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A neural pathway for social modulation of spontaneous locomotor activity (SoMo-SLA) in *Drosophila*

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Social enrichment or social isolation affects a range of innate behaviors, such as sex, aggression, and sleep, but whether there is a shared mechanism is not clear. Here, we report a neural mechanism underlying social modulation of spontaneous locomotor activity (SoMo-SLA), an internal-driven behavior indicative of internal states. We find that social enrichment specifically reduces spontaneous locomotor activity in male flies. We identify neuropeptides Diuretic hormone 44 (DH44) and Tachykinin (TK) to be up- and down-regulated by social enrichment and necessary for SoMo-SLA. We further demonstrate a sexually dimorphic neural circuit, in which the male-specific P1 neurons encoding internal states form positive feedback with interneurons coexpressing *doublesex* (*dsx*) and *Tk* to promote locomotion, while P1 neurons also form negative feedback with interneurons coexpressing *dsx* and *DH44* to inhibit locomotion. These two opposing neuromodulatory recurrent circuits represent a potentially common mechanism that underlies the social regulation of multiple innate behaviors.

social modulation | innate behavior | recurrent circuit | Diuretic hormone 44 | Tachykinin

Significance

Animals including humans behave differently if raised in isolation. How social isolation or social enrichment changes the brain in molecular and neuronal levels and further regulates multiple behaviors is still poorly understood. Here, we identify a neural pathway from sensory systems to central neuronal networks underlying social modulation of spontaneous locomotion in *Drosophila* males. The central pathway bilaterally controls locomotor activity, which involves two neuromodulatory recurrent circuits that respond to social isolation and social enrichment, respectively. We further show that this social-responsive circuit not only controls spontaneous locomotion but also social behaviors such as sex and aggression. The two opposing recurrent circuits and their neuromodulation may represent a potentially common mechanism that adjusts animal behaviors based on prior social experiences.

A fundamental and challenging question in neuroscience is to comprehend the general principles that govern the developmental construction and social modulation of innate behaviors across animal species. Over the past two decades, substantial studies have demonstrated neural circuit mechanisms controlling innate behaviors, such as sex, aggression, and sleep, in model organisms from fruit flies to mice (1–5). Of particular significance, these studies provide insights into the configurations of circuit modules linking internal states and innate social behaviors in flies and mice (1, 6). However, our current understanding of the mechanisms underlying the social modification of innate behaviors in these model organisms is relatively limited (7). Consequently, it becomes even more challenging to illustrate the potentially conserved genetic and neuronal mechanisms involved in this context.

Social experience has profound short or long-term effects on multiple innate behaviors in the fruit fly *Drosophila melanogaster* (1, 8, 9). For courtship behavior, group-housed (GH) males court less toward immobile females than single-housed (SH) males do, which depends on the function of neuropeptide Drosulfakinin (DSK) (10). Meanwhile, it is also reported that GH males show stronger pheromone response through Or47b olfactory receptor neurons and thus have reproductive advantages (11, 12). Strikingly, social experience can even restore courtship behavior in males lacking the male-specific *fruitless* (fru^M) but with the male-specific *doublesex* (dsx^M) (13, 14). Moreover, social experience can also modify sexual orientation and intensity in hypomorphic fru^M mutants (15, 16). As for aggressive behaviors, several studies show that social isolation promotes aggression through mechanisms involving pheromone sensitivity, neuromodulation, and circuit connectivity (17–22). For sleep, GH flies spend more daytime on sleep than SH flies do (23). Consistently, chronic isolation induces starvation and reduces sleep in *Drosophila* (24). Recently, an analytical framework indicates that SH males generally show more active approaching behaviors like turning, chasing, and touching while GH males display lower level of activities but form ordered social networks (25). Despite significant progress in understanding the role of social modulation in sex, aggression, and sleep in *Drosophila*, it remains unclear whether social enrichment or isolation would modulate these behaviors through common or shared mechanisms.

Spontaneous locomotor activity, in the absence of obvious sensory stimulation, may partially reflect an internal state that ultimately influences sex, aggression, and sleep. Indeed, studies in *Drosophila* have identified a subset of male-specific interneurons called P1, which express both dsx^M and fru^M, and encode a persistent internal state to regulate sex, aggression, sleep, as well as spontaneous locomotor activity (1, 10, 26–33). Motivated by this, we set out to investigate the impact of prior social experience, i.e., GH or SH, on spontaneous

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locomotor activity, aiming to identify more general mechanisms underlying the social modulation of innate behaviors.

In this study, we first established that social modulation of spontaneous locomotor activity (SoMo-SLA) was male-specific and dependent on *ppk23*-expressing gustatory M cells and *dsx*-expressing central neurons. We further revealed a sexually dimorphic neural circuit involving three central neural nodes crucial for SoMo-SLA. This neural circuit is centered on the male-specific P1 neurons and involves both positive feedback from Tachykinin (TK) neuromodulation and negative feedback from Diuretic hormone 44 (DH44) neuromodulation. We also found that social isolation and social enrichment enhanced expression of TK and DH44, respectively. Thus, our findings demonstrate a sexually dimorphic and social-responsive neural circuit that involves counteracting neuromodulation to tune flexibility of spontaneous locomotor activity, as well as innate social behaviors. These results highlight a potentially common mechanism underlying the social modulation of innate behaviors.

Results

SoMo-SLA Is Male-Specific and Dependent on *ppk23*-Positive M Cells. Previous studies found that social experience during waking time affected sleep need in *Drosophila* (23). Consistently, chronic social isolation reduced sleep in *Drosophila* through P2 neurons expressing neuropeitide F (NPF) (24). In our sleep experiments on GH and SH wild-type *Canton-S* (*CS*) flies (Fig. 1*A*), we observed that GH males indeed slept more than SH males during the daytime (Fig. 1*B*). However, we did not observe any difference of sleep amounts between GH and SH females (Fig. 1*C*). To investigate whether the change in sleep is due to locomotor activity, we recorded spontaneous locomotion of individual flies from GH and SH flies for 24 h. We found that GH males showed significantly lower locomotor activity than SH males, especially during the first hour (Fig. 1 *D* and *F*), while GH and SH females did not show any difference (Fig. 1 *E* and *F*). Such male-specific modulation of sleep and locomotion by social experience was further confirmed in the wild-type w^{1118} flies (*SI Appendix*, Fig. S1 *A–D*). As the social modulation of spontaneous locomotor activity may be the key factor underlying both sleep and locomotor changes in males, and is apparent during the 1-h test, we decided to test the 1-h locomotion from GH and SH males for further studies.

SoMo-SLA could be due to locomotor reduction from GH, or an increase from SH, or both. To discriminate these possibilities, we tested locomotion in 1- to 7-d-old males from GH or SH and found that it was the locomotor reduction from GH that resulted in SoMo-SLA (Fig. 1*G*). Such reduction is partially reversible as 1-d SH after 6-d GH significantly increased locomotor activity, although the level of locomotion was still lower than 7-d SH males (Fig. 1*G*). Moreover, we found that SoMo-SLA was independent of group size such that a group of 2, 25, 50, or 100 males showed indistinguishable locomotor activity (Fig. 1*H*). These results indicate that social experience during male-male GH reduces locomotor activity, and later SH experience can partially restore locomotor activity.

Sensory systems play key roles in perceiving and transmitting social information to modulate behavioral plasticity. To investigate the sensory pathway involved in SoMo-SLA, we first tested various sensory mutants and found that gustatory mutant $Poxn\Delta^{m22}$, but not mutants of other sensory modalities ($norpA^{33}$, iav^{1}, $Or83b^{2}$, Tmc^{1}, $Piezo^{KO}$) resulted in the same level of locomotor activity in GH and SH males, suggesting a vital function of gustatory inputs in SoMo-SLA (Fig. 1*I*). Note that locomotion levels varied in different genotypes. To avoid the potential effects of different genetic backgrounds on SoMo-SLA, we hereafter only compared locomotion between GH and SH flies of the same genotype. We next silenced specific gustatory receptor neurons that were previously identified to respond to conspecifics (34–37) or sugar (38) by expressing an inwardly rectifying potassium channel Kir2.1 (39). We found that silencing *ppk23*, but not *ppk25*, *Gr32a* or *Gr5a* neurons eliminated the locomotor difference from GH and SH males (Fig. 1*J*). Moreover, optogenetic activation of *ppk23* but not *ppk25* neurons expressing *CsChrimson* resulted in a prolonged locomotor reduction (*SI Appendix*, Fig. S2 *A–C*). As *ppk23* is expressed in both M cells and F cells in male forelegs and *ppk25* is expressed in F cells but not M cells (35), these results suggest that *ppk23*-positive M cells that respond to male pheromones are crucial to mediate the group-housing-induced locomotor inhibition.

Two Subsets of Sexually Dimorphic *dsx* Neurons Regulate SoMo-SLA. To determine which part of the central nervous system (CNS) would control SoMo-SLA, we first tried to silence subsets of CNS neurons including the sleep-regulating P2 neurons by expressing Kir2.1 or tetanus toxin (TNT) and failed to observe any significant effect (Fig. 2*A*). These results suggest that social modulation of sleep and locomotion may involve different mechanisms. As *ppk23* neurons act on mAL and vAB3 neurons that jointly control the activity of central P1 neurons (35, 40), which encode an internal state crucial for sexual and aggressive behaviors (1, 27), we then silenced a subset of P1 neurons ($P1^{a}$), mAL or vAB3 neurons, but still failed to see any effect (Fig. 2*A*). We next silenced all *dsx*-expressing neurons in the brain (dsx^{brain}) that are crucial for innate behaviors in *Drosophila* males and females (1, 26, 41), and observed a loss of SoMo-SLA. These results suggest that *dsx* neurons in the brain are crucial for SoMo-SLA in males. Indeed, the group-housing experience may act through dsx^{brain} neurons as we observed substantially more downstream neurons visualized by the trans-Tango technique (42) in GH males than those in SH males (*SI Appendix*, Fig. S3 *A* and *B*).

To further identify which subsets of *dsx* neurons are responsible for SoMo-SLA, we used two intersectional strategies, the FLP-out system and the split GAL4 system, to label and manipulate subsets of *dsx* neurons. Using such strategies, we identified two types of *dsx* neurons coexpressing neuropeptide DH44 ($DH44^{LexA} \cap dsx^{GAL4}$) and TK ($Tk^{AD} \cap dsx^{DBD}$), respectively that were important for SoMo-SLA. Silencing these neurons, but not other *dsx* subsets tested, abolished the locomotor difference from GH and SH males (Fig. 2*B*). However, these results do not exclude the potential involvement of neurons other than dsx^{DH44} and dsx^{Tk} in SoMo-SLA.

To visualize the two types of *dsx* neurons coexpressing DH44 or TK, we expressed *GFP* in $DH44^{LexA} \cap dsx^{GAL4}$ and $Tk^{AD} \cap dsx^{DBD}$ neurons, hereafter referred to as dsx^{DH44} and dsx^{Tk} neurons, respectively (Fig. 2*F*). We observed that the dsx^{DH44} driver labeled ~six pairs of pC1 and ~two pairs of αDN neurons in male brains and ~three pairs of pC1 neurons in female brains (Fig. 2 *C* and *D*). DH44 antibody staining confirmed its expression in most dsx^{DH44}-labeledpC1 neurons in both males and females (Fig. 2*E*). Similarly, we found that the dsx^{Tk} driver labeled ~nine pairs of pC2 neurons in male brains and ~three pairs of pC2 neurons in female brains, as well as 1 to 2 pairs of pCd neurons in both sexes (Fig. 2 *F* and *G*). Costaining with the TK antibody was observed in most dsx^{Tk}-labeled pC2 neurons in both sexes (Fig. 2*H*). Together, these results identify two types of sexually dimorphic

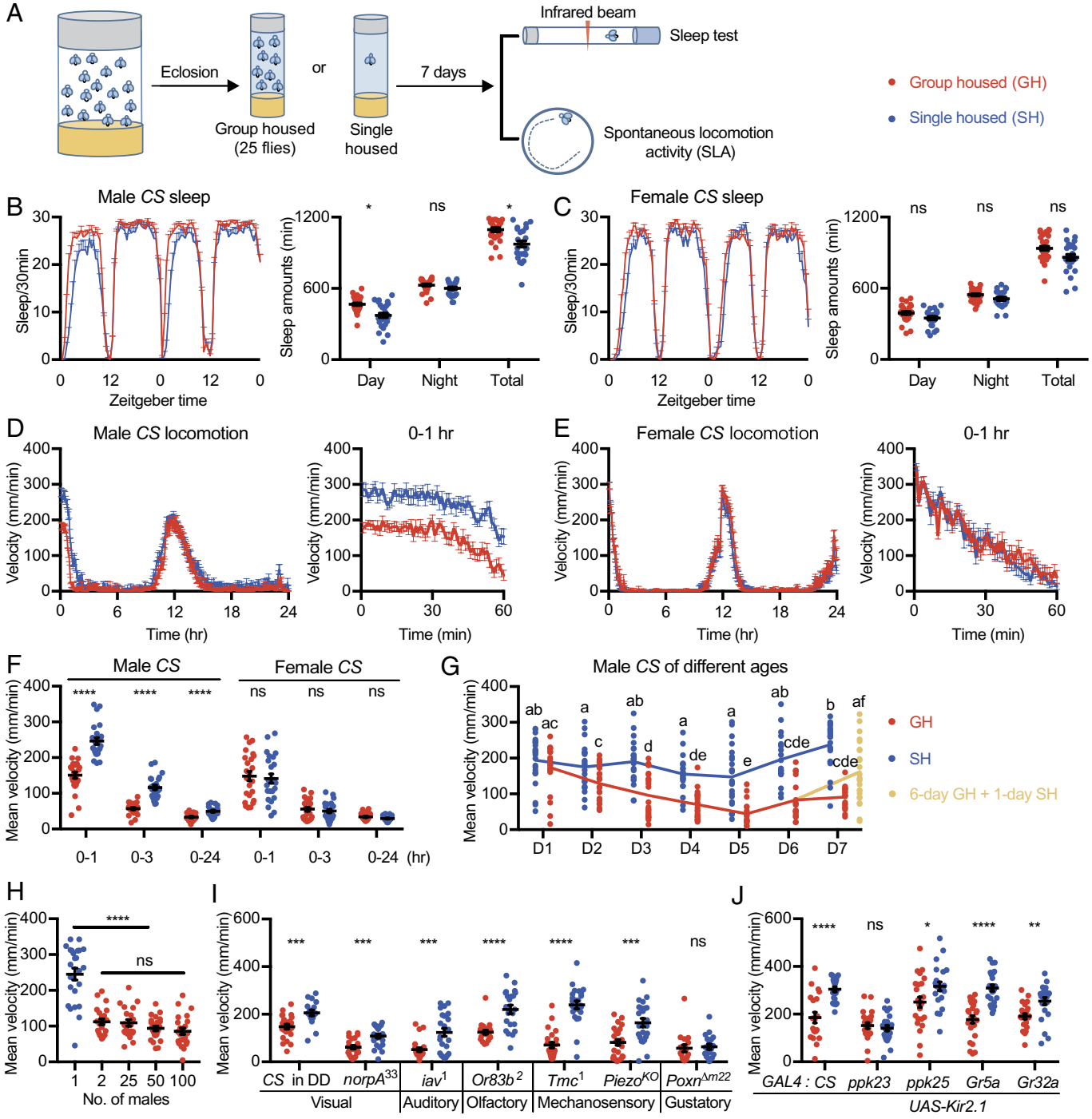

Fig. 1. Social modulation of spontaneous locomotor activity (SoMo-SLA) is sex-specific and dependent on gustatory sensory input. (*A*) Diagram of group- or single-housing conditions and behavioral assays. (*B*) Sleep profile (*Left*) and amounts (*Right*) of group-housed (GH, red) or single-housed (SH, blue) male CS flies. n = 32 and 28 for GH and SH males, respectively. (*C*) Sleep profile (*Left*) and amounts (*Right*) of GH or SH female CS flies. n = 32 and 25 for GH and SH females, respectively. (*D* and *E*) Spontaneous walking velocity of individual males (*D*) or females (*E*) from GH or SH. n = 24 for each. (*F*) Mean velocity of flies from *D* and *E* during 0-1, 0-3, or the total 24 h. (*G*) Mean velocity of GH (red) or SH (blue) 1- to 7-d-old CS males. Males that were GH for 6 d and then SH for 1 d showed intermediate velocity (brown). n = 20 to 24. (*H*) Mean velocity of CS males raising from different group sizes. n = 21 to 24. (*I*) Mean velocity of sensory mutant males from GH or SH. n = 18 to 24. (*J*) Mean velocity of males from GH or SH with specific sensory neurons expressing Kir2.1. n = 20 to 24. For *G*, all variables that have different letters are significantly different ($P < 0.05$), and all variables with the same letter are not significantly different, one-way ANOVA with Tukey's post hoc test. For *H*, ns, not significant, ****$P < 0.0001$, one-way ANOVA with Tukey's post hoc test. For all others, ns, not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, unpaired *t* test. Error bars indicate SEM throughout. All genotypes and sample sizes in this and all subsequent figures are listed in *SI Appendix*, Table S1.

dsx neurons expressing neuropeptide DH44 and TK, respectively, and reveal a crucial role of them in the male-specific SoMo-SLA.

Sexually Dimorphic dsx^{DH44}, dsx^{Tk}, and P1^{a} Neurons Differentially Regulate SoMo-SLA. The above results revealed crucial roles of dsx^{DH44} and dsx^{Tk} neurons in SoMo-SLA, but how they regulate locomotion is elusive. We utilized optogenetics by expressing *CsChrimson* in these neurons and assayed their acute function in locomotor control. Optogenetic stimulation with 30-s red-light pulses at either low (10 Hz) or high (40 Hz) frequency did not induce any change in walking velocity in control males (Fig. 3*A* and *SI Appendix*, Fig. S4*A*). Interestingly, optogenetic activation of

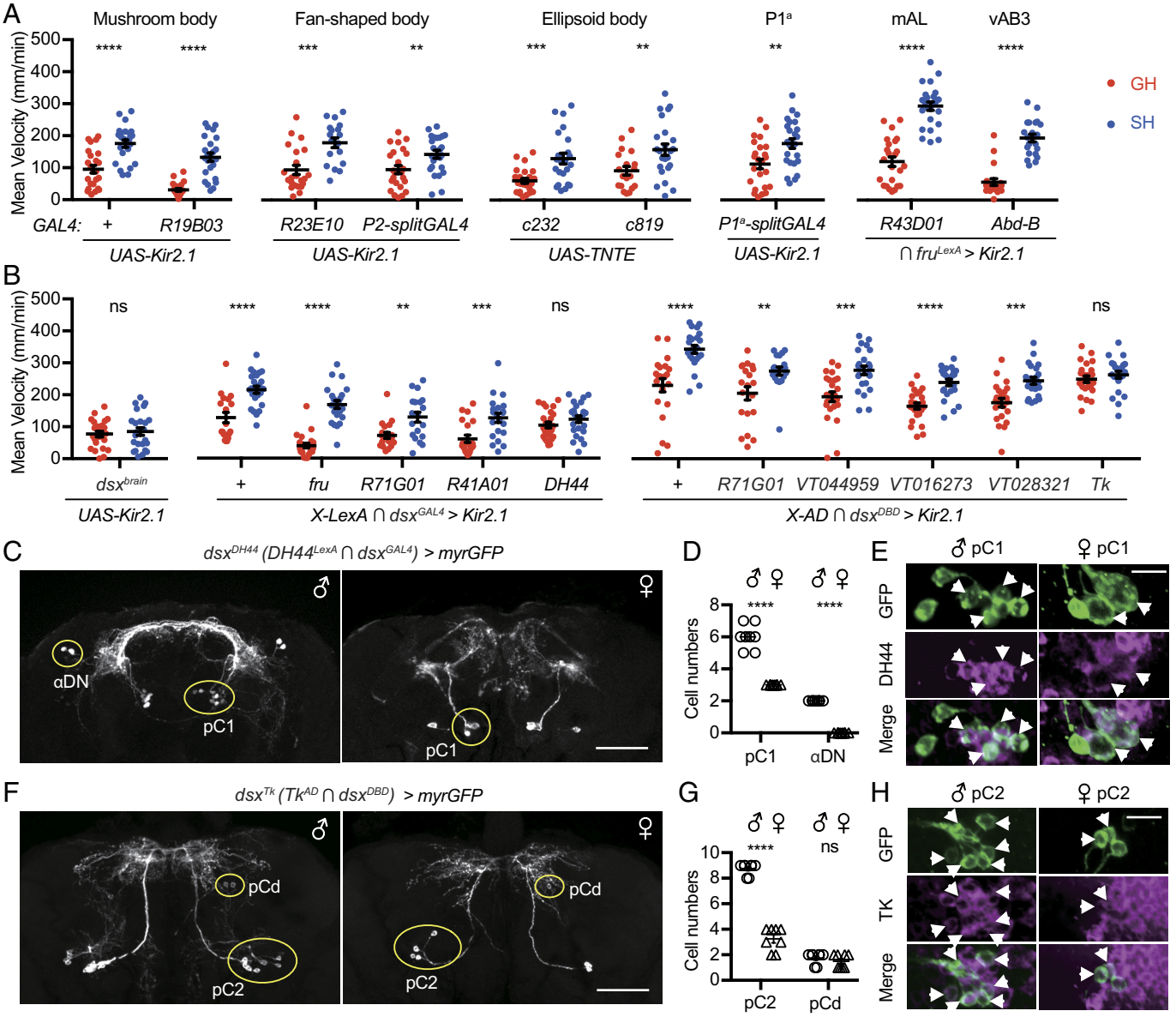

Fig. 2. Identification of sexually dimorphic peptidergic dsx^{Tk} and dsx^{DH44} neurons regulating SoMo-SLA. (*A*) Mean velocity of males from GH (red) or SH (blue) with specific neurons expressing Kir2.1 or TNTE. n = 18 to 24. (*B*) Mean velocity of males from GH or SH with subsets of dsx neurons expressing Kir2.1. n = 18 to 24. (*C*) Expression pattern of dsx^{DH44} neurons in male and female brains. Circles indicate cell bodies. Representative of eight samples for each. (Scale bar, 50 μm.) (*D*) Cell numbers of dsx^{DH44} neurons in males and females. n = 8 for each. (*E*) Costaining of dsx^{DH44} driving UAS-myrGFP (green) and anti-DH44 (magenta) in male and female brains. Arrowheads indicate colabeling cell bodies. Representative of five samples for each. (Scale bar, 10 μm.) (*F*) Expression pattern of dsx^{Tk} neurons in male and female brains. Circles indicate cell bodies. Representative of eight samples for each. (Scale bar, 50 μm.) (*G*) Cell numbers of dsx^{Tk} neurons in males and females. n = 8 for each. (*H*) Costaining of dsx^{Tk} driving UAS-myrGFP (green) and anti-TK (magenta). Arrowheads indicate colabeling cell bodies. Representative of five samples for each. (Scale bar, 10 μm.) ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, unpaired *t* test.

dsx^{DH44} neurons using low-frequency pulses significantly reduced walking speed in GH but not SH males (Fig. 3 *B*, *Left*), while activation of dsx^{DH44} neurons using high-frequency pulses reduced walking speed in both GH and SH males (Fig. 3 *B*, *Right*). We also found that the dsx^{DH44}-mediated suppression of locomotor activity persisted after optogenetic stimulation (*SI Appendix*, Fig. S4*B*). In contrast, optogenetic activation of dsx^{Tk} neurons using both low- and high-frequency pulses increased walking speed in both GH and SH males (Fig. 3*C*). Such an increase did not persist after red light was turned off (*SI Appendix*, Fig. S4*C*). Thus, dsx^{DH44} and dsx^{Tk} neurons oppositely regulate locomotor activity, and dsx^{DH44} but not dsx^{Tk} neurons may have different properties in GH and SH males.

In addition, we investigated the effect of optogenetic stimulation of the male-specific P1^{a} neurons as our previous studies indicated their involvement in locomotor control (10, 29, 43). Strikingly, optogenetic activation of P1^{a} neurons bilaterally regulates walking speed at low and high frequencies, respectively. Low-frequency stimulation of P1^{a} neurons promoted walking speed in both GH and SH males (Fig. 3*D*). The elevated walking speed persisted in GH males and further increased in SH males after light stimulation (*SI Appendix*, Fig. S4*D*). In contrast, high-frequency stimulation of P1^{a} neurons strongly suppressed walking in both GH and SH males (Fig. 3*D*). Such suppression persisted significantly in GH but not SH males (*SI Appendix*, Fig. S4*D*). Intersectional expression analysis revealed that a pair of P1^{a} neurons also express DH44 (*SI Appendix*, Fig. S4*E*), while none express TK (*SI Appendix*, Fig. S4*F*). Optogenetic activation of the pair of P1^{a}/DH44 neurons did not lead to a significant change in locomotion (*SI Appendix*, Fig. S4*G*), suggesting that

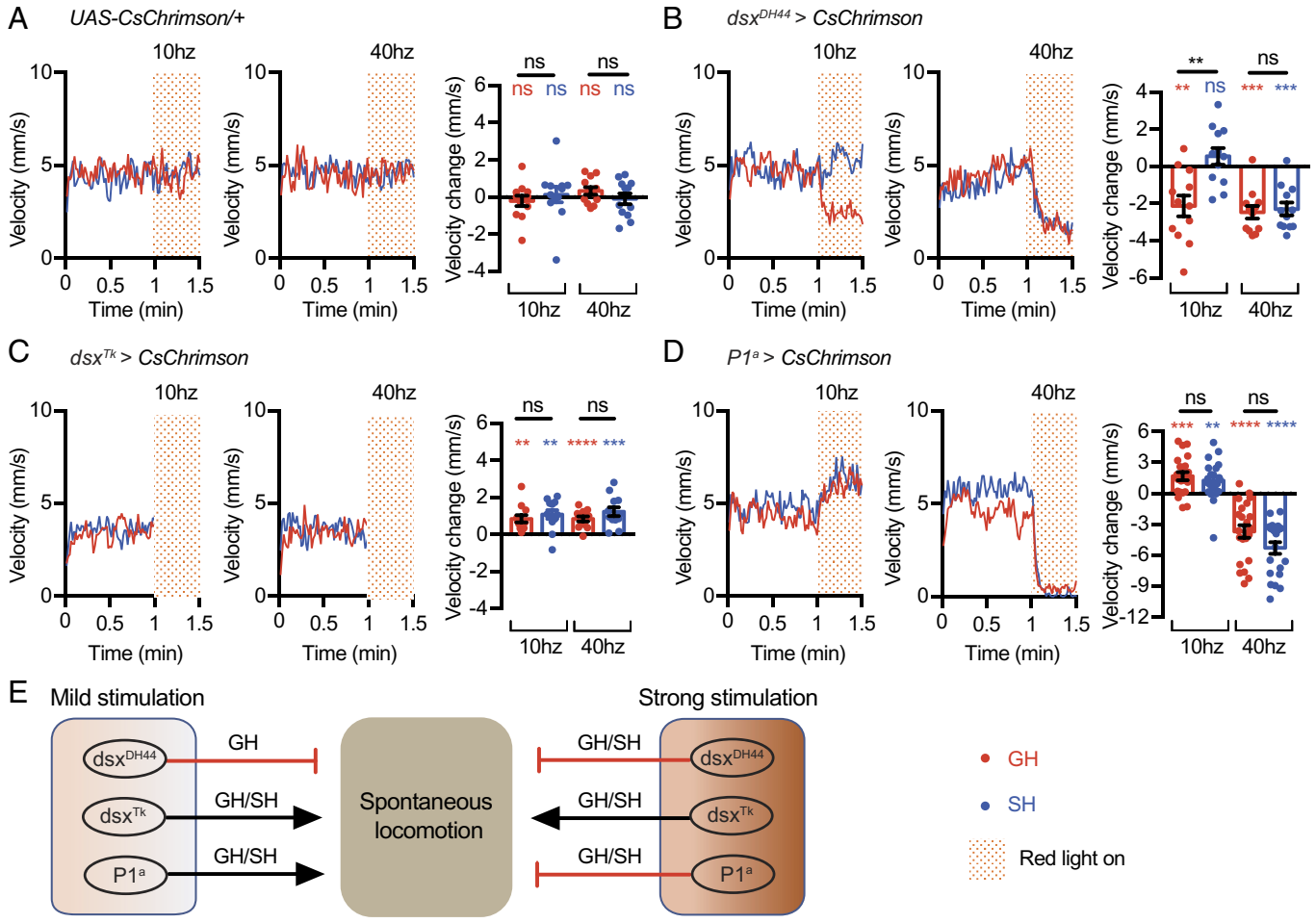

Fig. 3. dsx^{DH44}, dsx^{Tk}, and P1^{a} neurons differentially regulate SoMo-SLA. (*A–D*) Locomotion velocity profile (10 Hz or 40 Hz red-light stimulation for 30 s, *Left* and *Middle* panels) and velocity change (*Right* panels) of GH (red) and SH (blue) control males (*A*), males with optogenetic activation of dsx^{DH44} neurons (*B*), dsx^{Tk} neurons (*C*), or P1^{a} neurons (*D*). n = 12 for each in *A–C*. n = 22 for each in *D*. (*E*) Summary of behavioral effects on spontaneous locomotion by optogenetic activation of dsx^{DH44}, dsx^{Tk} or P1^{a} neurons in GH or SH males. Mild and strong activation refer to 10 Hz and 40 Hz red light stimulation, respectively. For *A–D*, ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. One sample *t* test for significance analysis between velocity change and zero (red for GH, blue for SH), and one-way ANOVA with Tukey's post hoc test for comparisons between velocity changes of GH and SH flies (black). Duration for each red-light stimulation is 15 ms, except for the mild activation of P1^{a} neurons (10 Hz, 5 ms). Full data with more light stimulation conditions are shown as *SI Appendix*, Fig. S4.

the above function of P1^{a} neurons is not from the pair of P1^{a}/DH44 neurons. In summary, dsx^{DH44} neurons inhibit, dsx^{Tk} neurons promote, and P1^{a} neurons bilaterally regulate locomotor activity, and these neurons may jointly control SoMo-SLA in males.

Social Experience Oppositely Regulates DH44 and TK Signaling to Mediate Spontaneous Locomotor Activity. The involvement of peptidergic dsx^{DH44} and dsx^{Tk} neurons in SoMo-SLA promoted us to speculate whether DH44 and TK peptides would respond to social experiences and modulate locomotor plasticity. We first used DH44 and TK antibodies to analyze their expression in GH and SH male brains and found generally higher expression of DH44 and lower expression of TK in GH male brains (Fig. 4 *A* and *C*). Moreover, quantitative real-time PCR (qPCR) showed that the relative mRNA expression levels of *DH44* were significantly higher in GH flies, while the levels of DH44 receptor genes *DH44R1* and *DH44R2* were lower in GH flies (Fig. 4*B*). On the contrary, the relative mRNA level of *Tk* was higher in SH flies, while the level of TK receptor gene *TkR99D* was lower in SH flies (Fig. 4*D*). These results indicate that housing experiences oppositely regulate *DH44* and *Tk* expression levels.

To validate whether DH44 and TK peptides are necessary for SoMo-SLA, we tested the 1-h locomotor activity in *DH44* and *Tk* mutant males, respectively, and found that the difference in locomotion from GH and SH experiences was lost (Fig. 4*E*). We next used RNA interference (RNAi) to knock down *DH44*, *Tk* and their receptor genes. Immunostaining and qPCR analysis confirmed the efficiency of RNAi knockdown (*SI Appendix*, Fig. S5 *A–C*). However, knocking down *DH44* or *Tk* in *dsx* neurons did not eliminate the locomotor difference in GH and SH males (*SI Appendix*, Fig. S5*D*), possibly due to the incomplete ablation of their expression or their potential roles in non-*dsx* neurons. We also performed a RNAi screen driven in *dsx^{brain}* neurons for neurotransmission signaling potentially involved in SoMo-SLA but failed to observe a complete loss of SoMo-SLA (*SI Appendix*, Fig. S5*E*). To further confirm the role of DH44 and TK peptides in SoMo-SLA, we tried to optogenetically activate dsx^{DH44} and dsx^{Tk} neurons while knocking down *DH44* and *Tk* expression, respectively. We found that knocking down *DH44* expression eliminated the locomotor inhibition under low-frequency (Fig. 4*F*) but not high-frequency stimulation (Fig. 4*G*). These results indicated that the inhibition of velocity partially depended on the expression of *DH44*. Furthermore, knocking down *Tk* expression blocked the increase in walking speed under both low- and high-frequency stimulation (Fig. 4 *H* and *I*). These results reveal acute roles of DH44 in suppressing and TK in promoting locomotion. Taken together, the above findings demonstrate that housing

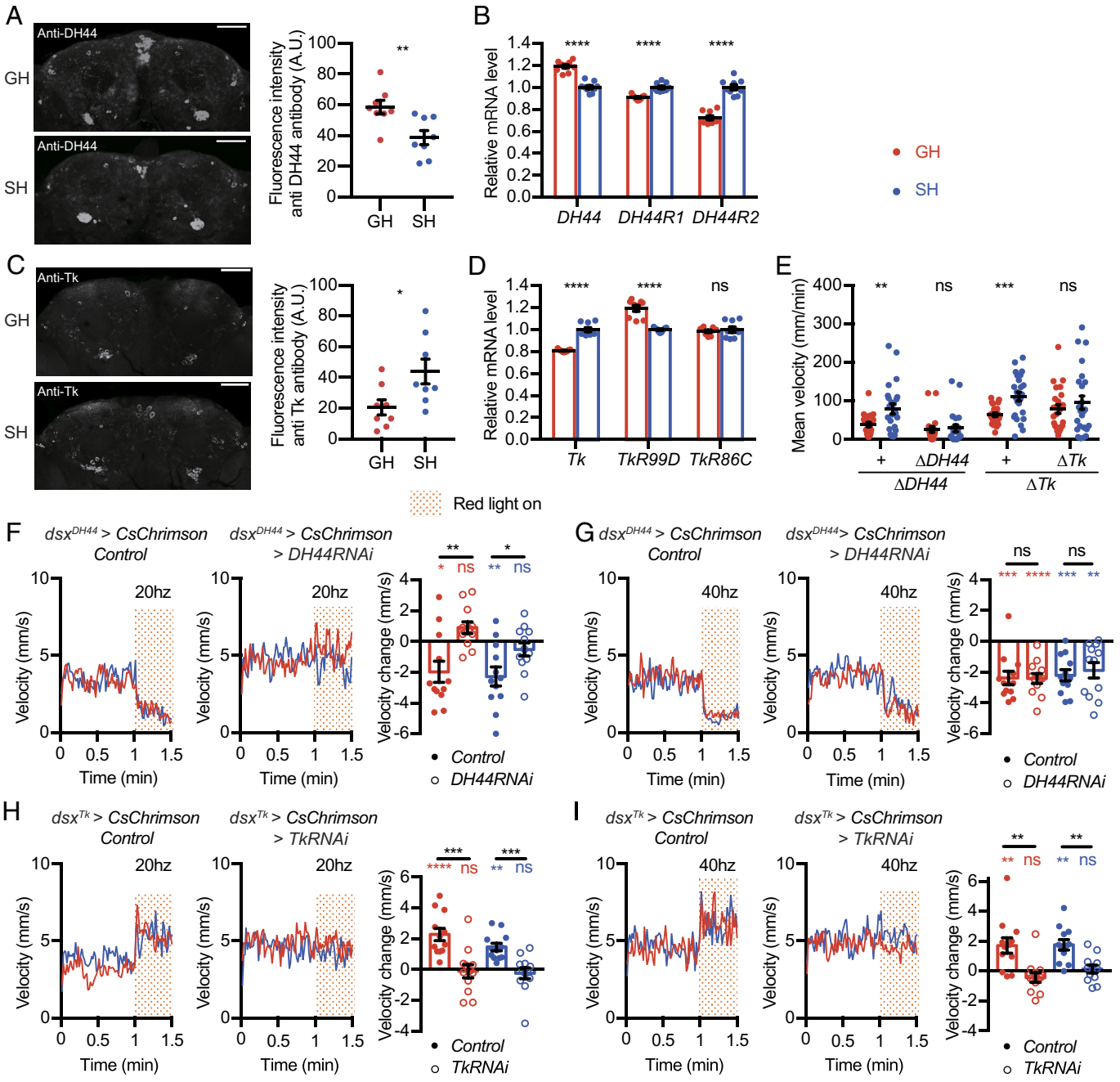

Fig. 4. Group- or single-housing conditions oppositely regulate DH44 and TK to mediate spontaneous locomotor activity. (*A–D*) Expression analysis of DH44 and TK signalings in GH (red) and SH (blue) male brains. For *A* and *C*, n = 8 for each; for *B* and *D*, n = 9 based on three replicates for each. (*E*) Mean velocity of *DH44* mutant, *Tk* mutant, and control males from GH or SH. n = 20 to 24 (*SI Appendix*, Table S1). ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, unpaired *t* test. (*F* and *G*) Locomotion velocity profile and velocity change of GH and SH males with optogenetic activation of dsx^{DH44} neurons (*F*, 20 Hz, 15 ms; *G*, 40 Hz, 15 ms) with or without interfering DH44 expression. n = 12 for each. (*H* and *I*) Locomotion velocity profile and velocity change of GH and SH males with optogenetic activation of dsx^{Tk} neurons (*H*, 20 Hz, 15 ms; *I*, 40 Hz, 15 ms) with or without interfering *Tk* expression. n = 12 for each. ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. For *A–D*, Mann–Whitney *U* test. For *F–I*, one-sample *t* test for significance analysis between velocity change and zero (red for GH, blue for SH), and one-way ANOVA with Tukey's post hoc test for comparisons between velocity changes of GH and SH flies (black).

experiences oppositely regulate *DH44* and *Tk* expression to mediate locomotor plasticity.

Social Enrichment Enhances the Basal Activity of dsx^{DH44} Neurons but Reduces the Excitability of dsx^{Tk} and $P1^{a}$ Neurons. The above results showed that dsx^{DH44}, dsx^{Tk}, and $P1^{a}$ neurons differentially regulate SoMo-SLA, and the function of dsx^{DH44} and dsx^{Tk} neurons depended on social-responsive DH44 and TK peptides. We hypothesized that social experiences may not only affect DH44 and TK expression but also modulate the basal activity and/or neuronal excitability in these neurons. We first recorded basal activity in dsx^{DH44}, dsx^{Tk}, and $P1^{a}$ neurons expressing GCaMP6 in GH and SH males. We found that the Ca^{2+} concentration in dsx^{DH44} neurons was significantly higher in GH males than that in SH males, while Ca^{2+} levels in dsx^{Tk} and $P1^{a}$ neurons were indistinguishable in GH and SH males (Fig. 5 *A* and *B*). To investigate changes in neuronal excitability in GH and SH males, we employed a fast temperature-control system to rapidly activate neurons via dTrpA1 and simultaneously recorded GCaMP6 signals (44). We found that the same

temperature-raising stimulation induced stronger Ca^{2+} signals in dsx^{Tk} and $P1^a$ neurons, but not dsx^{DH44} neurons, in SH males than those in GH males (Fig. 5 *C* and *D*). Thus, the group-housing experience enhances the basal activity in dsx^{DH44} neurons and reduces excitability in dsx^{Tk} and $P1^a$ neurons.

Based on the above findings, we proposed that chronic grouping/isolation may influence the properties of dsx^{DH44}, dsx^{Tk}, and $P1^a$ neurons and have prolonged effects on spontaneous locomotor activity. We designed acute thermal activation and 24-h chronic thermal activation experiments to further dissect the function of these neurons (Fig. 5*E*). Consistent with the above optogenetic activation experiments, acutely activating dsx^{DH44} neurons via dTrpA1 at 30 °C during the 1-h test inhibited walking speed in SH males, compared to the temperature-induced increase of walking speed in control SH males (Fig. 5 *E*1 and *E*2). Moreover, 24-h chronic activation of dsx^{DH44} neurons at 30 °C resulted in a reduction in walking speed in SH males in a later 1-h test at 22 °C (Fig. 5*E*2). In contrast, acute activation of dsx^{Tk} and $P1^a$ neurons increased walking speed, which could be due to their function in promoting walking speed as above described and/or the temperature effect. Chronic activation of dsx^{Tk} neurons had no effect on walking speed in SH males in the later test at 22 °C (Fig. 5*E*3), while chronic activation of $P1^a$ neurons inhibited walking speed in the later test at 22 °C (Fig. 5*E*4). Consistently, 1-d GH experience after 6-d SH significantly decreased walking speed (Fig. 5*F*). In summary, GH experience chronically activates dsx^{DH44} neurons to inhibit locomotor activity both during and after that social experience (Fig. 5*G*). In addition, GH experience also decreases the excitability of dsx^{Tk} and $P1^a$ neurons, which may interact with dsx^{DH44} neurons (see below) to inhibit locomotor activity (Fig. 5*G*).

$P1^a$ Neurons form Positive and Negative Feedbacks with dsx^{Tk} and dsx^{DH44} Neurons to Bilaterally Regulate Locomotor Activity. The above results established how social experience acts through dsx^{DH44}, dsx^{Tk}, and $P1^a$ neurons to modulate locomotor activity. These neurons may interact with each other and coordinate locomotor control. Thus, we next investigated the functional connections among these neurons. First, we found that at least some $P1^a$ neurons may express DH44 receptors DH44R1 and DH44R2 by intersectional immunostaining of *$P1^a$-splitGAL4* with *$DH44R1^{LexA}$* and *$DH44R2^{LexA}$*, respectively (Fig. 6*A*). Second, we identified *dsx^{Tk}*-labeled pC2 neurons coexpressing *DH44R2* from the intersection between *dsx^{Tk}* and *$DH44R2^{LexA}$* drivers (Fig. 6*B*). Furthermore, subsets of pC1 neurons may express TK receptors TkR86C and TkR99D as indicated by intersection of *dsx^{LexA}* with *$TkR86C^{GAL4}$* and *$TkR99D^{GAL4}$*, respectively (Fig. 6*C*), but whether these neurons could overlap with $P1^a$ [most $P1^a$ are *dsx*-positive pC1 (26)] or *dsx^{DH44}*-labeled pC1 neurons was not determined due to the lack of proper reagents. These results suggest that the $P1^a$ neurons could be the downstream of dsx^{DH44} and dsx^{Tk} neurons through the DH44 and TK receptors, *dsx^{Tk}*-labeled pC2 neurons could receive signals from dsx^{DH44} neurons by the DH44 receptor DH44R2, and *dsx^{DH44}*-labeled pC1 neurons may respond to dsx^{Tk} neurons through TK receptors. Moreover, we conducted directional syb-GRASP (45) experiments and observed specific synaptic signals from $P1^a$ to dsx^{DH44} (Fig. 6*D*1) and dsx^{Tk} neurons (Fig. 6*E*1), as well as reciprocal signals from dsx^{DH44} and dsx^{DH44} to $P1^a$ neurons (Fig. 6 *D*2 and *E*2).

To further establish such potential connections between these neurons, we knocked down DH44 receptors or TK receptors in specific neurons by corresponding RNAi. We found that knocking down *DH44R1* or *DH44R2* driven by *$P1^a$-splitGAL4* or *Tk^{GAL4}* did not affect SoMo-SLA, while knocking down *TkR99D* in $P1^a$ neurons abolished locomotor difference in GH and SH males (Fig. 6*F*). These results support that $P1^a$ neurons express TkR99D and respond to TK, and such interaction is crucial for SoMo-SLA. Note that silencing $P1^a$ neurons did not eliminate SoMo-SLA (Fig. 2*A*). We propose that $P1^a$ neurons are heterogeneous and contain functional opposite neurons such that the two strategies, silencing all $P1^a$ neurons or knocking down *TkR99D* in a subset of $P1^a$ neurons, produced different effects on SoMo-SLA.

In addition to the above anatomical and behavioral investigations, we further tested functional connectivity among dsx^{Tk}, dsx^{DH44}, and $P1^a$ neurons by epistasis experiments, in which we activated one type of neurons expressing ATP-dependent $P2X_2$ (46) and recorded the Ca^{2+} signals in another type of neurons expressing GCaMP6 using both GAL4/UAS and LexA/LexAop systems. We use the sparse *R15A01-LexA* driver to label $P1^a$ neurons as previously (10). We found that activation of dsx^{Tk} neurons expressing $P2X_2$ with a puff of ATP increased GCaMP6 signals in $P1^a$ neurons, while activation of dsx^{DH44} in the same way decreased GCaMP6 signals in $P1^a$ neurons (Fig. 6*G*). We then examined the functional pathway from $P1^a$ to dsx^{Tk} and dsx^{DH44} neurons. We found that activation of $P1^a$ by ATP induced a significant increase of Ca^{2+} in both dsx^{Tk} and dsx^{DH44} neurons (Fig. 6 *H* and *I*). Control brains without the *GAL4*- or *LexA*-driven $P2X_2$ expression did not show any calcium responses. These results demonstrate that $P1^a$ and dsx^{Tk} neurons act upon each other to form an excitatory recurrent circuit, while $P1^a$ and dsx^{DH44} neurons form a negative feedback circuit.

The above results suggested that $P1^a$ neurons may act through dsx^{Tk} neurons to up-regulate, and dsx^{DH44} neurons to down-regulate, locomotor activity, depending on $P1^a$ activation levels and phases (*SI Appendix*, Fig. S4*D*). To further validate this, we activated $P1^a$ neurons via *CsChrimson* driven by *R15A01-LexA* and silenced dsx^{Tk} or dsx^{DH44} neurons via Kir2.1. We found that silencing dsx^{Tk} neurons prevented the locomotor increase by activation of $P1^a$ neurons under low-frequency red light stimulation (Fig. 6*J*). Furthermore, silencing dsx^{DH44} neurons prevented the locomotor reduction by activation of $P1^a$ neurons under high-frequency stimulation (Fig. 6*K*). These results indicate that $P1^a$ neurons bilaterally regulate locomotor activity through dsx^{Tk} and dsx^{DH44} neurons. In summary, we identified a sexually dimorphic dsx^{DH44}-$P1^a$-dsx^{Tk} circuit that would respond to social experience to modify spontaneous locomotor activity in *Drosophila* (Fig. 7).

Substantial studies found that *dsx*-positive P1/pC1 neurons may encode a persistent internal state important for sexual and aggressive behaviors (1, 26, 27, 47). We wondered whether the dsx^{DH44}-$P1^a$-dsx^{Tk} circuit we identified in mediating SoMo-SLA would also regulate social modulation of sexual and aggressive behaviors. Indeed, we found that at least a part of this circuit was crucial for male courtship and aggression, as well as their differences from GH and SH conditions (*SI Appendix*, Fig. S6).

Discussion

Innate behaviors, despite the nature being genetically determined, are also flexible in an ever-changing environment. In this study, we focused on a simple behavioral output, the spontaneous locomotor activity, which may reflect an internal state that ultimately influences other innate behaviors. We identified the sexually dimorphic dsx^{DH44}-$P1^a$-dsx^{Tk} circuit that can respond to social enrichment and social isolation to modulate spontaneous locomotor activity. However, the absence of adequate genetic controls in our study precludes a definitive conclusion as to whether the

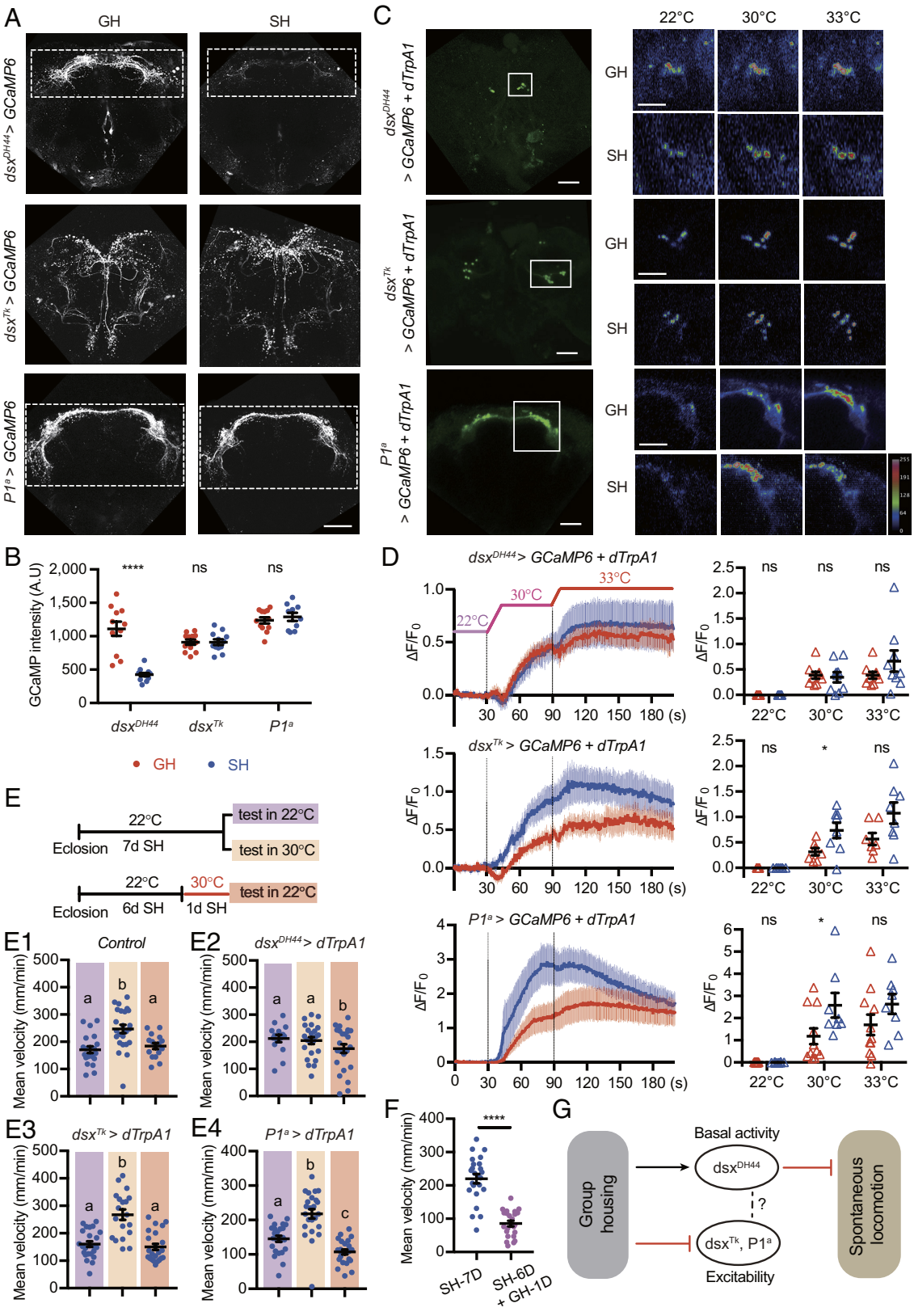

Fig. 5. Social enrichment enhances the basal activity of dsx^{DH44} neurons but reduces the excitability of dsx^{Tk} and P1^{a} neurons. (*A* and *B*) Basal GCaMP6 signals in dsx^{DH44}, dsx^{Tk}, and P1^{a} neurons in GH or SH males (*A*), which are quantified in (*B*). (Scale bar, 50 μm.) n = 10 to 13. (*C* and *D*) Changes of GCaMP6 signals in dsx^{DH44}, dsx^{Tk}, and P1^{a} neurons in GH or SH males after dTrpA1-mediated activation (*C*), which are quantified in (*D*). n = 7 to 11. (*E*) Schematic of various activation conditions. (*E1–E4*) Mean velocity of SH males with dsx^{DH44}, dsx^{Tk}, and P1^{a} neurons activated at different phases. n = 15 to 24. (*F*) Mean velocity of CS males with different GH and SH experiences. One-day GH was sufficient to reduce male spontaneous locomotion. n = 24 and 23 from left to right. (*G*) A model of how group-housing affects the basal activity of dsx^{DH44}, excitability of dsx^{Tk}, and P1^{a} neurons to modulate spontaneous locomotion. For *B, D,* and *F*, ns, not significant, *P < 0.05, ****P < 0.0001, unpaired t test. For *E*, all variables that have different letters are significantly different (P < 0.05), one-way ANOVA with Tukey's post hoc test.

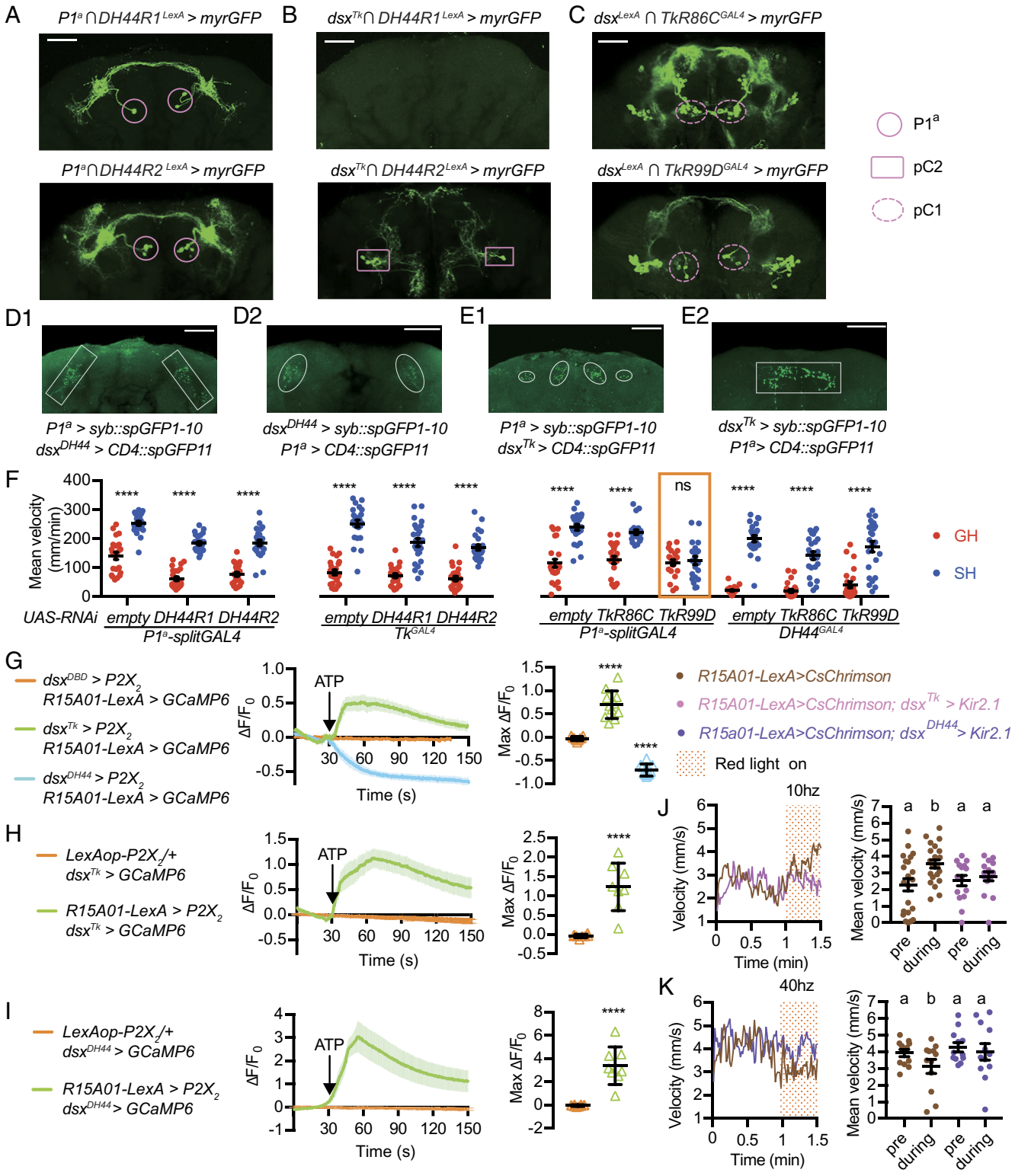

Fig. 6. P1^a neurons form positive and negative feedbacks with dsx^{Tk} and dsx^{DH44} neurons, respectively, to regulate locomotor activity. (*A*–*C*) Intersectional expression between P1^a-splitGAL4 and DH44R1^{LexA}/DH44R2^{LexA} (*A*), dsx^{Tk}-splitGAL4 and DH44R1^{LexA}/DH44R2^{LexA} (*B*), and dsx^{LexA} and TkR86D^{GAL4}/TkR99C^{GAL4} (*C*) in male brains. Representative of five samples for each. Circles indicate P1^a cell bodies, rectangles indicate dsx-positive pC2 cell bodies, and dashed circles indicate dsx-positive pC1 cell bodies. (Scale bar, 50 μm.) (*D1* and *D2*) Directional syb-GRASP signals between P1^a and dsx^{DH44} (*D1*, from P1^a to dsx^{DH44}; *D2*, from dsx^{DH44} to P1^a). (*E1* and *E2*) syb-GRASP signals between P1^a and dsx^{Tk} (*E1*, from P1^a to dsx^{Tk}; *E2*, from dsx^{Tk} to P1^a). (Scale bar, 50 μm.) (*F*) Mean velocity of GH (red) and SH (blue) males with receptors for DH44 or TK knocked down. n = 21 to 24. ****$P < 0.0001$, unpaired *t* test. (*G*) Mean GCaMP6 response traces of R15A01-LexA neurons with activation of dsx^{DH44} or dsx^{Tk} neurons in male brains. n = 9 (orange), 11 (green), and 10 (blue). (*H*) Mean GCaMP6 response traces of dsx^{Tk} neurons with activation of R15A01-LexA neurons. n = 8 for each. (*I*) Mean GCaMP6 response traces of dsx^{DH44} neurons with activation of R15A01-LexA neurons. n = 10 (orange) and 8 (green). (*J*) Locomotion velocity profile (*Left*) and mean velocity (*Right*) of GH males with R15A01-LexA neuronal activation (10 Hz) and dsx^{Tk} neuronal inactivation. n = 22 (brown) and 15 (magenta). (*K*) Locomotion velocity profile (*Left*) and mean velocity (*Right*) of GH males with R15A01-LexA neuronal activation (40 Hz) and dsx^{DH44} neuronal inactivation. n = 12 for each. For *G*–*I*, black arrows indicate ATP application. The max $\Delta F/F_0$ for each genotype is plotted on the *Right* panel. All samples are GH males. ****$P < 0.0001$, unpaired *t* test. For *J* and *K*, pre refers to 30 s before red light on and during refers to 30 s after red light on. All samples are GH males. All variables that have different letters are significantly different ($P < 0.05$), one-way ANOVA with Tukey's post hoc test.

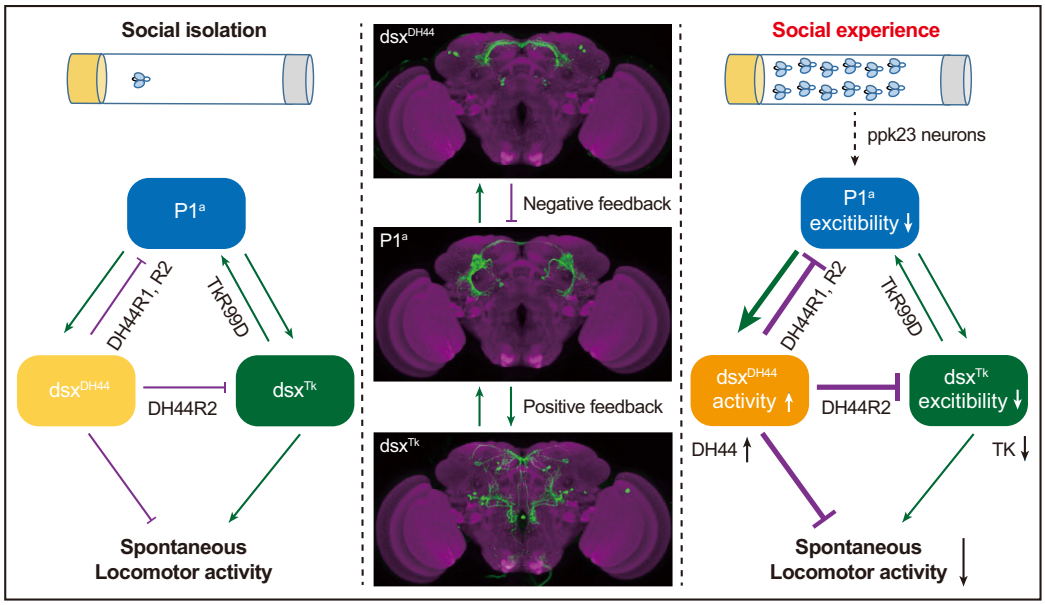

Fig. 7. A model of how social experience modulates spontaneous locomotor activity through P1^a, dsx^{Tk}, and dsx^{DH44} circuit. At the neuronal level, male–male group-housing, as detected by ppk23 neurons, elevates the basal activity of dsx^{DH44} neurons and decreases the excitability of P1^a and dsx^{Tk} neurons. Concurrently, at the molecular level, it up-regulates DH44 expression and down-regulates TK expression in the brain. dsx^{DH44} neurons negatively and dsx^{Tk} neurons positively regulate locomotion, while P1^a neurons form negative feedback with dsx^{DH44} neurons, and positive feedback with dsx^{Tk} neurons, to bilaterally regulate locomotion, depending on the intensity and phase of activation.

observed changes in locomotion are attributable primarily to social isolation or social enrichment.

Previous studies have shown that group-housing experience generally reduces sexual and aggressive behaviors in *Drosophila*, but these studies mostly use male flies (10, 17–21, 33, 48, 49). Similarly in rodents, social isolation induced hyperactivity and enhanced aggressive behaviors in males but not females (50–52). Our results support that social enrichment or social isolation may have stronger effects on male innate behaviors. Male–male group-housing experience increased day-time sleep amount and decreased spontaneous locomotor activity in individual male flies, while female–female group-housing experience did not significantly affect female locomotor activity or sleep. Regarding to the mechanism underlying such male-biased control, we found a sexually dimorphic neural circuit underlying SoMo-SLA. The primary sensory input for SoMo-SLA is the *ppk23*-expressing gustatory M cells that specifically respond to male pheromones (35), and the central integrative P1^a neurons are male-specific. It has been found that the M cells can transmit the male pheromone stimulation to the male-specific P1^a neurons via two parallel pathways (35, 53), although it is also possible that M cells may act on other subsets of P1 neurons including P1^{DH44}. Moreover, the two peptidergic neurons in this circuit, dsx^{Tk} and dsx^{DH44}, are both sexually dimorphic. Why does social regulation on locomotor activity and innate social behaviors show a bias toward males? One potential explanation is that it would be more beneficial for males than females to modify their behaviors accordingly, either living in isolation or in groups, to enhance their chances to find potential mates and gain successful reproduction.

Our results identified two social-responsive neuromodulatory systems. On one hand, social enrichment increases *DH44* expression and enhances the basal activity of the dsx^{DH44} neurons to suppress spontaneous locomotor activity. On the other hand, social enrichment decreases *Tk* expression and reduces the neuronal excitability of dsx^{Tk} and P1^a neurons, resulting in higher responding thresholds to their inputs in GH males. A caveat of this finding is the lack of appropriate reagents to further refine the subsets of dsx^{DH44} and dsx^{Tk} neurons. Remarkably, dsx^{DH44}, dsx^{Tk}, and P1^a neurons interact with each other to fine-tune locomotor activities in a state-dependent manner. First, P1^a neurons can activate dsx^{DH44} and dsx^{Tk} neurons, possibly through Acetylcholine (Ach) as previously found (29). Second, dsx^{DH44} can inhibit P1^a neurons through both DH44R1 and DH44R2 receptors, while dsx^{Tk} neurons can activate P1^a neurons through the TkR99D receptor. Third, dsx^{DH44} neurons may act on dsx^{Tk} neurons via the DH44R2 receptor, although a direct functional study is still needed. The positive feedback between P1^a and dsx^{Tk} neurons amplifies the activity of this circuit and ensures a persistent effect, which is crucial for the long-lasting social modulation. Indeed, knocking down TkR99D in P1^a neurons eliminated SoMo-SLA. Meanwhile, the negative feedback between P1^a and dsx^{DH44} neurons prevents overexcitation and thus maintains circuit stability. Note that dsx^{DH44} neurons also exert a persistent inhibitory effect on locomotion, which may be due to the nature of DH44 as a neuropeptide that acts on its metabotropic receptors. Future studies would investigate how DH44 functions, possibly as an internal coordinator, to regulate locomotion, sleep, feeding, and perhaps more innate behaviors.

Studies in the last decade have shown that P1^a neurons encode a persistent internal state and regulate multiple social behaviors (10, 26–33, 43). The two neuromodulatory recurrent circuits we identified in this study, which is centered on P1^a neurons, could thus serve as a potentially common mechanism for generating flexibility of these innate behaviors. We propose that this circuit functions adaptively in both GH and SH males to not only allow long-term modification of spontaneous locomotor activity but can also potentially tune locomotor-based social behaviors rapidly, e.g., speeding up to follow a potential mate or pause to play courtship song based on the activation levels of P1^a neurons at a particular social context. It has been proposed that P1 neurons in flies and VMHvl neurons in mice share conserved circuit configurations to control internal states and multiple innate behaviors (1, 6). Furthermore, the mammalian Corticotropin-Releasing Factor (CRF) and Tachykinin 2 (Tac2), orthologs of the fly DH44 and TK, have both been shown to respond to social isolation and regulate innate social behaviors (50, 54, 55). Thus, the two neuromodulatory recurrent circuit mechanism we identified would

also provide insights into how social experiences modulate locomotor and innate social behaviors in higher organisms.

Materials and Methods

Fly Stocks. Flies were maintained at 22 or 25 °C in a 12 h:12 h light: dark cycle. *Canton-S* flies were used as the wild-type strain. Transgenic strains were mainly from the Tsinghua Fly Center and Bloomington Drosophila Stock Center. Tk^{AD}, Tk^{LexA}, and other chemoconnectome lines were from Yi Rao. Full genotypes of the flies are listed in *SI Appendix*, Table S1.

***CsChrimson* Activation.** For all the *CsChrimson* experiments, crosses were set up on standard fly food. Tester flies were collected as virgins after eclosion and reared in single (1 male) or group (25 males) condition for 5 d on standard food in 12 h:12 h light:dark condition and then transferred onto 1 mM all-trans-retinal (ATR) (116-31-4, Sigma-Aldrich) food in vials that covered by opaque foil paper for 2 d before the optogenetic test. The spontaneous locomotion experiment was performed in dark on an infrared-light (850 nm) and red-light (620 nm, 2 mW) coemitter (30 × 30 cm, Vanch Technology, Shanghai, China) and recorded by an infrared-sensitive camera. Light intensity was measured by placing an optical power meter nearby the chambers. Light frequency and pulse width were controlled by a programmable logic controller (Youli Electronic Technology, Jinan, China).

***dTrpA1* Activation.** Tester flies were maintained for single/group housing for 7 d after eclosion at 22 °C, cold anesthetized and loaded into behavioral chambers, and allowed to recover in control (22 °C) or experimental temperatures (30 °C) for at least 30 min before behavioral tests. For the chronic thermal activation experiment (e.g., Fig. 5*E*), flies were maintained at 22 °C for 6 d and then transferred to 30 °C for 1 d before tested at 22 °C.

Sleep Test and Analysis. Individual 7-d-old males or females from single/group-housed environment were placed in locomotor activity monitor tubes (DAM2, TriKinetics Inc.) with sleep food (2% agar+5% sugar). The break times of the infrared beam induced by the fly crossing in the middle of the tube were recorded. Consistent 5 min of no beam break was considered as one sleep bout. Sleep data were analyzed using custom-designed Matlab software (56). The sleep amounts (e.g., Fig. 1*A*) are the average sleep amount in two testing days.

SoMo-SLA and Analysis. For the 24-h locomotion test, single/group-housed 7-d-old flies, if not otherwise stated, were transferred individually into round wells (2 cm diameter and 3 mm height) covered by transparent food (2% agar + 5% sucrose) on an infrared light (850 nm) emitter (20 × 30 cm), and recorded by an infrared-sensitive camera for 24 h starting from 9 am under 12 h:12 h light:dark condition. The average walking velocity during the 24-h recordin was quantified using the ZebraLab software system (ViewPoint Life Sciences, Montreal, QC, Canada) as previously used (29). For 1-h locomotion test, experiments were conducted between 9 am to 12 am and recorded by a camera (Sony FDR-AX40) in light, while the other operations and analysis were the same as above described.

Tissue Dissection, Immunostaining, and Imaging. Brains of 4- to 6-d-old flies were dissected in Schneider's insect medium (S2) and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 30 min at room temperature. After four washes of 15 min (4 × 15 min) in PAT3 (0.5% Triton X-100, 0.5% bovine serum albumin in PBS), brains were blocked in 3% normal goat serum (NGS) for 60 min before incubated in primary antibodies diluted in 3% NGS at room temperature for 4 h and at 4 °C overnight, then washed four times (4 × 15 min) again in PAT3, and incubated in secondary antibodies diluted in 3% NGS at room temperature for 4 h and at 4 °C overnight. Brains were then washed four times (4 × 15 min) in PAT3 and mounted in Vectorshield (Vector Laboratories, H-1000) for imaging. Samples were imaged at 10× or 20× objective magnification on Zeiss 700 confocal microscopes and processed with ImageJ. Antibodies used in this study were listed in *SI Appendix*, Materials and Methods.

Ex Vivo Calcium Imaging. The GCaMP6m calcium indicator was expressed in specific neurons of flies to measure intracellular calcium levels. Fly brains were dissected and mounted in hemolymph-like saline (AHLS, pH 7.5). The brain was secured on a glass slide with AHLS for imaging. GCaMP signals were captured within 5 min using a Bruker confocal microscope (Ultima Investigator) and analyzed with ImageJ. For details, see *SI Appendix*, Materials and Methods.

Brain Image Registration. The standard brain used in this study was described previously (57). In this study, confocal images of dsx^{Tk}, dsx^{DH44}, and $P1^{a}$ neurons were registered onto the standard brain with a Fiji graphical user interface as described previously (58).

Quantitative Real-Time PCR. Approximately 100 flies from single- or group-housing condition were collected into a 15-mL tube and chilled on liquid nitrogen, followed by vigorous vortex and filtering with metal sieves to obtain the fly heads. Total RNA was extracted from the head with the RNA isolation kit (RC112-01 Vazyme) and purified with the DNA-free kit (AM1906 Ambion). The cDNA was synthesized using the Prime Script reagent kit (18091050, Invitrogen). Quantitative PCR was performed on the LightCycler 96 Real-Time PCR System (Roche) using FastStart Universal SYBR Green Master /ROX qPCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA). *Rp49* was used as control for normalization.

Statistics. Prism GraphPad was used for statistical analysis. Data presented in this study were first verified for normal distribution by the D'Agostino–Pearson normality test. If normally distributed, Student's *t* test was used for pairwise comparisons, and one-way ANOVA was used for comparisons among multiple groups, followed by Tukey's multiple comparisons. If not normally distributed, the Mann–Whitney *U* test was used for pairwise comparisons, and the Kruskal–Wallis test was used for comparisons among multiple groups, followed by Dunn's multiple comparisons. For comparison between the change in velocity and zero (Figs. 3 *A–D* and 4 *F–I*), the one-sample *t* test was performed.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*. This study does not involve new code or sequence data. Fly stocks and reagents used in this study are available from the corresponding author upon reasonable request.

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