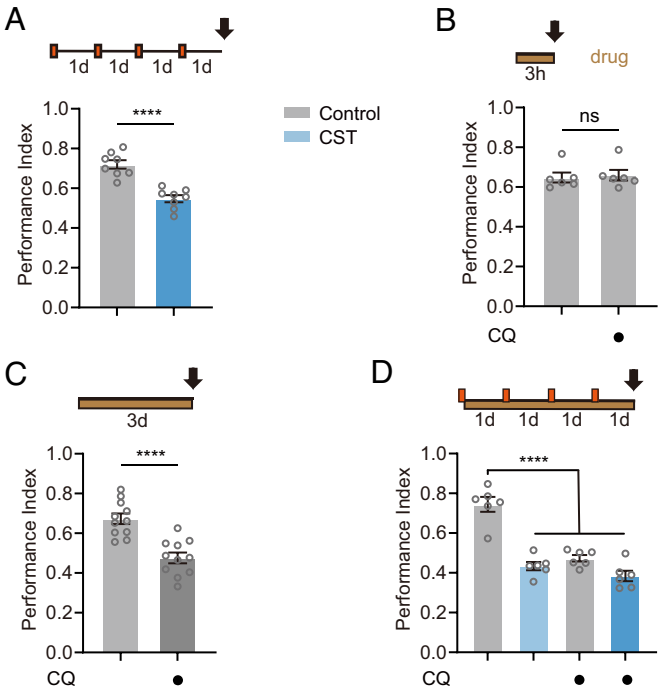

Fig. 2. Chronic disruption of autophagic flux results in a learning deficit. (*A*) Chronic stress induced a learning deficit (*t* test, ****$P < 0.0001$; n = 8). (*B*) Three hours of CQ feeding did not affect olfactory learning (Welch's corrected *t* test, $P > 0.05$, n = 6). (*C*) Three days of CQ feeding resulted in a significant learning deficit (*t* test, ****$P < 0.0001$, n = 11). (*D*) CQ feeding did not further decrease the learning performance of chronically stressed flies (one-way ANOVA, ****$P < 0.0001$; Tukey's test, $P > 0.05$, n = 6). Data are represented as mean ± SEM. The stars indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$); ns indicates not significant ($P > 0.05$). See also *SI Appendix*, Fig. S3.

autophagy levels might be able to ameliorate the stress-induced cytotoxicity, and thus prevent the development of CSDA. To raise the neuronal autophagic activity, we used the TARGET system to spatiotemporally overexpress Atg1, a kinase essential for autophagy induction (28, 29). TARGET system uses a ubiquitously expressed temperature-sensitive GAL80 ($GAL80^{ts}$) that represses GAL4's activity at low temperatures (e.g., 18 °C) but permits it at high temperatures (e.g., 30 °C) (30).To elevate the basal autophagy level in the adult brain, flies were raised to the adult stage at 18 °C and then shifted to 30 °C 2 d before CST to release the overexpression of Atg1. Although CST increased both the Atg8a-II/Atg8a-I ratio and Ref(2)p level in *UAS-Atg1*/+ controls, these CSDA phenotypes were abolished in *elav-GAL4; Tub-GAL80^{ts}/UAS-Atg1* flies (Fig. 3 *A–C*). This preventive effect on CSDA was not detected when flies were maintained under 18 °C to constantly silence the Atg1 expression (Fig. 3 *D–F*). These data suggested that up-regulating neuronal basal autophagy levels can prevent CSDA. In line with this finding, we noticed that pan-neural overexpression of *UASp-GFP-mCherry-Atg8a*, a commonly used reporter for monitoring autophagic flux, is sufficient to prevent CSDA as well (*SI Appendix*, Fig. S4). We then tested the CSLD phenotype of Atg1 overexpression. As shown in Fig. 3*G*, Atg1 pan-neural overexpression was sufficient to protect against CSLD, whereas keeping flies under 18 °C did not show a protective effect against CSLD (Fig. 3*H*). Thus, a higher basal autophagy level in neurons not only prevents CSDA but also protects against CSLD. Consistently, feeding flies with rapamycin (Rapa), a potent autophagy inducer (31), during CST ameliorated the learning deficit induced by chronic stress (Fig. 3*I*). Furthermore, CQ feeding during CST abolished the protective effect against CSLD by Atg1 overexpression (Fig. 3*J*), whereas shock reactivity and odor acuity stayed normal in all groups (*SI Appendix*, Fig. S5). Together, these data are in agreement with the notion that up-regulating basal autophagy levels protect against the development of CSLD by preventing the disruption of autophagy. To examine the effect of Atg1 overexpression on the reversal of chronic stress–induced phenotypes, flies were kept under 18 °C until CST was finished, then shifted to 30 °C to release the overexpression of Atg1. Interestingly, 2 d of Atg1 overexpression was not sufficient to rescue either the upregulation of Ref(2)p level (Fig. 3 *K–M*) or CSLD (Fig. 3*N*), indicating that augmentation of basal autophagy level might not be effective in reversing chronic stress–induced abnormalities. Based on the above findings, we conclude that preventing the disruption of autophagic flux promotes resilience to CSLD.

Dopamine Neural Activity Is Necessary for CSDA. In our previous study, we reported that DAergic activity mediates chronic stress signals to drive neuronal maladaptations and promote the development of CSLD (12). Given that CSDA accounts for CSLD, we predicted that DAergic activity should also be important for CSDA. To test this hypothesis, we used Shibire-based thermogenetic technique to acutely block neurotransmission. *TH-GAL4* that expresses in the majority of dopamine neurons (DANs) was used to drive *UAS-shi^{ts1}* which encodes a temperature-sensitive and dominant-negative mutant form of dynamin. Synaptic release from DANs could be conditionally inhibited by shifting from the permissive temperature (e.g., 18 °C) to the restrictive temperature (e.g., 30 °C) (32). As shown in Fig. 4 *A–C*, blocking the synaptic release from TH-labeled DANs only during the daily mechanical shock in the course of CST was sufficient to prevent CSDA. In contrast, the preventive effect on CSDA was absent if the flies were treated at the permissive temperature (Fig. 4 *D–F*). Thus, stress-induced hyperactivity of DANs is essential for chronic stress to disrupt autophagic flux. Together with all the above findings, we propose that DANs activity mediates chronic stress signals to induce the disruption of autophagic flux that precipitates susceptibility to CSLD.

NPFergic Activity Maintains Autophagic Flux and Prevents CSDA. Neuropeptide functions are important for both autophagy and depression (33–35). To identify genes that influence resilience to CSLD, we screened the neuropeptide mutants of the chemoconnectome knockout collection(36). These mutants cover most of the neuropeptide genes in the *Drosophila melanogaster* genome (37 out of 40, *SI Appendix*, Table S1). We first screened for abnormal autophagic flux in the adult head and identified 4 mutants, including NPF^{attP}, $AstA^{attP}$, ETH^{attP}, and $CAPA^{attP/+}$. Both the Atg8a-II/Atg8a-I ratio and Ref(2)p level were significantly up-regulated in each of these mutants compared with wild-type (WT) controls (Fig. 5 *A–C* and *SI Appendix*, Fig. S6 and Table S2), indicating that autophagic flux was dampened in these mutants. We reasoned that these mutants should be more susceptible to CSLD. To test this idea, instead of the standard 4-d CST, a 3-d chronic stress treatment (3-d CST) was applied. As expected, the 3-d CST did not affect olfactory learning in WT (*SI Appendix*, Fig. S7). However, 3-d CST induced a significant learning deficit in NPF^{attP} but not the other three mutants, suggesting that NPF might promote resilience to CSLD (*SI Appendix*, Fig. S7). We thus focused on the NPF in the rest of this study.

We verified that NPF is required to maintain autophagic flux by transgenic RNAi approach. As shown in Fig. 5 *D–F*, RNAi knockdown of NPF with NPF-GAL4 is sufficient to up-regulate both the Atg8a-II/Atg8a-I ratio and Ref(2)p level in the fly head. Furthermore,

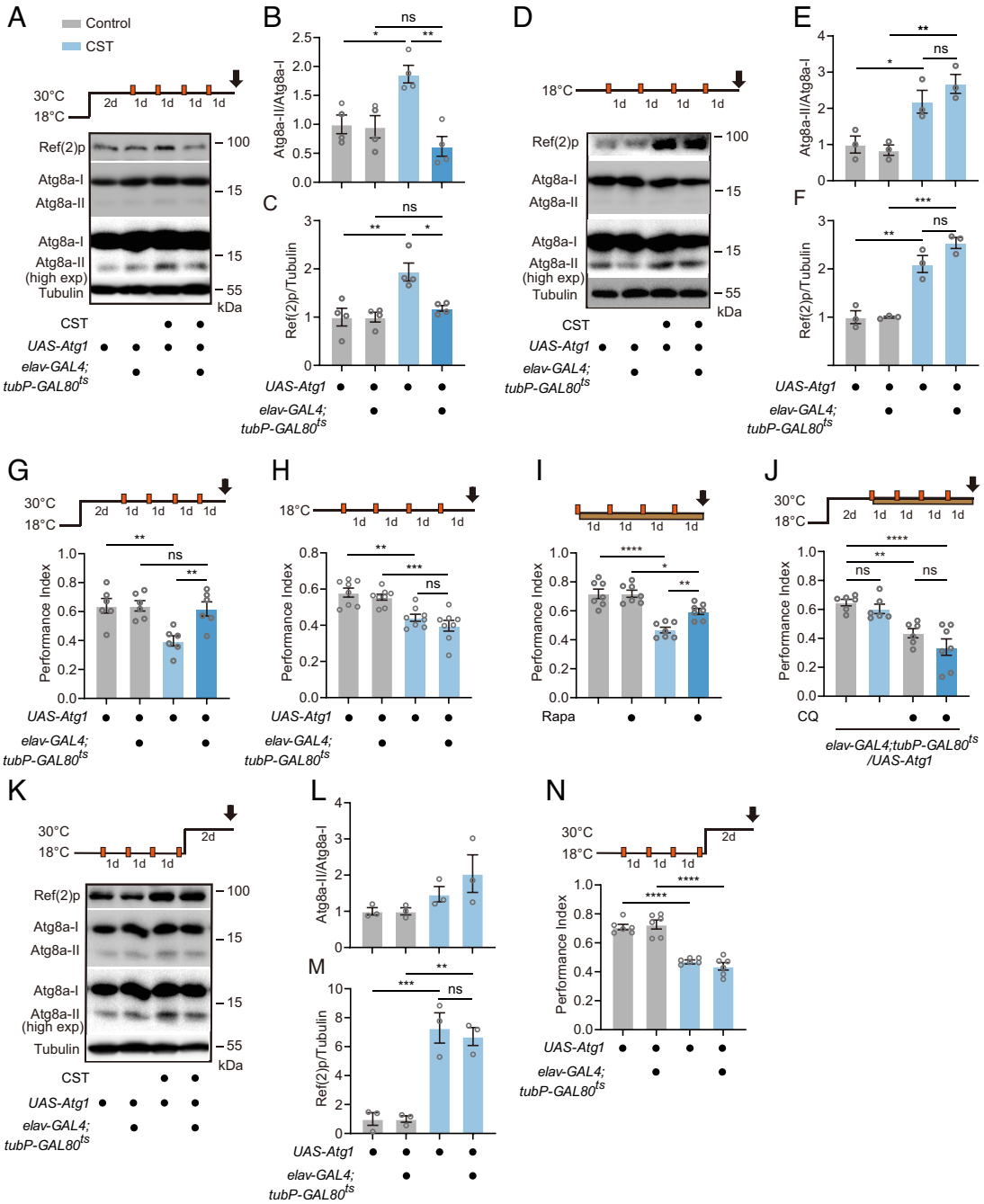

Fig. 3. Preventing CSDA is sufficient to protect against CSLD. (*A–C*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio and Ref(2)p protein levels to examine the effects of Atg1 pan-neural overexpression on CSDA phenotypes. Flies were shifted to 30 °C 2 d before CST to release the overexpression of Atg1. Chronic stress induced the increase of the Atg8a-II/Atg8a-I ratio in *UAS-Atg1/+* (one-way ANOVA, $^{**}P < 0.01$; Tukey's test, $^{*}P < 0.05$, n = 4), but not *elav-GAL4; tubP-GAL80^{ts}/UAS-Atg1* (Tukey's test, $P > 0.05$, n = 4) (*B*). Similarly, chronic stress also induced the increase of Ref(2)p level in *UAS-Atg1/+* (one-way ANOVA, $^{**}P < 0.01$; Tukey's test, $^{**}P < 0.01$, n = 4), but not *elav-GAL4; tubP-GAL80^{ts}/UAS-Atg1* (Tukey's test, $P > 0.05$, n = 4) (*C*). (*D–F*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio and Ref(2)p protein level for the temperature controls of (*A–C*). Flies were maintained under 18 °C to constantly silence the Atg1 expression. Under this condition, chronic stress significantly increased both the Atg8a-II/Atg8a-I ratio (one-way ANOVA, $^{**}P < 0.01$; Tukey's test, $^{**}P < 0.01$, n = 3) (*E*) and Ref(2)p level (one-way ANOVA, $^{****}P < 0.0001$; Tukey's test, $^{***}P < 0.001$, n = 3) (*F*) in *elav-GAL4; tubP-GAL80^{ts}/UAS-Atg1*. (*G*) Atg1 overexpression protects against CSLD. While chronic stress induced a learning deficit in *UAS-Atg1/+* (one-way ANOVA, $^{**}P < 0.01$; Tukey's test, $^{**}P < 0.01$, n = 6), the learning performance of *elav-GAL4; tubP-GAL80^{ts}/UAS-Atg1* was not affected (Tukey's test, $P > 0.05$, n = 6). (*H*) Learning performances of the temperature controls for (*G*). Chronic stress induced learning deficits in both *UAS-Atg1/+* (one-way ANOVA, $^{****}P < 0.0001$; Tukey's test, $^{**}P < 0.01$, n = 6) and *elav-GAL4; tubP-GAL80^{ts}/UAS-Atg1* (Tukey's test, $^{***}P < 0.001$, n = 6). (*I*) Rapa feeding during the course of CST ameliorated CSLD. After CST, the learning performance of the Rapa feeding group was significantly better than the no feed control (one-way ANOVA, $^{****}P < 0.0001$; Tukey's test, $^{**}P < 0.01$, n = 7). (*J*) CQ feeding abolished the protective effect against CSLD of Atg1 overexpression. The learning performance of *elav-GAL4; tubP-GAL80^{ts}/UAS-Atg1* was not affected by CST (one-way ANOVA, $^{****}P < 0.0001$; Tukey's test, $P > 0.05$, n = 6), while feeding these flies with CQ during CST resulted in significant learning deficit (Tukey's test, $^{****}P < 0.0001$, n = 6). (*K–M*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio and Ref(2)p protein level to show that Atg1 overexpression was less effective in reversing the chronic stress–induced Ref(2)p upregulation. Flies were shifted to 30 °C after CST to release the overexpression of Atg1 for 2 d. After CST, the Atg8a-II/Atg8a-I ratio showed a nonsignificant trend of increase in both *UAS-Atg1/+* and *elav-GAL4; tubP-GAL80^{ts}/UAS-Atg1* (one-way ANOVA, $P > 0.05$, n = 3 for both) (*L*), while Ref(2)p levels were significantly increased in both *UAS-Atg1/+* (one-way ANOVA, $^{***}P < 0.001$; Tukey's test, $^{***}P < 0.001$, n = 3) (*M*) and *elav-GAL4; tubP-GAL80^{ts}/UAS-Atg1* (Tukey's test, $^{**}P < 0.01$, n = 3) (*M*). (*N*) Atg1 overexpression after CST was less effective in reversing CSLD. Chronic stress induced learning deficit in both *UAS-Atg1/+* (one-way ANOVA, $^{****}P < 0.0001$; Tukey's test, $^{****}P < 0.0001$, n = 6) and *elav-GAL4; tubP-GAL80^{ts}/UAS-Atg1* (Tukey's test, $^{****}P < 0.0001$, n = 6). Tubulin was the internal control in all western blot experiments. Data are represented as mean ± SEM. The stars indicate significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$); ns indicates not significant ($P > 0.05$). See also *SI Appendix*, Figs. S4 and S5.

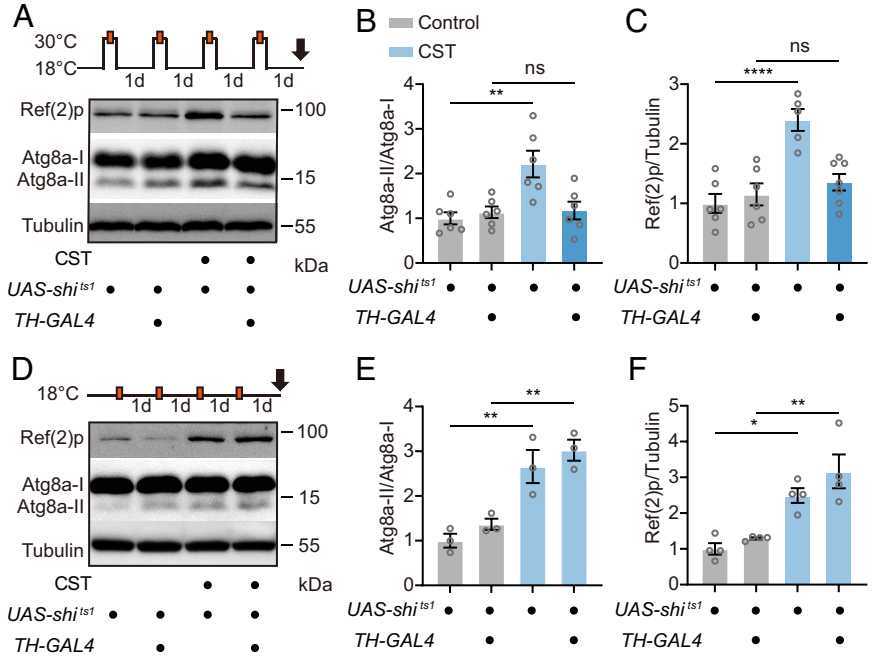

Fig. 4. Dopamine neural activity is necessary for CSDA. (*A–C*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio and Ref(2)p to show the necessity of DAN synaptic transmission for CSDA. Flies were shifted to 30 °C during the daily vibration treatment to block synaptic release from DANs. Chronic stress induced the increase of the Atg8a-II/Atg8a-I ratio in *UAS-shi^{ts1}/+* (one-way ANOVA, $^{**}P < 0.01$; Tukey's test, $^{**}P < 0.01$, n = 6), but not *TH-GAL4/UAS-shi^{ts1}* (Tukey's test, $P > 0.05$, n = 6) (*B*). Similarly, chronic stress also induced the increase of Ref(2)p level in *UAS-shi^{ts1}/+* (one-way ANOVA, $^{****}P < 0.0001$; Tukey's test, $^{****}P < 0.0001$, n ≥ 5), but not *TH-GAL4/UAS-shi^{ts1}* (Tukey's test, $P > 0.05$, n ≥ 5) (*C*). (*D–F*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio and Ref(2)p for the temperature controls of *A–C*. Flies were maintained under 18 °C during CST. Under this condition, chronic stress significantly increased both the Atg8a-II/Atg8a-I ratio (one-way ANOVA, $^{***}P < 0.001$; Tukey's test, $^{**}P < 0.01$, n = 3) (*E*) and Ref(2)p level (one-way ANOVA, $^{***}P < 0.001$; Tukey's test, $^{**}P < 0.01$, n = 4) (*F*) in *TH-GAL4/UAS-shi^{ts1}*. Tubulin was the internal control in all western blot experiments. Data are represented as mean ± SEM. The stars indicate significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$); ns indicates not significant ($P > 0.05$).

feeding CQ did not further enhance the Atg8a-II/Atg8a-I ratio and Ref(2)p level in the *NPF^{attP}* mutant (Fig. 5 *G–I*). Although there were no significant differences in ubiquitin-conjugated protein levels between the WT and *NPF^{attP}* flies (Fig. 5 *J* and *K*), we observed a significant accumulation of ubiquitin-conjugated proteins in *NPF^{attP}* flies following a 3-d CST (Fig. 5 *L* and *M*). Consistent with the idea that autophagic flux is disrupted in *NPF^{attP}*, abnormal LysoTracker signals were observed in the central brain. Both the puncta counts and total puncta area exhibited a significant increase in *NPF^{attP}* (Fig. 5 *N–P*). Additionally, the active or mature form of cathepsin L, m-CTSL, displayed a significant downregulation in *NPF^{attP}* (Fig. 5 *Q* and *R*). Collectively, these findings provide evidence of aberrant autophagic flux resulting from lysosome dysfunction in the *NPF* mutant, indicating a crucial role for NPF in maintaining normal autophagic flux.

Based on the above genetic evidence, we hypothesized that NPFergic neural activity might promote resilience to CSDA. To test this hypothesis, we acutely activated NPF neurons with another thermogenetic tool, the heat-activated ion channel transient receptor potential channel A1 (TrpA1) (37, 38). Activating NPF neurons only during mechanical shock treatments prevented the development of CSDA (Fig. 5 *S–U*), whereas CSDA phenotypes were not affected in low temperature (18 °C) (Fig. 5 *V–X*). Together, these data suggested that NPFergic activity maintains normal autophagic flux and therefore prevents CSDA.

Neuropeptide F Signaling Promotes Resilience to CSLD by Preventing CSDA. Given that NPFergic activity prevents CSDA and preventing CSDA promotes resilience to CSLD, NPFergic activity should also promote resilience to CSLD. To test this idea, we first showed that *NPF^{sk1}*, another NPF knockout mutant, reproduced the CSLD-susceptible phenotype of *NPF^{attP}* (Fig. 6*A*), whereas shock reactivity and odor acuity were normal in all groups (*SI Appendix*, Fig. S8 *A–C*). Importantly, the CSLD-susceptible phenotype of *NPF^{sk1}* was fully rescued by restoring NPF function with *NPF*-GAL4 driving *UAS-NPF* (Fig. 6*A*). Furthermore, knockdown of NPF expression with *NPF*-GAL4 driving *UAS-NPF-RNAi* also exhibited the CSLD-susceptible phenotype (Fig. 6*B*), and yet shock reactivity and odor acuity were not affected (*SI Appendix*, Fig. S8 *D–F*). These data corroborated the idea that NPF promotes resilience to CSLD. We then activated NPF neurons with *NPF*-GAL4 driving *UAS-TrpA1*. Activation of NPF neurons only during mechanical shock treatments protected against the development of CSLD (Fig. 6*C*), whereas CSLD stayed significant if the flies were kept at 18 °C (Fig. 6*D*). Remarkably, CQ feeding during CST abolished the protective effect of NPF activation against CSLD (Fig. 6*E*), while shock reactivity and odor acuity were not affected (*SI Appendix*, Fig. S8 *G–I*). These data support the idea that NPFergic activity promotes resilience to CSLD by preventing the disruption of autophagic flux. Besides NPF, NPF neurons express many other neuropeptides, as well as neurotransmitters and neuromodulators, as indicated by cell-type-specific transcriptome analyses (39). To validate that NPF per se involves in the protection against CSLD, we used *NPF*-GAL4 to simultaneously drive *UAS-TrpA1* and *UAS-NPF-RNAi*. As shown in Fig. 6*F*, RNAi knockdown of NPF abrogated the protective effect against CSLD of NPF neural activation, while shock reactivity and odor acuity were not affected (*SI Appendix*, Fig. S8 *J–L*), suggesting that NPF is indispensable for the protection against CSLD. Interestingly, activating NPF neurons for 2 d after CST failed to reverse the learning deficit induced by chronic stress (Fig. 6*G*). Thus, NPFergic activity could effectively prevent the onset of CSLD but is less effective in reversing CSLD.

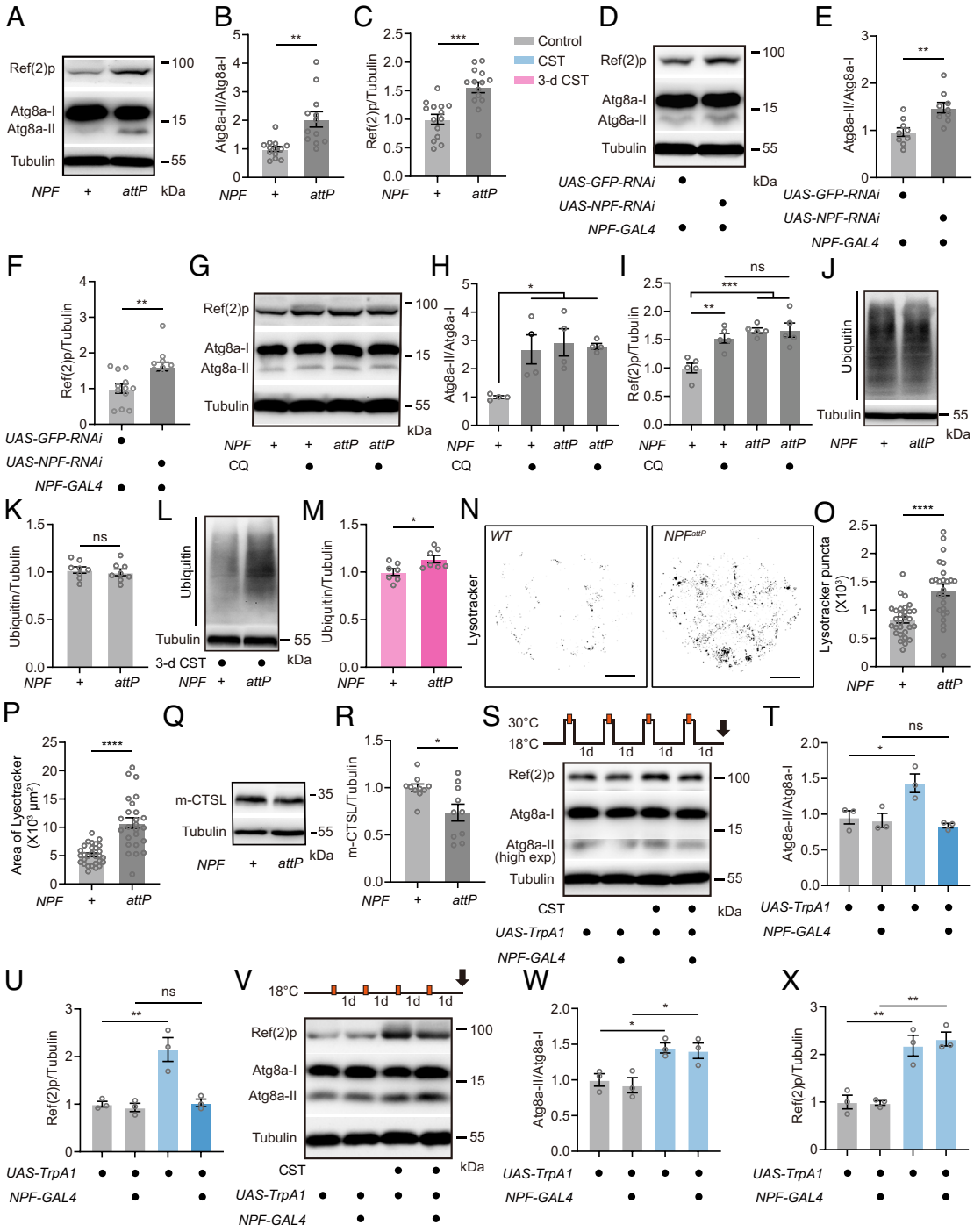

Fig. 5. Neuropeptide F signaling maintains autophagic flux and alleviates CSDA. (*A–C*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio (Welch's corrected *t* test, $^{**}P < 0.01$; n ≥ 12) (*B*) and Ref(2)p (*t* test, $^{***}P < 0.001$; n = 14) (*C*) in head extracts from WT and NPF^{attP}. (*D–F*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio (*t* test, $^{**}P < 0.01$; n = 9) (*E*) and Ref(2)p (*t* test, $^{**}P < 0.01$; n = 12) (*F*) in head extracts from *NPF-GAL4/UAS-GFP-RNAi* and *NPF-GAL4/UAS-NPF-RNAi*. (*G–I*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio and Ref(2)p. CQ feeding significantly increased the Atg8a-II/Atg8a-I ratio in WT (one-way ANOVA, $^{**}P < 0.01$; Tukey's test, $^{*}P < 0.05$, n = 4) (*H*), but not NPF^{attP} (Tukey's test, $P > 0.05$, n = 4) (*H*). Similarly, CQ feeding also significantly increased Ref(2)p level in WT (one-way ANOVA, $^{***}P < 0.001$; Tukey's test, $^{**}P < 0.01$, n = 5) (*I*), but not NPF^{attP} (Tukey's test, $P > 0.05$, n = 5) (*I*). (*J* and *K*) Western blot images and quantification of ubiquitinated proteins in adult head extracts from WT and NPF^{attP} (*t* test, $P > 0.05$; n = 8). (*L* and *M*) Western blot images and quantification of ubiquitinated proteins in adult head extracts. The amount of ubiquitin-conjugated proteins was significantly increased in NPF^{attP} after 3-d CST (*t* test, $^{*}P < 0.05$; n = 7). (*N–P*) Images and quantification of LysoTracker staining. Representative images of the central brain region of adult flies were presented. (Scale bars represent 100 μm.) Both puncta number (Welch's corrected *t* test, $^{****}P < 0.0001$, n ≥ 25) (*O*) and area (Welch's corrected *t* test, $^{****}P < 0.001$, n ≥ 25) (*P*) were significantly increased in *NPF* mutant. (*Q* and *R*) Western blot images and quantification of mature CTSL. m-CTSL was significantly down-regulated in NPF^{attP} (Welch's corrected *t* test, $^{*}P < 0.05$, n = 10). (*S–U*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio and Ref(2)p to examine the effects of activating NPF neurons on CSDA phenotypes. Flies were shifted to 30 °C during the daily vibration treatment to activate NPF neurons. Chronic stress induced the increase of the Atg8a-II/Atg8a-I ratio in *UAS-TrpA1/+* (one-way ANOVA, $^{**}P < 0.01$; Tukey's test, $^{*}P < 0.05$, n = 3), but not *NPF-GAL4/UAS-TrpA1* (Tukey's test, $P > 0.05$, n = 3) (*T*). Similarly, chronic stress also induced the increase of Ref(2)p level in *UAS-TrpA1/+* (one-way ANOVA, $^{***}P < 0.001$; Tukey's test, $^{**}P < 0.01$, n = 3), but not *NPF-GAL4/UAS-TrpA1* (Tukey's test, $P > 0.05$, n = 3) (*U*). (*V–X*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio and Ref(2)p for the temperature controls of *S–U*. Flies were maintained under 18 °C during CST. Under this condition, chronic stress significantly increased both the Atg8a-II/Atg8a-I ratio (one-way ANOVA, $^{**}P < 0.01$; Tukey's test, $^{*}P < 0.05$, n = 3) (*W*) and Ref(2)p level (one-way ANOVA, $^{***}P < 0.001$; Tukey's test, $^{**}P < 0.01$, n = 3) (*X*) in *NPF-GAL4/UAS-TrpA1*. Tubulin was the internal control in all western blot experiments. Data are represented as mean ± SEM. The stars indicate significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$); ns indicates not significant ($P > 0.05$). See also *SI Appendix*, Figs. S6 and S7.

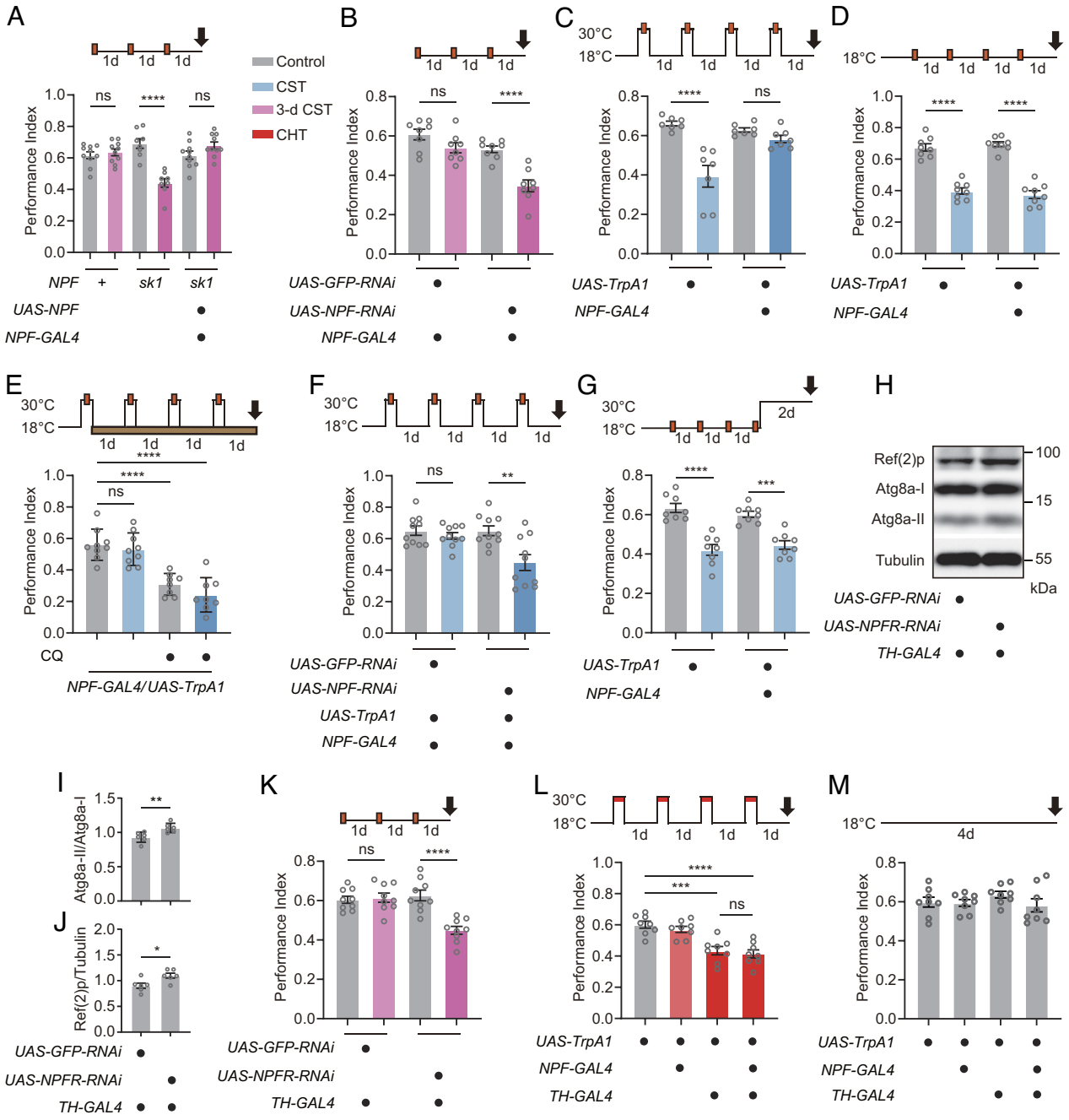

Fig. 6. Neuropeptide F signaling promotes resilience to CSLD by preventing CSDA. (*A*) NPF knockout mutant, NPF^{sk1}, exhibited a CSLD-susceptible phenotype that can be rescued by restoring NPF function. Olfactory learning was not affected by 3-d CST in WT (one-way ANOVA, ****$P < 0.0001$; Tukey's test, $P > 0.05$, n = 10). Although 3-d CST induced a learning deficit in NPF^{sk1} (Tukey's test, ****$P < 0.0001$, n = 8), olfactory learning was not affected in *NPF-GAL4/UAS-NPF;* NPF^{sk1}/NPF^{sk1} (Tukey's test, $P > 0.05$, n = 10). (*B*) NPF RNAi knockdown exhibited the CSLD-susceptible phenotype. 3-d CST induced a learning deficit in *NPF-GAL4/UAS-NPF-RNAi* (one-way ANOVA, ****$P < 0.0001$; Tukey's test, ****$P < 0.0001$, n = 8), but not *NPF-GAL4/UAS-GFP-RNAi* (Tukey's test, $P > 0.05$, n = 8). (*C*) Thermogenetic activation of NPF neurons during the daily vibration treatments protected against CSLD. Flies were shifted to 30 °C to activate NPF neurons. Chronic stress induced learning deficit in *UAS-TrpA1/+* (one-way ANOVA, ****$P < 0.0001$; Tukey's test, ****$P < 0.0001$, n = 7), but not *NPF-GAL4/UAS-TrpA1* (Tukey's test, $P > 0.05$, n = 7). (*D*) Learning performances of the temperature controls for (*C*). Chronic stress induced learning deficit in both *UAS-TrpA1/+* (one-way ANOVA, ****$P < 0.0001$; Tukey's test, ****$P < 0.0001$, n = 8) and *NPF-GAL4/UAS-TrpA1* (Tukey's test, ****$P < 0.0001$, n = 8). (*E*) CQ feeding abolished the protective effect against CSLD of NPF neuronal activation. The learning performance of *NPF-GAL4/UAS-TrpA1* was not affected by CST (one-way ANOVA, ****$P < 0.0001$; Tukey's test, $P > 0.05$, n = 9), while feeding with CQ during CST significantly diminished learning performance (Tukey's test, ****$P < 0.0001$, n ≥ 8). (*F*) NPF RNAi knockdown abrogated the protective effect against CSLD of NPF neural activation. The learning performance of *NPF-GAL4/UAS-TrpA1; UAS-GFP-RNAi* was not affected by CST (one-way ANOVA, ***$P < 0.001$; Tukey's test, $P > 0.05$, n = 10), while the learning performance of *NPF-GAL4/UAS-TrpA1; UAS-NPF-RNAi* was significantly diminished after CST (Tukey's test, **$P <$ 0.01, n = 10). (*G*) Activating NPF after CST was not effective in reversing CSLD. Chronic stress induced learning deficit in both *UAS-TrpA1/+* (one-way ANOVA, ****P < 0.0001; Tukey's test, ****$P < 0.0001$, n = 8) and *NPF-GAL4/UAS-TrpA1* (Tukey's test, ***$P < 0.001$, n = 8). (*H–J*) Western blot images and quantification of the Atg8a-II/Atg8a-I ratio and Ref(2)p level to examine the effects of NPFR downregulation in DANs. Tubulin was the internal control in all western blot experiments. Both Atg8a-II/Atg8a-I ratio (*t* test, **$P < 0.01$, n = 6) (*I*) and Ref(2)p level (*t* test, *$P < 0.05$, n = 6) (*J*) were significantly higher in *TH-GAL4/UAS-NPFR-RNAi* than *TH-GAL4/UAS-GFP-RNAi*. (*K*) NPFR knockdown in DANs also exhibited the CSLD-susceptible phenotype. 3-d CST induced a learning deficit in *TH-GAL4/UAS-NPFR-RNAi* (one-way ANOVA, ****$P < 0.0001$; Tukey's test, ****$P < 0.0001$, n = 9), but not *TH-GAL4/UAS-GFP-RNAi* (Tukey's test, $P > 0.05$, n = 9). (*L*) Thermogenetic activation of TH-labeled DANs for 1 h per day over a span of 4 d was sufficient to induce a learning deficit (one-way ANOVA, ****$P < 0.0001$; Tukey's test, ***$P < 0.001$, n = 8). This deficit could not be prevented by the simultaneous activation of NPF neurons (Tukey's test, ****$P < 0.0001$, n = 8). (*M*) Learning performances of the temperature controls for (*L*) (one-way ANOVA, $P > 0.05$, n = 8). Data are represented as mean ± SEM. The stars indicate significant differences (*$P < 0.05$, **$P <$ 0.01, ***$P < 0.001$, and ****$P < 0.0001$); ns indicates not significant ($P > 0.05$). See also *SI Appendix*, Fig. S8.

NPF function has been linked with DAN activity in previous reports (40–42). Since NPF neurons and DANs had opposite effects on CSLD, we speculated that inhibitory NPF signaling to DANs might involve in resilience to CSLD. To test this idea, we down-regulated the expression of NPF receptor (NPFR) in DANs with TH-GAL4 driving *UAS-NPFR-RNAi*. As shown in Fig. 6 *H–J*, RNAi knockdown of NPFR in DANs led to the upregulation of both Atg8a-II/Atg8a-I ratio and Ref(2)p level in the fly head, suggesting that NPFR function is required in DANs to maintain autophagic flux. Moreover, NPFR knockdown in DANs also exhibited the CSLD-susceptible phenotype (Fig. 6*K*), while shock reactivity and odor acuity were not affected (*SI Appendix*, Fig. S8 *M–O*), indicating that NPFR activity in DANs promotes resilience to CSLD. These data demonstrated that inhibitory NPF signaling to DANs maintains autophagic flux and promotes resilience to CSLD. To validate that NPF signaling protects against CSLD via DANs, we tested whether the learning deficit induced by chronic activation of DANs would be ameliorated by activating NPF neurons. As reported previously, chronic thermogenetic activation of DANs induced a learning deficit (Fig. 6*L*) (12), whereas the learning of permissive temperature controls stayed normal (Fig. 6*M*). Activating DANs and NPF neurons simultaneously resulted in a learning deficit similar to that of activating DANs alone (Fig. 6*L*), suggesting that NPF neurons function upstream of DANs. These findings thus corroborate our hypothesis that NPF signaling promotes resilience to CSLD by inhibiting DA neurons.

Collectively, our data support a model that NPFergic activity maintains autophagic flux by inhibiting stress-induced excess DAergic activity, thus promoting resilience to CSLD.

Discussion

In the current study, we investigated the cellular signaling mechanisms underlying the etiology of CSLD, one of the behavioral aberrations associated with depression-like states. With a combination of transcriptomic, molecular biological, genetic, pharmacological, and behavioral studies, we demonstrated that chronic stress treatment induces the disruption of autophagic flux and that chronic disruption of autophagic flux facilitates the development of CSLD. Moreover, we tracked down a neuropeptide gene, *NPF*, the *Drosophila* homolog of *NPY,* whose function is indispensable for maintaining normal autophagic flux and thus promotes resilience to CSLD. Remarkably, thermogenetically activating NPF neurons during CST is sufficient to protect against CSLD by preventing the disruption of autophagic flux. In line with our previous report that DAN activity mediates chronic stress signals to promote susceptibility to CSLD (12), DAN activity is required for the development of CSDA. Furthermore, NPFR activity in DANs is required to maintain autophagic flux and promote resilience to CSLD. Taken together, our data reveal a model whereby stress-induced excessive DAergic activity precipitates the disruption of autophagy and chronic autophagic disruption leads to CSLD. On the other hand, NPFergic activity maintains autophagic flux and promotes resilience to CSLD by inhibiting DAN neural activity (*SI Appendix*, Fig. S9).

Protective autophagy is an evolutionarily conserved stress response mechanism. Autophagy-lysosome pathway maintains cellular homeostasis that is crucial for stress adaptation by degrading and recycling abnormal proteins and damaged organelles (17, 43). Multiple lines of evidence from the current study support that chronic stress induces the disruption of autophagic flux in the fly head. First, time-series transcriptome profiling identified as many as 58 DEGs that are annotated as autophagy-lysosomal genes, suggesting that the autophagy-lysosome pathway involves in the development of depression-like states. Second, both Atg8a-II/Atg8a-I ratio and Ref(2)p protein level are accumulated after CST, and blocking lysosomal degradation with CQ does not further increase the level of these markers, strongly suggesting that autophagy is completely blocked at the late stages of autophagy. Consistently, the accumulation of ubiquitin-conjugated proteins after CST also denotes impaired degradation. Third, lysosome dysfunction after CST is evidenced by the aberrant LysoTracker signal and diminished m-CTSL level. It is not clear yet how chronic stress dampens lysosomal function and blocks autophagy. Nevertheless, raising basal autophagy level is sufficient to prevent the upregulation of the Atg8a-II/Atg8a-I ratio and Ref(2)p level, indicating that the accumulation of stress-induced aberrant proteins and damaged organelles might overload lysosomes and cause damage. Collectively, our findings suggest that chronic stress targets lysosomes and interferes with lysosome function, which in turn leads to the disruption of autophagic flux. In agreement with this hypothesis, promoting the induction of autophagy is not effective in reversing CSDA.

To link the disruption of autophagic flux with CSLD, we fed flies with CQ. The finding that chronic but not acute disruption of autophagic flux by CQ led to a learning deficit supports the idea that accumulated autophagy intermediates are cytotoxic and responsible for CSLD. CST plus CQ feeding did not show a severer learning deficit compared with either CST or CQ feeding alone, further suggesting that chronic disruption of autophagic flux is the underlying cause of CSLD. Moreover, raising basal autophagy level by Atg1 overexpression not only prevents CSDA but also protects against CSLD. This CSLD protective effect can be abolished by CQ, suggesting that normal autophagic flux is indispensable for CSLD protection. Thus, chronic disruption of autophagic flux is both necessary and sufficient for CSLD. We conclude that chronic stress–induced disruption of autophagic flux is the underlying cause of CSLD. This mechanism might be evolutionarily conserved, as aberrant autophagy has been linked to many neural disorders including depression (15, 16). However, in rodent models, heterogeneous results of the impact of chronic stress on autophagy have been reported (44). Reasons for the discrepancy could be that different chronic stress paradigms were used, different brain areas were investigated and different markers were examined. In our case, our data strongly support that blockage of the late stages of autophagy accounts for the etiology of a cognitive symptom associated with the depression-like state, which remains to be demonstrated in mammalian models.

The finding that chronic disruption of autophagic flux lies behind CSLD allows a two-step forward genetic screening approach to identify CSLD-resilient genes. In the primary screening, we identified four neuropeptide mutants that showed upregulation of the Atg8a-II/Atg8a-I ratio and Ref(2)p level in the fly head, suggesting that the functions of these neuropeptides are required for maintaining autophagic flux. Surprisingly, out of four mutants, only NPF^{attP} showed the CSLD-susceptible phenotype, which reflects that these neuropeptides might regulate the autophagy of different brain cell subsets. We then focused on NPF, and provided compelling genetic evidence, including additional mutant allele, RNAi knockdown, and genetic rescue, to corroborate that NPF indeed promotes resilience to CSLD, a core depression-like behavioral symptom. This finding indicates that NPF has a role in regulating the development of depression-like states, which, however, disagrees with a previous report (10), which found that depression-like state is independent of NPF. Possible explanations for the disagreement could be different depression-like behavioral symptoms were examined and NPF

might not regulate the gap climbing circuitry. With thermogenetic tools, we showed that NPF neuronal activity during CST is sufficient to protect against both CSDA and CSLD. Moreover, this CSLD protective effect is NPF-dependent and could be blocked by CQ feeding. These data thus link the function of the *NPF* gene with that of NPF neurons and suggest that NPFergic activity protects CSLD by maintaining normal autophagic flux. As predicted by this hypothesis, thermogenetic activation of NPF neurons is less effective in reversing CSDA and CSLD.

We have previously reported that DAergic activity mediates stress signals to precipitate susceptibility to CSLD (12). To link the findings in the current study with the DAergic system, we showed that DAN neural activity is required for the development of CSDA. Among the PPL1 DANs that project to the mushroom body lobes, a pair of PPL1-γ1pedc DANs has been identified as the key CSLD-regulating DANs (12). However, blocking the synaptic release from PPL1-γ1pedc neurons does not effectively prevent CSDA (*SI Appendix*, Fig. S10). Since CSDA was evaluated using western blot analysis of protein samples obtained from the entire heads of adult flies, these negative results may be explained by the possibility that a pair of PPL1-γ1pedc neurons may not have the capacity to exert sufficient nonautonomous control over the autophagy of the entire brain. Whether the activity of PPL1-γ1pedc could induce the disruption of autophagic flux in specific subsets of the mushroom bodies needs to be further addressed in future studies. Furthermore, we demonstrated that NPFR activity is necessary in DANs for maintaining autophagic flux and promoting resilience to CSLD. Consistent with these findings, NPF neurons act upstream of DANs to protect against CSLD. Thus, inhibitory NPF signaling to DANs might alleviate the stress-induced disruption of autophagic flux and promote resilience to CSLD. Although the impact of NPF on DANs could be complicated and cell-specific, our findings are in line with previous studies indicating that NPF can inhibit the activity of DANs to regulate olfactory memory and other olfactory behaviors (40–42, 45). This mechanism could potentially be evolutionarily conserved, as NPY is a potent anxiolytic neuropeptide that involves in stress response and depression-like states (46, 47). Moreover, NPY could inhibit a subset of VTA dopamine neurons, which involve in motivated behaviors toward food (48). Interestingly, NPY has been reported to induce autophagy via its receptors (49, 50). Since elevating basal autophagy level is protective against CSLD, our data in the current study do not exclude the possibility that besides inhibiting stress-induced DAN activity, NPF might also induce autophagy that would contribute to CSLD protection, which awaits to be clarified in the future. In either case, one would expect that NPY/NPF should be more effective in preventing the development of chronic stress–induced depression-like states than in reversing these depression-like states.

In sum, our investigations into the etiology of CSLD have found that the autophagy-lysosome pathway and NPFergic signaling might have evolutionarily conserved roles in regulating the development of chronic stress–induced depression-like states. Together with our previous finding that DAergic activity promotes susceptibility to CSLD, our work reinforces the idea that the *Drosophila* chronic stress paradigm is a valid depression-like animal model for studying conserved underlying molecular and neural circuit mechanisms.

Materials and Methods

Flies were cultured in cornmeal fly food at 23 °C unless otherwise indicated. Details of *Drosophila* husbandry and strains are included in *SI Appendix*. Methods for chronic stress treatment, RNA-seq and data analysis, aversive olfactory learning, western blot assay, qRT-PCR, LysoTracker staining, pharmacology, and statistical analysis are described in *SI Appendix*.

Data, Materials, and Software Availability. All data needed to evaluate the conclusions in the paper are present in the paper and supporting information [SRA ID: PRJNA921886 (51) (RNA-seq and analyzed data)].

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