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1 # The neuropeptide Drosulfakinin regulates social isolation-induced
2 aggression in *Drosophila*

3 Pavan Agrawal[1], Damian Kao, Phuong Chung, Loren L. Looger
4 Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA, USA
5 [1]Correspondence: pavan.janelia@gmail.com
6

7 ## ABSTRACT

8 Social isolation strongly modulates behavior across the animal kingdom. We utilized the fruit fly
9 *Drosophila melanogaster* to study social isolation-driven changes in animal behavior and gene
10 expression in the brain. RNA-seq identified several head-expressed genes strongly responding to social
11 isolation or enrichment. Of particular interest, social isolation downregulated expression of the gene
12 encoding the neuropeptide *Drosulfakinin* (*Dsk*), the homologue of vertebrate cholecystokinin (CCK),
13 which is critical for many mammalian social behaviors. *Dsk* knockdown significantly increased social
14 isolation-induced aggression. Genetic activation or silencing of *Dsk* neurons each similarly increased
15 isolation-driven aggression. Our results suggest a U-shaped dependence of social isolation-induced
16 aggressive behavior on *Dsk* signaling, similar to the actions of many neuromodulators in other contexts.

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20 **Data availability:** The raw sequence data from RNA-seq experiments has been deposited into the
21 Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) with accession number: PRJNA481582.
22 Supplementary files and figures accompany this article.
23
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30 **Corresponding author**
31 Pavan Agrawal
32 Howard Hughes Medical Institute, Janelia Research Campus
33 19700 Helix Drive
34 Ashburn, VA 20147
35 USA
36 Phone: +1 571 209 4000 (ext. 3208)
37 Email: pavan.janelia@gmail.com
38

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39 **INTRODUCTION**

40 Social isolation is a passive stressor that profoundly influences the behavior of social animals
41 (Grippo et al., 2007; Hall et al., 1998; Wallace et al., 2009). Social isolation increases aggression in
42 humans (Ferguson et al., 2005), rodents (Luciano and Lore, 1975; Ma et al., 2011; Wallace et al., 2009),
43 and fruit flies (Hoffmann, 1989; Wang et al., 2008).

44 *Drosophila melanogaster* has been a successful model system for identifying the neural
45 substrates of aggressive behavior (Asahina, 2017; Baier et al., 2002; Chen et al., 2002; Kravitz and
46 Huber, 2003). Several conserved neuromodulators have been identified as key players in regulating
47 aggression, including biogenic amines such as dopamine (Alekseyenko et al., 2013; Kayser et al., 2015),
48 octopamine (Certel et al., 2007; Hoyer et al., 2008; Kayser et al., 2015; Williams et al., 2014; Zhou et
49 al., 2008), and serotonin (Alekseyenko et al., 2010, 2014; Dierick and Greenspan, 2007); and
50 neuropeptides including neuropeptide F (NPF; Asahina et al., 2014; Dierick and Greenspan, 2007) and
51 tachykinin (Asahina et al., 2014). The associated receptors (Asahina et al., 2014) and neural circuits have
52 been identified in some cases (Koganezawa et al., 2016).

53 Flies display aggression in a variety of settings, including male-male competition for females,
54 territorial disputes, etc. (Asahina, 2017; Dow and Schilcher, 1975; Hoffmann, 1987; Jacobs, 1960;
55 Kravitz and Fernandez, 2015). In this study, we sought to elucidate the circuit and genetic underpinnings
56 of male aggression induced by deprivation of social interactions. Using an RNA-seq screen, we identified
57 several candidate genes, most notably the neuropeptide Drosulfakinin (Dsk) (Chen and Ganetzky, 2012;
58 Chen et al., 2012; Nichols et al., 1988; Söderberg et al., 2012), the homologue of the vertebrate
59 cholecystokinin (CCK). CCK is well documented as a critical modulator of anxiety and aggression in a
60 number of settings (Katsouni et al., 2013; Li et al., 2007; Panksepp et al., 2004; Vasar et al., 1993;

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61 Zwanzger et al., 2012). Dsk has been reported to modulate aggression in *Drosophila* (Williams et al.,
62 2014), but many mechanistic details are lacking.
63 Here we use modulation of group size and isolation duration, RNA-seq, RNA interference
64 (RNAi), and genetic activation or silencing of target neural populations to further elucidate the
65 involvement of Dsk in aggressive behavior.
66
67 **RESULTS**

68 **Social isolation induces transcriptional changes in male *Drosophila* heads**

69 To probe the molecular mechanisms involved in regulating social isolation-induced behaviors, we
70 performed RNA-seq on male flies that were housed either individually (single-housed, SH) or in groups
71 of 20 (group-housed, GH) for four days in vials containing standard fly food. Flies were flash-frozen,
72 whole heads isolated, RNA prepared and sequenced (N=2 biological replicates) (**Figure 1A**). Both SH
73 and GH flies showed strong inter-replicate concordance: r = 0.964 and 0.965, respectively
74 (**Supplementary Figure S1A,B**). Commonly used RNA-seq analysis methods utilize diverse models for
75 dispersion, normalization and differentially-expressed gene (DEG) calling. To increase stringency of our
76 DEG calling, we utilized three separate techniques, DEseq2 (Love et al., 2014), edgeR (Robinson et al.,
77 2010), and EBseq (Leng et al., 2013), and compared their results (**Material and Methods**). We focused
78 on genes identified by all three methods, which we considered to be robust hits.

79 Using stringent criteria for differential expression, *i.e.* fold-change ≥ 2 and false discovery rate ≤
80 0.05 (**Figure 1B, Supplementary Table S1**), 90 genes were selected by at least one method, and 25 by
81 all three (**Table 1**). Most DEGs were related to the immune response (**Table 1, Supplementary Tables**
82 **S1, S2**), which is consistent with the observation that social isolation leads to immune upregulation across

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83 the animal kingdom (Cole et al., 2015; Ellen et al., 2004; Powell et al., 2013). Many of these immune-
84 related genes are commonly seen as DEGs in fly microarray and RNA-seq experiments (Carney, 2007;
85 Ellis and Carney, 2011; Mohorianu et al., 2017; Wang et al., 2008) (**Supplementary Table S3**). We
86 compared 90 social isolation-induced DEGs in whole fly heads with similar data generated specifically
87 from FACS-purified dopaminergic neurons (Agrawal et al., 2019). Depending on the particular method
88 used to identify DEGs, we found between 3 to 9 genes common between these two datasets, suggesting
89 that social isolation regulates somewhat different set of genes in whole heads relative to dopaminergic
90 neurons (**Supplementary Table S3**). Along these lines, perturbation of neural activity stimulates
91 expression of distinct sets of genes in whole brain *versus* dopaminergic neurons (Chen et al., 2016).

92 In our data, very few (7 down-regulated, 4 up-regulated) not-obviously-immune transcripts were
93 identified by all three RNA-seq analysis methods as being significantly modulated by social experience
94 (**Table1, Supplementary Table S1)**. Examples of genes down-regulated in SH males include the male-
95 specific odorant-binding protein *Obp99b* (Anholt et al., 2003), hydroxysteroid dehydrogenase *CG6012*,
96 and the neuropeptide *Drosulfakinin* (*Dsk*) (**Figure 1B, Table1**). Genes upregulated in SH males included
97 the sensory cilium structural protein *Artichoke* (*Atk*), cathepsin-like protease *CG11459*, long non-coding
98 RNA *CR44404*, and secreted peptide *CG43175* **(Table1)**. Several of these transcripts have been
99 identified in previous studies (**Supplementary Table S3**), albeit under different conditions (*e.g.*
100 courtship, social defeat) (Barajas-Azpeleta et al., 2018; Carney, 2007; Ellis and Carney, 2011; Mohorianu
101 et al., 2017; Wang et al., 2008) or by different techniques (*e.g.* microarray, RNA-seq). Of these
102 stringently selected transcripts, only *Dsk* is central nervous system-specific and was selected for further
103 study. *Dsk* expression differences were validated with qPCR on head-extracted RNA isolated from SH
104 and GH males (**Supplementary Figure S2A**).

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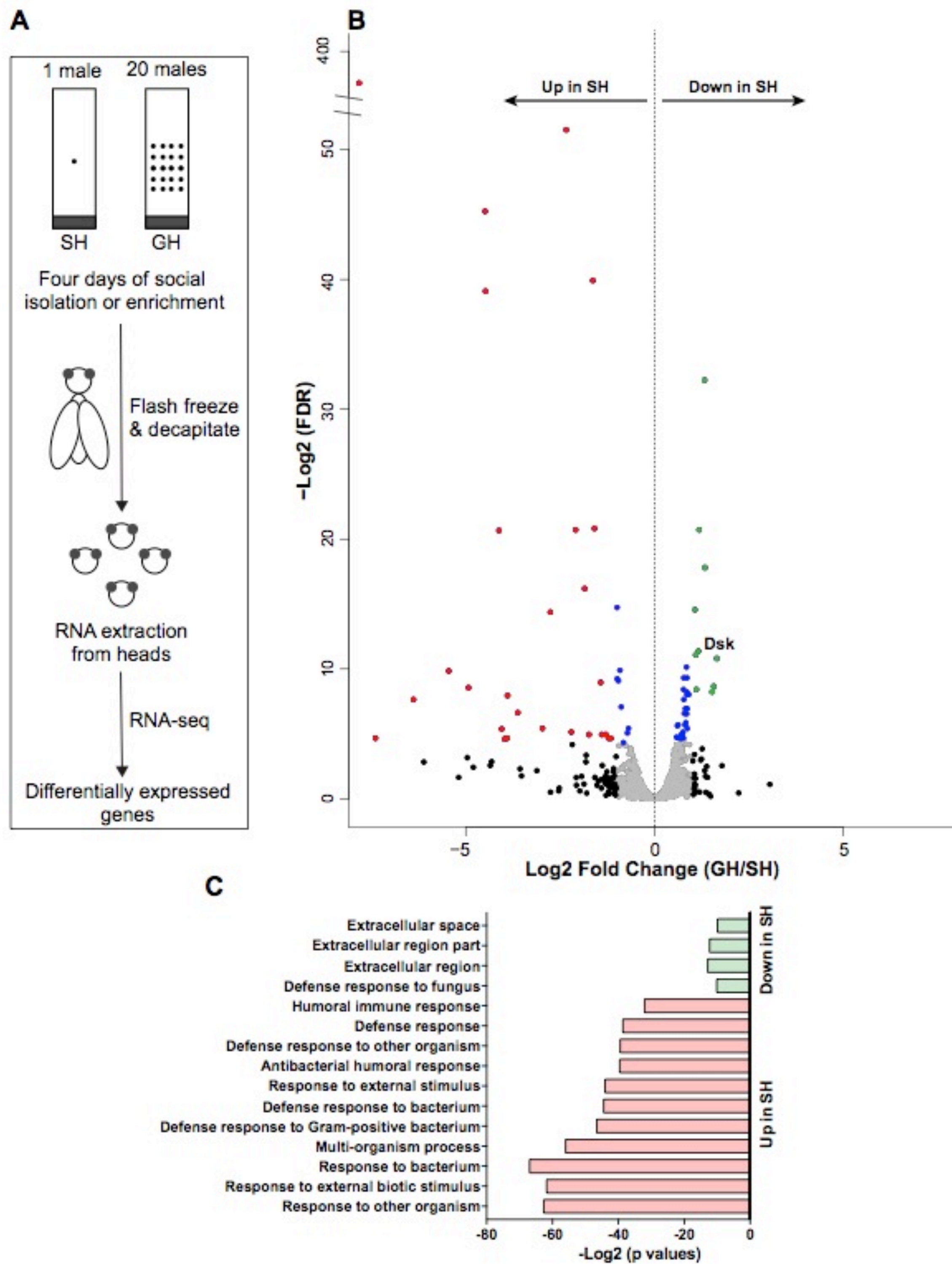

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106 **Figure 1. Transcriptional differences in male heads after single housing (SH) or group housing (GH).** (A)

107 Outline of the experimental paradigm. (B) Volcano plot of RNA-seq profile using DEseq2 for individual genes.

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108 Gray: FC<2; black: FC>2; blue: P<0.05; green: P <0.05 and +FC>2, red: P <0.05 and -FC>2; FC = fold change.

109 (C) Enriched Gene Ontology categories for differentially-expressed genes obtained from DEseq2 analysis.

Flybase Gene ID	Gene symbol	DESeq2	edgeR	EBseq	Annotation/known/predicted function
Genes down-regulated in response to social isolation					
FBgn0039685	Obp99b	Down	Down	Down	sensory perception of chemical stimulus
FBgn0000500	Dsk	Down	Down	Down	neuropeptide
FBgn0032615	CG6012	Down	Down	Down	hydroxysteroid dehydrogenase
FBgn0036589	CG13067	Down	Down	Down	cuticle protein
FBgn0030398	Cpr11B	Down	Down	Down	cuticle protein
FBgn0032507	CG9377	Down	Down	Down	epithelial protease
FBgn0040629	CG18673	Down	Down	Down	carbonic anhydrase 2
FBgn0052282	Drsl4	Down	Down	Down	antifungal peptide
FBgn0035434	Drsl5	Down	Down	Down	antifungal peptide
Genes up-regulated in response to social isolation					
FBgn0000278	CecB	Up	Up	Up	antibacterial humoral response
FBgn0005660	Ets21C	Up	Up	Up	defense response to bacterium
FBgn0032638	SPH93	Up	Up	Up	defense response to Gram-positive bacterium
FBgn0032639	CG18563	Up	Up	Up	epithelial protease
FBgn0265577	CR44404	Up	Up	Up	long non-coding RNA
FBgn0014865	Mtk	Up	Up	Up	antifungal peptide
FBgn0036995	Atk	Up	Up	Up	sensory cilium structural protein
FBgn0038299	Spn88Eb	Up	Up	Up	fungal-induced protease inhibitor
FBgn0052185	Edin	Up	Up	Up	defense response to Gram-negative bacteria
FBgn0037396	CG11459	Up	Up	Up	cathepsin-like protease
FBgn0010381	Drs	Up	Up	Up	antifungal peptide
FBgn0043578	PGRP-SB1	Up	Up	Up	innate immune response
FBgn0010388	Dro	Up	Up	Up	antibacterial peptide
FBgn0039593	Sid	Up	Up	Up	response to bacterium
FBgn0262794	CG43175	Up	Up	Up	unannotated secreted peptide
FBgn0044812	TotC	Up	Up	Up	response to bacterium

110

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111 **Table 1.** Genes identified as differentially expressed upon social isolation by three different RNA-seq analysis
112 methods. The last column shows gene function from Flybase, or predicted from Pfam (http://pfam.xfam.org)
113 classification or annotation of homologues in other species if not annotated in Flybase. A complete list of genes
114 and GO analysis is included in Supplementary Tables S1 and S2.

115 ***Dsk* knock-down affects social isolation-induced aggression**

116 As CCK is known to regulate aggression, anxiety, and social-defeat responses in rodents (Katsouni et
117 al., 2013; Li et al., 2007; Panksepp et al., 2004; Vasar et al., 1993; Zwanzger et al., 2012), we next tested
118 specific phenotypic effects of *Dsk* modulation. Dsk localizes to the pars intercerebralis, parts of the
119 protocerebrum and the sub-esophageal ganglion (Nichols, 1992; Nichols and Lim, 1996; Söderberg et
120 al., 2012). In the pars intercerebralis, Dsk is expressed in the insulin-like peptide Dilp2-producing
121 neurons (Söderberg et al., 2012). Faithful GAL4 driver lines are available for both *Dsk* (Asahina et al.,
122 2014) and *Dilp2* (Rulifson, 2002) (**Figure 2A, B**). To quantify aggression, we counted lunges using the
123 software package CADABRA (Dankert et al., 2009). Lunging, *i.e.* a fly rearing on its hind legs and
124 snapping downward on its opponent, is a prominent aggressive behavior in *Drosophila* males (Hoffmann,
125 1987; Hoyer et al., 2008; Nilsen et al., 2004). Knockdown of *Dsk* in *Dsk-GAL4* neurons using RNA
126 interference (RNAi) significantly increased lunges in SH flies relative to controls without RNAi insert
127 (**Figure 2C**). Similar effects were observed upon *Dsk* knock-down using the *Dilp2-GAL4* driver (**Figure
128 2E**). Successful knockdown using pan-neuronal *elav*-GAL4 ^{c155} was confirmed by qRT-PCR
129 (**Supplementary Figure S2B**). Thus, lowering *Dsk* expression in the pars intercerebralis increased
130 aggressive lunging following social isolation.

131 It was previously suggested that aggressive behaviors should be normalized to overall locomotor
132 activity (Hoyer et al., 2008). Isolated wild-type flies sleep less and show greater levels of overall daytime
133 activity (Ganguly-Fitzgerald et al., 2006) than GH flies. In contrast, isolated *Dsk*-knockdown flies show

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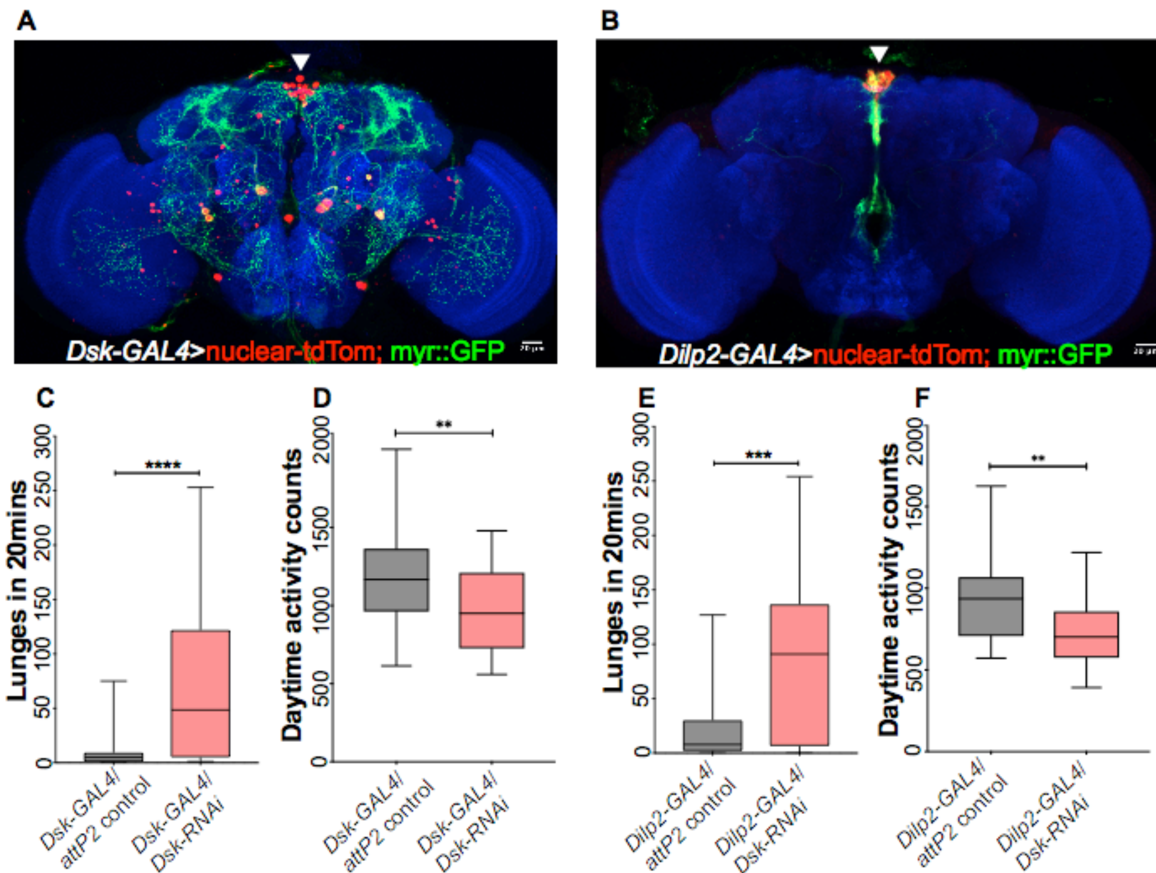
134 significantly reduced overall daytime activity (**Figure 2D, F**; for *Dsk-GAL4* and *Dilp2-GAL4*,
135 respectively), with no effect in GH flies (**Supplementary Figure S3**). Thus, the observed increase in
136 aggression in SH males upon *Dsk* knockdown arises despite decreased overall activity.



137

138 **Figure 2. *Dsk* knockdown increases social isolation-induced aggression independently of overall activity**
139 **levels.** *Dsk* levels were reduced by driving the expression of *UAS-Dsk*-RNAi with *Dsk-GAL4* or *Dilp2-GAL4*.
140 These drivers overlap in the pars intercerebralis region (white arrowhead, A and B). RNAi-mediated *Dsk*
141 knockdown increased aggression in SH flies relative to the *attP2* background controls without RNAi insert (C,
142 *Dsk-GAL4*, P<0.0001, N=38; E, *Dilp2-GAL4*, P=0.0004, N=26; Mann-Whitney U-test). *Dsk* knockdown
143 significantly reduced the overall daytime activity of SH males (D, *Dsk-GAL4*, P=0.004, N=32; F, *Dilp2-GAL4*,
144 P=0.002, N=32; Student's t-test).

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145 In *Drosophila*, Dsk has two receptors, CCK-like receptor (CCKLR)-17D1 and CCKLR-17D3
146 (Kubiak et al., 2002). Signaling through CCKLR-17D1, but not CCKLR-17D3, is responsible for larval
147 neuromuscular junction growth and muscle contraction (Chen and Ganetzky, 2012; Chen et al., 2012).
148 Since *GAL4* driver reagents are not available for several neuropeptide receptors, we used a MiMiC
149 reporter line (Nagarkar-Jaiswal et al., 2015) available for *CCKLR-17D1* (**Material and Methods**) to
150 ascertain its expression in the brain. We found reporter expression in several brains regions including in
151 the pars intercerebralis (PI), which overlapped with *Dilp2* expression (**Supplementary Figure S4A-C,**
152 **A'-C')**. Successful knockdown for both *CCKLR-17D1* and *CCKLR-17D3* was confirmed using *elav*-
153 GAL4 ^{c155} by qRT-PCR (**Supplementary Figure S4D**). However, in *Dsk-GAL4* or *Dilp2*-GAL4 flies,
154 knockdown of *CCKLR-17D1*, but not *CCKLR-17D3*, increased aggression of SH flies (**Supplementary**
155 **Figure S4E, F**). This is consistent with results for the ligand *Dsk* and suggests that signaling of Dsk
156 through its receptor CCKLR-17D1 in PI increases isolation-driven aggression.

157

158 **Social isolation is essential for *Dsk*-mediated aggression**

159 To more precisely determine the interaction between social isolation and *Dsk*, we varied group
160 size and isolation length from 1-20 flies and from 1-4 days, respectively. The presence of even a single
161 other fly almost eliminated *Dsk* knockdown-evoked aggression, and aggression remained suppressed as
162 group size increased (**Figure 3 A, B**). As few as 1-2 days of isolation modestly but significantly increased
163 aggression in SH males in which *Dsk* was knocked down (**Supplementary Figure S5A, B**); the effect
164 increased for up to 4 days.

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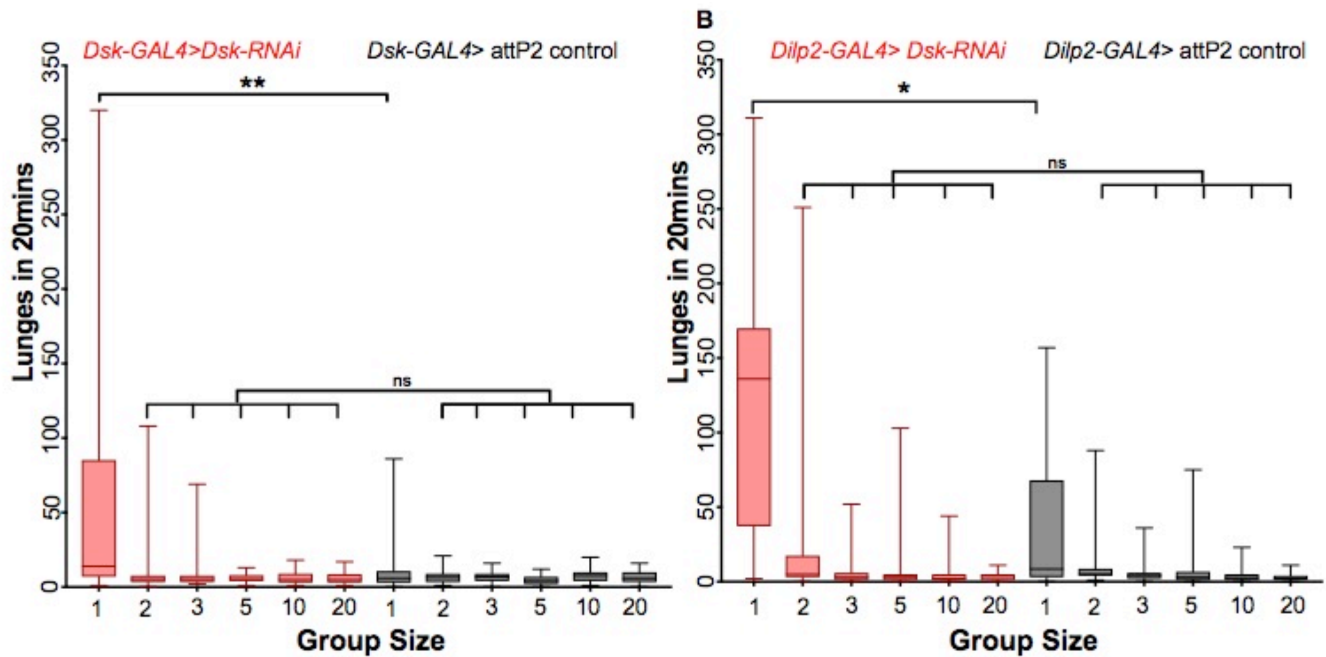

165

166 **Figure 3. Social isolation is essential for aggression mediated by *Dsk* knockdown.** (A) *Dsk-GAL4*/*Dsk-RNAi*
167 *vs.* *Dsk-GAL4*/*attP2*-control, group size 1, **: P=0.0037. N=33-36. (B) *Dilp2-GAL4*/*Dsk-RNAi* *vs.* *Dilp2-*
168 *GAL4*/*attP2*-control, group size 1, *: P=0.013. N=24-36. Presence of other males drastically reduced aggression
169 as seen in group size 2 or greater. Kruskal-Wallis ANOVA with Dunn's multiple comparison test.

170

171 **Both activation and silencing of *Dsk* neurons increase aggression**

172 Having established the contribution of *Dsk* and its receptor *CCKLR-17D1* to aggression in SH
173 males, we next explored the function of the neurosecretory cells themselves. Silencing of *Dsk* neurons
174 with the inward rectifying potassium channel Kir2.1 (Baines et al., 2001) significantly increased lunging
175 (**Figure 4A**), which is consistent with the involvement of *Dsk* and *CCKLR-17D1* signaling for promoting
176 aggression in SH males. Surprisingly, genetic activation of *Dsk* neurons with the bacterial sodium
177 channel NaChBac (Nitabach, 2006) also increased aggression (**Figure 4B**). GH flies showed very few
178 lunges in all cases, indicating that social isolation is critical for aggression in our assays (**Figure 4 A, B**).

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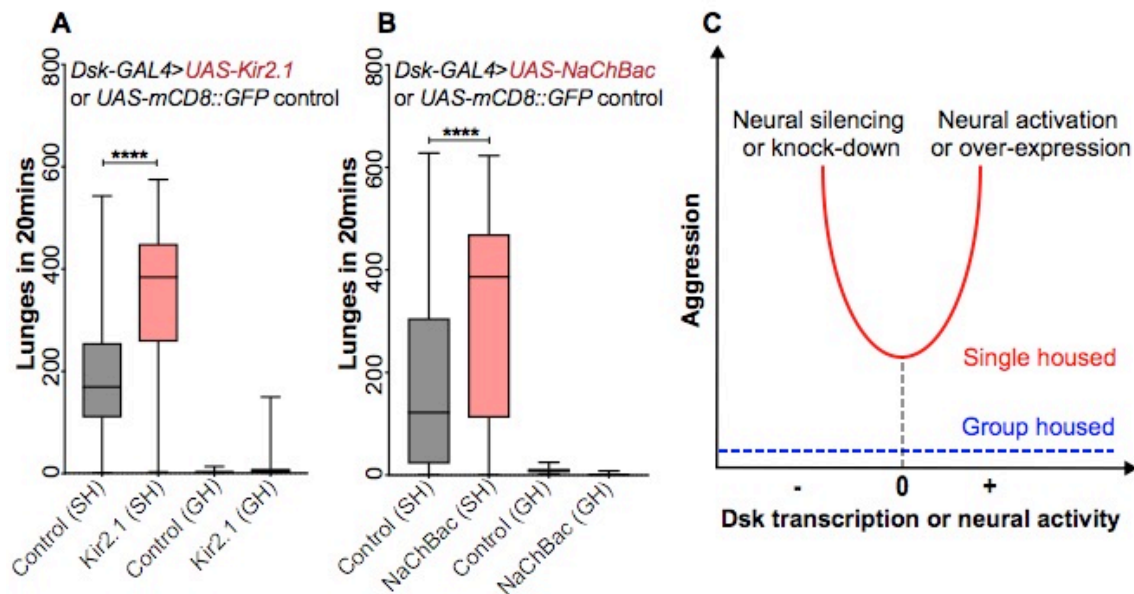

179

180 **Figure 4. Genetic activation and silencing of *Dsk* neurons both increase aggression.** (A) Silencing of *Dsk*
181 neurons with *UAS-Kir2.1-EGFP* by *Dsk-GAL4* driver increases aggression in SH males ($P < 0.0001$, Mann-
182 Whitney U-test, N=48). (B) Activation of *Dsk* neurons with *UAS-NaChBac* by *Dsk-GAL4* driver also increases
183 aggression in SH males ($P < 0.0001$, Mann-Whitney U-test, N=52). Controls in both (A) and (B) are *UAS-*
184 *mCD8::GFP* transgene driven by *Dsk-GAL4*. (C) Schematic of putative U-shaped effect of *Dsk* neuron activity
185 and transcript levels on aggression.

186

187 ## DISCUSSION

188 We have shown that knockdown of the neuropeptide *Dsk* or its receptor *CCKLR-17D1* in the pars
189 intercerebralis (PI) increases social isolation-driven aggression of male flies. Moreover, *Dsk* appears to
190 act in a U-shaped fashion (**Figure 4C**), with both knockdown (our results) and overexpression (Williams
191 et al., 2014) increasing aggression. We also showed that *Dsk* neuronal activity follows a similar trend,
192 with both activation and silencing increasing aggression. This suggests that the primary role of these
193 neurons in this context is indeed production and secretion of Dsk (Söderberg et al., 2012). Transcription

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194 factors in the fly PI neurons regulating aggression were recently identified (Davis et al., 2014), and it
195 was shown that activation of PI neurons increases aggression. However, the downstream neuropeptides
196 were not known (Thomas et al., 2015). Our findings identify *Dsk* as a key neuropeptide expressed in the
197 PI region that regulates aggression. Further work will be required to delineate the aggression-modulating
198 functions, if any, of other neuropeptides also secreted from the PI region.

199 A recent neural activation screen (Asahina et al., 2014) explored the role of neuropeptides in
200 aggression in *Drosophila*, but investigated only group-housed flies. Intriguingly, Asahina *et al.* identified
201 tachykinin signaling in the lateral protocerebrum and explicitly ruled out involvement of *Dsk* neurons
202 (Asahina et al., 2014) in GH fly aggression. Thus, male-male aggression in GH and SH flies appears to
203 be controlled by different neuropeptides in different brain regions. The absence of *Dsk* neurons from the
204 screen results in GH flies (Asahina et al., 2014), combined with our results showing suppressed
205 aggression in GH flies regardless of *Dsk* transcription or neural activity, suggests a mechanism that
206 overrides *Dsk* function.

207 Downregulation of Dsk receptor *CCKLR-17D1* in *Dsk/Dilp2* neurons also increased aggression,
208 consistent with the observation that some neuropeptidergic neurons, *e.g.* those for neuropeptide F (Shao
209 et al., 2017; Wen et al., 2005), neuropeptide Y (Qi et al., 2016) and FMRFamide (Ravi et al., 2018) have
210 receptors to modulate their signaling in an autocrine manner. However, pan-neuronal down-regulation
211 of *CCKLR-17D1* receptor did not affect aggression (data not shown), suggesting potential antagonistic
212 effects outside *Dsk/Dilp2* neurons.

213

214 **Hormetic regulation of behaviors**

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215 The U-shaped ("hormetic") response of the aggression phenotype to both Dsk levels and *Dsk*$^+$ neuronal
216 activity are similar to such responses seen for NPF (Asahina et al., 2014; Dierick and Greenspan, 2007)
217 and dopamine (Alekseyenko et al., 2013) neurons in *Drosophila* aggression. Such effects are not
218 unexpected, given the ubiquity of such hormetic responses in neuromodulator signaling pathways (Baldi
219 and Bucherelli, 2005; F.Flood et al., 1987; Monte-Silva et al., 2009) and receptors in general (Calabrese
220 and Baldwin, 2001). At the level of individual G-protein coupled receptors, such U-shaped responses
221 (low-dose agonism, high-dose antagonism) arise directly from equations considering receptor expression
222 level and the effects of receptor activation on downstream signaling pathways (Kohn and Melnick, 2002).
223 At the circuit level, it is thought that such U-shaped responses help to maintain neuronal activity patterns,
224 and the resulting behaviors, near homeostatic optima, with deviations resulting in negative feedback
225 (Arnsten et al., 2012; Brunel and Wang, 2001; Herman, 2013).
226

227 **Social experience modulates gene expression in *Drosophila* heads**

228 There have been a number of prior studies on the genetic basis of aggression in *Drosophila*, many
229 of them performed with DNA microarrays – which record counts for specific transcripts of interest –
230 rather than with RNA-seq, which counts all transcripts within cells. Four such studies have been
231 performed in recent years, each identifying a large number of putative aggression-related genes:
232 (Edwards et al., 2006) found 1672 such transcripts, (Dierick and Greenspan, 2006) found 149, (Wang et
233 al., 2008) found 183, and (Tauber, 2010) found 339. It should be noted that these four studies used very
234 different experimental methods: (Edwards et al., 2006) and (Dierick and Greenspan, 2006) bred flies for
235 aggressive behavior over several generations, and isolated mRNA for microarray analysis at a time
236 unrelated to aggression events; thus, these genes are generally high in the selected flies. (Wang et al.,
237 2008) analyzed single-housed and group-housed flies, again irrespective of specific aggression events.

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238 (Tauber, 2010), meanwhile, isolated pairs of aggressive flies and obtained mRNA for sequencing directly
239 following bouts, looking for aggression bout-driven gene expression. Given the substantial differences
240 in experimental design, and the imperfect reliability of microarray quantification, it is perhaps
241 unsurprising that of the 1672, 149, 183, and 339 differentially expressed genes in each study,
242 respectively, there were only 2 in common to all four studies: the olfactory binding protein Obp99b and
243 CG13794, an unannotated transporter. Obp99b appeared amongst our 25 most significant hits, whereas
244 CG13794 did not appear to be differentially expressed at all in our assays. Given that the involvement of
245 Dsk in aggression is quite context-specific, for instance Asahina *et al.* explicitly ruled out of involvement
246 in aggression of group-housed flies (Asahina et al., 2014), it is perhaps unsurprising that it was not found
247 in several of the screens. In fact, the only one of these four studies to uncover Dsk was the one that
248 utilized socially isolated flies (Wang et al., 2008), strengthening the notion that Dsk specifically links
249 social isolation to aggression. It was this link with social behavior that drew our attention to Dsk, and
250 indeed our experiments bear out that this function is mediated through activity in the central brain. The
251 pars intercerebralis has been shown to be the seat of regulation of many other social and sexually
252 dimorphic behaviors (Belgacem and Martin, 2002; Luo et al., 2014; Mattaliano et al., 2007; Terhzaz et
253 al., 2007).

254 At the other end of the spectrum, olfactory inputs are ubiquitous and odor processing through
255 olfactory receptors factors into essentially every fly action. Along with Dsk, Obp99b was the most down-
256 regulated gene in our single-housed males **(Table 1, Supplementary Table S1)**. Obp99b was also picked
257 up in two studies of other social behaviors: courtship-exposed males (Carney, 2007) and male
258 competition for mates (Mohorianu et al., 2017). Olfactory binding proteins (OBPs) are secreted by
259 support cells in the antennal trichoid sensilla to assist in odorant binding and recognition by olfactory
260 receptors (Galindo and Smith, 2001; Larter et al., 2016). A critical role for Obp76a (Lush) in recognition

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261 of the pheromone cis-vaccenyl acetate (cVA) by olfactory receptor Or67d, and in driving aggression
262 following acute pheromone exposure, has been established (Billeter and Levine, 2015; Wang and
263 Anderson, 2010). However, following chronic cVA exposure, the pheromone also activates a second
264 receptor, Or65a, which then inhibits Or67d glomeruli and decreases aggression (Liu et al., 2011). The
265 OBP mediating cVA recognition by Or65a is currently unknown, but appears not to be Lush (Laughlin
266 et al., 2008). It is possible that the OBP identified in all the screens discussed, *i.e.* Obp99b, recognizes
267 cVA for signaling through Or65a; it is also possible that it recognizes other odorants. Given its ubiquity
268 in screens for social behaviors, we speculate that the molecules recognized are likely pheromones.
269 Obp99b is one of the most male-specific transcripts identified (Fujii and Amrein, 2002), and indeed it
270 had been previously discovered as a gene in the sex-determination cascade, under its previous name Turn
271 on Sex-Specificity (Tsx) (Wolfner, 2003). In this set of experiments, we selected Dsk for investigation
272 because of its link to social context; however, the precise function of Obp99b warrants closer study,
273 given its probable role in pheromone detection.

274 Other prominent hits from our screen appear interesting, as well **(Table 1, Supplementary Table**
275 **S1)**. The third most isolation-driven down-regulated transcript, *CG6012*, appears to encode a
276 hydroxysteroid dehydrogenase. Such enzymes have been shown to be critical for pheromone production
277 in insects, with the related enzyme CG1444 (Spidey) processing both ecdysone and related cuticular
278 hydrocarbons (Chiang et al., 2016). The Turandot peptides, annotated as stress-response genes, also
279 appear to play sex-specific roles in behaviors such as courtship. Turandot A and Turandot C, both up-
280 regulated in isolated males in our study **(Supplementary Table S1)**, are greatly female-enriched, and
281 Turandot C, in particular, is up-regulated in female flies following playing of an attractive, conspecific
282 courtship song over a speaker (Immonen and Ritchie, 2012). Finally, we would note that new roles have
283 recently been proposed for transcripts annotated as encoding antimicrobial peptides (*e.g.* Diptericin B,

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284 DptB), specifically the modulation of long-term memory (Barajas-Azpeleta et al., 2018). It is possible
285 that some of the transcripts annotated as antimicrobial peptides are instead (or in addition) memory
286 regulators with roles in social behaviors. Indeed, playing synthetic attractive, conspecific *versus* aversive,
287 heterospecific courtship song dramatically lowered expression of the ostensibly antimicrobial peptides
288 Attacin-A, Attacin-C, DptB, Drosocin, and Immune-induced molecule 18 (Immonen and Ritchie, 2012).
289 Intriguingly, all of these molecules were increased in males following aversive social isolation in our
290 study **(Figure 1 B, C, Supplementary Table S1)**. The only other molecule shared between the two
291 studies, Methuselah-like 8, showed the opposite pattern: up-regulated in females hearing attractive song
292 and down-regulated in males after aversive isolation. Of course, given the wealth of bacteria, fungi,
293 viruses and other microbes present in and on flies and their food, it is probable that many annotated
294 antimicrobial peptides are indeed responding to differences in pathogen load composition between SH
295 and GH flies. But the observation that many putatively antimicrobial molecules respond strongly to
296 stimuli (*e.g.* synthetic courtship song) that do not involve alteration of their physical environment in any
297 way indicates that these molecules have more sophisticated functions in the brain, and may encode
298 valence (attractive, aversive) of social interactions.
299

300 **Dsk and its homologue CCK have evolutionarily conserved role in regulating aggression**

301 In mammals, the Dsk homologue cholecystokinin (CCK) and its receptors regulate aggression, anxiety,
302 and social-defeat responses (Katsouni et al., 2013; Li et al., 2007; Panksepp et al., 2004; Vasar et al.,
303 1993; Zwanzger et al., 2012). For instance, intravenous injection of the smallest isoform, CCK-4, in
304 humans reliably induces panic attacks (Bradwejn et al., 1991; Tõru et al., 2010) and is often used to
305 screen anxiolytic drug candidates. However, in other contexts, such as in mating (Bloch et al., 1987) and
306 juvenile play (Burgdorf et al., 2006), CCK encodes strong positive valence. CCK colocalizes with

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307 dopamine in the ventral striatum, and microinjection of CCK into the rat nucleus accumbens phenocopies
308 the effects of dopamine agonists, increasing attention and reward-related behaviors (Vaccarino, 1994),
309 further supporting its role in positive valence encoding. CCK actions differ across brain regions, in a
310 context-dependent manner. For instance, time pinned (negative-valence) during rough-and-tumble play
311 correlated with increased CCK levels in the posterior cortex and decreased levels in hypothalamus
312 (Burgdorf et al., 2006). However, lower hypothalamic CCK also correlated with positive-valence play
313 aspects including dorsal contacts and 50 kHz ultrasonic vocalizations. Thus, CCK can encode both
314 positive- and negative-valence aspects of complex behaviors differentially across the brain. As with
315 many neuromodulators (Calabrese, 2001; Cools and D'Esposito, 2011; Joëls, 2006), CCK appears to act
316 in a U-shaped fashion, with increases and decreases of signaling from baseline levels often producing
317 similar phenotypes (Burgdorf et al., 2006; Calabrese and Baldwin, 2003; Ding and Bayer, 1993; Kõks et
318 al., 1999; Kulkosky et al., 1976).

319 Taken together, our results suggest an evolutionarily-conserved role for neuropeptide signaling
320 through the drosulfakinin pathway (homologue of cholecystokinin) in promoting aggression.
321 Intriguingly, this pathway only seems active in socially-isolated flies; in socially-enriched flies,
322 aggression is controlled by tachykinin (*a.k.a.* Substance P) signaling. The PI region, in which the
323 *Dsk*/*Dilp2* neurons reside, has considerable similarities with the hypothalamus (Hartenstein, 2006), a
324 brain region crucial for regulating aggression in mammals (Gregg and Siegel, 2001; Haller, 2013; Kruk
325 et al., 1984; Lin et al., 2011; Lipp and Hunsperger, 1978; Toth et al., 2010), with the most relevant
326 activity localized to the ventrolateral subdivision of the ventromedial hypothalamus (Lin et al., 2011),
327 where CCK neurons reside (Fulwiler and Saper, 1985). Thus, the predominant aggression-regulating
328 mechanism in rodents bears strong homology to the fly pathway regulating aggression of socially-
329 deprived, but not socially-enriched, individuals.

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330

331 **MATERIALS AND METHODS**

332 **Fly stocks & rearing**

333 Flies were reared on standard food at 25ºC and 65% relative humidity with a 12-h light/dark cycle. For
334 behavioral and molecular experiments, flies were collected within 24-48 hours of eclosion and group
335 housed (GH) or single housed (SH) for four days, unless mentioned otherwise. The following fly strains
336 were obtained from the Bloomington stock center: *Dsk*-GAL4 (#BL51981; Asahina et al., 2014); *Dilp2*-
337 GAL4 (*Dilp2 a.k.a. Ilp2*; #BL37516); *elav*-GAL4^{c155} (#BL458). For *Dsk*- and *Dilp2*-GAL4 expression
338 analysis a fluorescent reporter (Etheredge et al., 2018) carrying *pJFRC105-10XUAS-IVS-NLS-*
339 *tdTomato* in VK00040 (a gift of Barret D. Pfeiffer, Rubin lab, Janelia Research Campus) and *pJFRC29-*
340 *10XUAS-IVS-myr::GFP-p10* in attP40 (Pfeiffer et al., 2012) was used. For examining CCKLR-17D1
341 expression a MiMIC reporter (Nagarkar-Jaiswal et al., 2015) (#BL 61771; y[1] w[*] Mi{PT-
342 GFSTF.2}CCKLR-17D1[MI03679-GFSTF.2]) was used. For aggression and qPCR assays, comparisons
343 were made between equivalent genetic backgrounds. The stock used for neural silencing *pUAS-Kir2.1-*
344 *EGFP* and its corresponding control *pJFRC2-10XUAS-IVS-mCD8::GFP* were obtained from the Fly
345 Facility Shared Resource at Janelia Research Campus. *Dsk-GAL4*, *Dilp2-GAL4* and *elav-GAL4*^{c155} and
346 stocks used for neural activation (*UAS-NaChBac*, #BL9466 and control *UAS-mCD8::GFP*, #BL5130)
347 were outcrossed for 6-7 generations into *w*; Berlin background. The following Transgenic RNAi Project
348 (TRiP) RNAi lines (Perkins et al., 2015) were obtained from the Bloomington stock center: *Dsk*-RNAi
349 (#BL25869); *CCKLR-17D1*-RNAi (#BL27494); *CCKLR-17D3*-RNAi (#BL28333) and the *attP2*
350 background control without RNAi insert (#BL36303). To negate effects of the mini-white gene on
351 aggression (Hoyer et al., 2008), male progenies containing (*w* y[1] v[1]) were obtained by crossing virgin

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352 females of various GAL4 drivers and males of TRiP RNAi for *Dsk*, *CCKLR-17D1* and *CCKLR-17D3* or
353 corresponding background controls.
354

355 **Immunohistochemistry and imaging**

356 Fly brains were dissected in cold 1X PBS and fixed in 2% paraformaldehyde (in 1X PBS) at room
357 temperature for one hour on a Nutator, washed 4 times for 20 min each in PAT (1X PBS, 0.5% PBS
358 Triton, 1% BSA) at room temperature, blocked for one hour at room temperature with blocking buffer
359 (PAT + 3% Normal Goat Serum) and incubated with primary antibodies, diluted in blocking buffer,
360 overnight on a Nutator at 4°C. The primary antibodies used were: mouse anti-GFP (Sigma-Aldrich,
361 #G6539, 1:200 dilution); Rabbit anti-DsRed (Clontech, 632496, 1:500 dilution), rat anti-DN-cadherin
362 (Developmental Studies Hybridoma Bank, DNEX#8, 1:50 dilution), mouse anti-Flag (Sigma-Aldrich,
363 #F1804, 1:100 dilution); and Rat anti-Dilp2 (1:800 dilution, Eric Rulifson). This was followed by 4
364 washes for 20 min each in PAT, and incubation overnight on a Nutator at 4°C with secondary antibodies
365 diluted in blocking buffer. The secondary antibodies were all from Molecular Probes and used at 1:500
366 dilution:
367 Alexa Fluor 488 anti-mouse (A11029), Alexa Fluor 568 anti-rabbit (A11036), Alexa Fluor 568 anti-
368 rat (A11077) and Alexa Fluor 633 anti-rat (A21094). Brains were then washed 4 times for 20 min each
369 in PAT at room temperature, 1 time for 20 min in 1X PBS and mounted with VECTASHIELD mounting
370 medium (Vector Laboratories, H-1000). Samples were imaged on a Zeiss 800 confocal laser-scanning
371 microscope.

372

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373 **RNA extraction, library preparation and sequencing**

374 Male Canton-S flies collected within 24-48 hours of eclosion were group housed (GH) or single housed
375 (SH) for four days and flash-frozen during the afternoon and stored at -80°C until RNA extraction. 10-
376 15 flies were vortex-decapitated and heads were collected on dry ice. Heads were lysed in Trizol, and
377 total RNA was extracted using a Zymo Direct Zol kit (Zymo Research, USA: #R2051), in-tube DNAse
378 digestion was performed using a Turbo DNA free kit (Thermo Fisher Scientific, USA: #AM1907), and
379 RNA was purified using a Zymo RNA Clean and Concentrator kit (Zymo Research, USA: #R1013) as
380 per manufacturer's instructions. External RNA Controls Consortium (ERCC) spike-ins were added, and
381 RNA was processed for sequencing using Ovation RNA-Seq System V2 (Nugen Technologies, USA:
382 #7102-32) and Ovation Rapid DR Multiplex System 1-8 (Nugen Technologies, USA: #0319-32) as per
383 manufacturer's instructions. Two biological replicates were performed for each condition. Paired-end
384 100 bp sequencing reads were obtained using Illumina Hi-seq 2500 (Illumina, San Diego, CA).

385

386 **RNA-seq analysis**

387 All reads were trimmed with Trimmomatic 0.36 at a minimum read length of 50 and average read quality
388 across a sliding window of 15. Trimmed reads were mapped to *Drosophila* genome version r6.03 with
389 STAR (Dobin et al., 2013) with default settings. Pairwise differential expression analysis was performed
390 with DESeq2, EBseq, and edgeR following instructions given in the respective R package's workflow.
391 Genes that were differentially expressed at stricter than an adjusted (corrected for multiple testing using
392 the Benjamini-Hochberg method) P-value of 0.05 and fold-change greater than 2 were used for further
393 analysis. Gene Ontology (GO) analysis was performed on enriched genes using GOrilla (http://cbl-
394 gorilla.cs.technion.ac.il/). ~5,000 genes expressed in fly heads were used as background for GO analysis

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395 and obtained from FlyBase (http://www.flybase.org). The raw data from RNA-seq experiments has been
396 deposited into the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) with accession number:
397 PRJNA481582.
398

399 **qPCR validation**

400 RNA was extracted from heads of flies as described in previous sections. Genotype and age of flies used
401 for qPCR was matched to their corresponding behavioral assay. After RNA extraction, cDNA was
402 prepared using a SuperScript VILO Master Mix kit (Thermo-Fisher Scientific, USA: #11755050). qPCR
403 was performed using Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies: #600880) on a
404 StepOne Plus Real-Time PCR System (Thermo-Fisher Scientific, USA: #4376600). The following
405 hydrolysis probes (Applied Biosystems, Life Technologies, USA) were used: RPL32 (*Dm02151827_g1*:
406 #4331182) as an endogenous control, *Dsk* (*Dm02152147_s1*: #4351372), CCKLR-17D1
407 (*Dm01813942_g1*: # 4448892) and CCKLR-17D3 (*Dm01813944_m1*: # 4448892) as test probes.
408

409 **Aggression assay**

410 The assay was performed essentially as described before (Dankert et al., 2009; Kim et al., 2018). In brief,
411 males of a given genotype were introduced as a pair by gentle aspiration into single wells (16 mm
412 diameter and 10mm height) of 12-well chambers. Chamber floors were made from 2.25% w/v agarose
413 in commercial apple juice and 2.5% (w/v) sucrose. Walls of the arena were covered with Fluon (BioQuip)
414 to prevent flies from climbing the walls. Flies were allowed to acclimatize to the arena for 5 minutes,
415 and then fights were recorded for 20 minutes at 30 frames per second. All fights were performed during
416 the morning activity peak within 2.5 hours of lights on, at 25°C and ~40% relative humidity. Lunges

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417 were counted by CADABRA (Caltech Automated Drosophila Aggression-Courtship Behavioral
418 Repertoire Analysis) software (Dankert et al., 2009).
419
420 **Locomotor activity analysis**
421 Flies of various genotypes that were previously SH or GH for 4 days were anesthetized briefly by carbon
422 dioxide and transferred into 5mm × 65mm transparent plastic tubes with standard cornmeal dextrose agar
423 media. For recording locomotion levels, *Drosophila* activity monitors (Trikinetics, Waltham, USA) were
424 kept in incubators at 25°C with 65% relative humidity on a 12-h light/dark cycle. Flies were allowed one
425 night to acclimatize to the activity monitor, and then data was collected in 1-minute bins for 24 hours
426 (day-time plus night-time activity) as described before (Donelson et al., 2012).
427
428 **Statistical analysis**
429 Statistical analysis of behavioral data was performed using Prism 7 (Graph pad software). Aggression
430 data is usually non-normally distributed and appropriate non-parametric tests were chosen. For activity
431 data parametric test were chosen. Unless specified, we used ANOVA (non-parametric or parametric, as
432 appropriate), followed by appropriate *post hoc* tests of significance. We used Mann-Whitney U (*a.k.a.*
433 Wilcoxon rank-sum), Kruskal-Wallis and Student's t-tests of significance, as appropriate.
434
435

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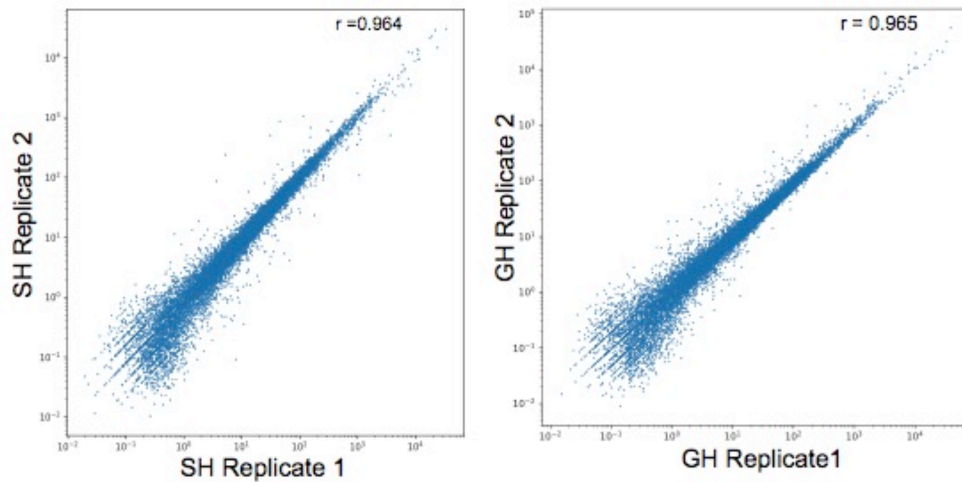
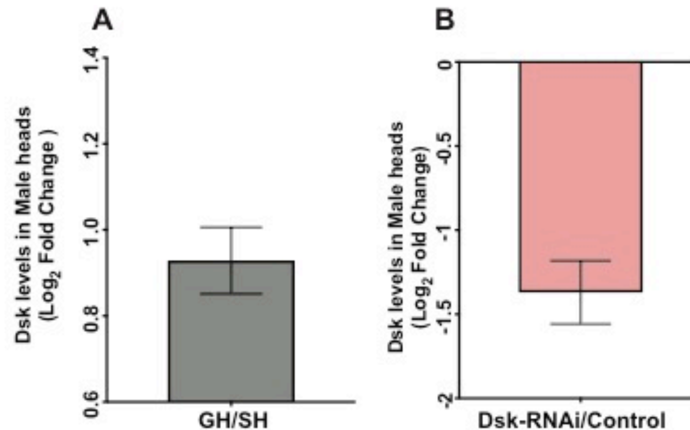
436 # Supplementary Figures with legends



437

438 **Supplementary Figure S1**. **Replicate concordance of RNA-seq for GH and SH datasets.** Pearson r-coefficients
439 were calculated on the Transcripts Per Million (TPM) values for independent biological replicates (N=2) for RNA-
440 seq data obtained from heads of (A) single-housed (SH) and (B) group-housed (GH) flies.



441

442 **Supplementary Figure S2. qPCR confirmation of Dsk upregulation upon group-housing and RNAi-**
443 **mediated knockdown.** (A) qPCR confirmation of *Dsk* transcriptional up-regulation in GH fly heads compared to
444 SH fly heads. (N= 6 biological replicates). (B) qPCR confirmation of *Dsk* knockdown. *Dsk-RNAi* was driven pan-
445 neuronally by *elav-GAL4*^{c155} and compared with controls without RNAi insert driven by *elav-GAL4*^{c155}. (N= 5
446 biological replicates). Y-axis shows Log_2 Fold change of *Dsk* expression calculated using the ΔΔCt method; error
447 bars show mean ± SEM. *Rpl32* was used as an endogenous control.

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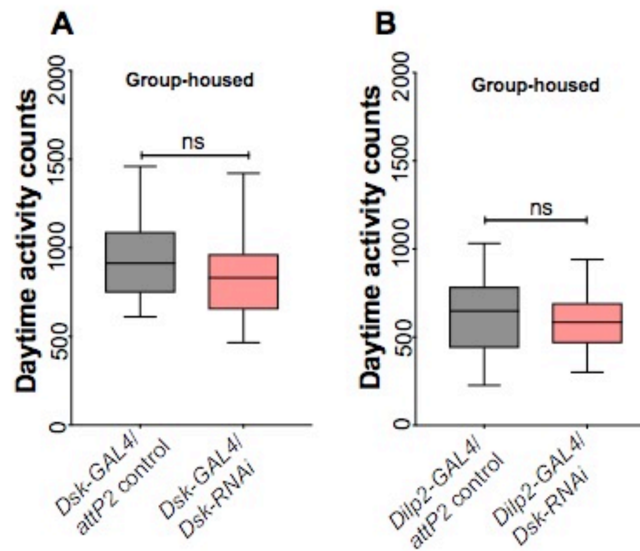

448

449 **Supplementary Figure S3. Group-housed flies do not show differences in overall daytime activity upon *Dsk***

450 **knockdown.** Daytime activity is not significantly affected when *Dsk* was down-regulated using (A) *Dsk*-GAL4

451 driver GH, Student's t-test; N=32; and (B) *Dilp2*-GAL4 driver GH, Student's t-test; N=32.

452

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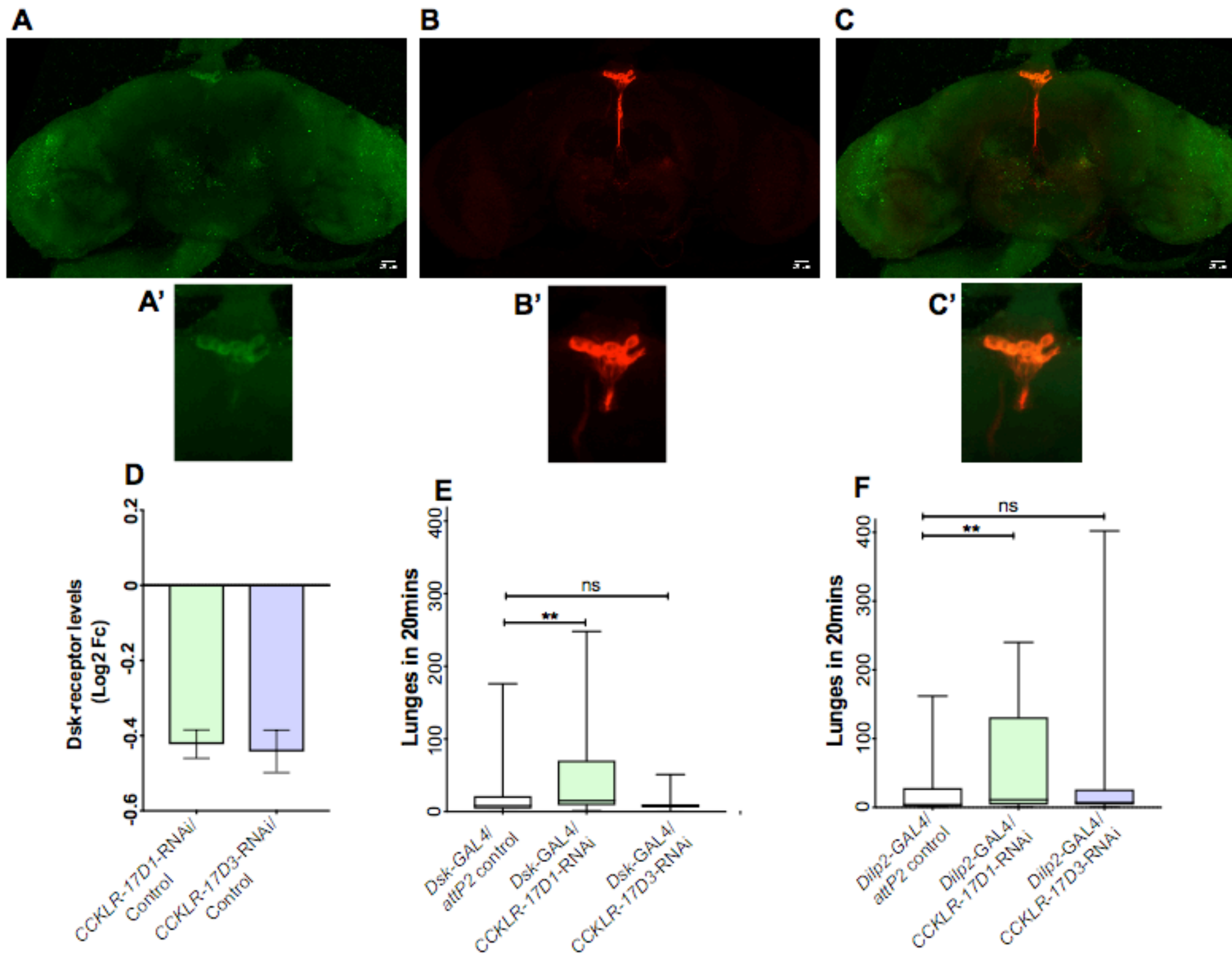

453

454 **Supplementary Figure S4. Aggression is mediated by the CCKLR1-17D1 receptor in peptidergic neurons.**
455 *CCKLR-17D1* expression in the brain ascertained using a MiMiC reporter line. (A) *CCKLR-17D1* reporter (green)
456 is expressed in various brain regions including in PI region, where (B) *Dilp2* is expressed (red). (C) Overlap in PI
457 region is shown. Scale bar is 20 µm. A', B' and C' show zoomed images from PI region. (D) qPCR confirmation
458 of *CCKLR-17D1* and *CCKLR-17D3* knockdown. RNAi constructs against *CCKLR* were driven pan-neuronally by
459 *elav-GAL4*^{c155} and compared with controls without RNAi insert driven by *elav-GAL4*^{c155} (N=3 biological
460 replicates). Y-axis shows Log_2 Fold change of *Dsk* expression calculated using the ΔΔCt method; error bars show
461 mean ± SEM. *Rpl32* probe was used as an endogenous control. (E,F) Knockdown of *CCKLR-17D1* led to increased
462 aggression in both (E) *Dsk*-GAL4> *CCKLR-17D1*-RNAi (P= 0.003, Kruskal-Wallis ANOVA with Dunn's
463 multiple comparison test, N= 44-48) and (F) *Dilp2*-GAL4> *CCKLR-17D1*-RNAi (P= 0.0045, Kruskal-Wallis

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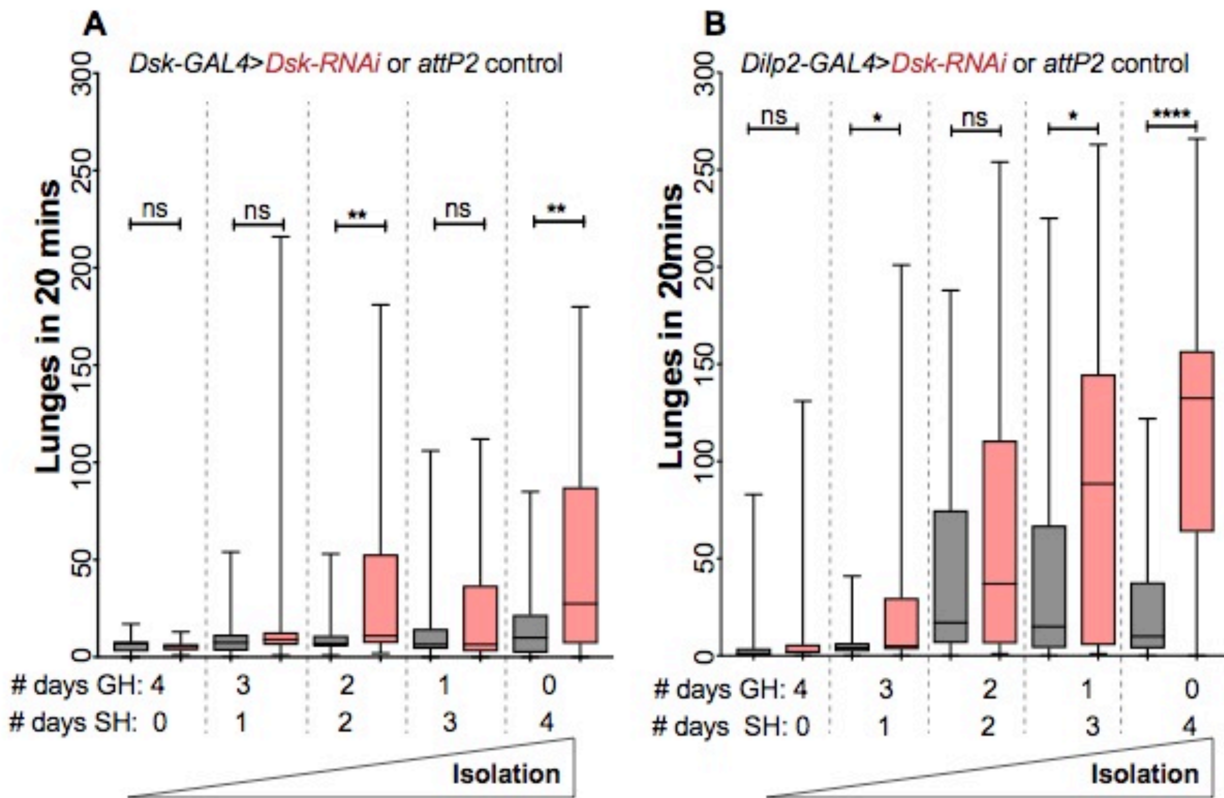
464 ANOVA with Dunn's multiple comparison test, N=47-48). Aggression was not significantly affected when
465 *CCKLR-17D3* was down-regulated in either GAL4 driver line.



466

467 **Supplementary Figure S5. Effect of increased degree of social-isolation on *Dsk* knockdown-mediated**
468 **aggression.** Lunge numbers versus degree of isolation, GAL4 driver line, and *Dsk*-RNAi *vs*. *attP2* background
469 control. Flies were group housed (20 males/vial) followed by single-housing. for varying degree. (A) *Dsk*-GAL4.
470 **, P =0.008 for 2-days GH> 2-days SH; **; P = 0.0096 for 4 days of SH. N= 25-41. Mann-Whitney U-test. (B)
471 *Dilp2*-GAL4. *; P = 0.043 for 3-days GH > 1-day SH; *; P = 0.013 for 1-day GH > 3-day SH and ****; P <0.0001
472 for 4 days of SH. N= 31-49. Mann-Whitney U-test.

473

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478 seq analysis. We thank Karen Hibbard and Fly Facility Shared Resource at Janelia for help with fly
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481 for helpful discussions.

482

483 **COMPETING INTERESTS**

484 The authors declare no competing interests exist.

485 **AUTHOR CONTRIBUTIONS**

486 PA conceptualized the project, designed and performed experiments, analyzed data and wrote the
487 manuscript. DK performed RNA-seq analysis. PC performed immunostaining and imaging. LLL
488 designed experiments, analyzed the data and wrote the manuscript. All authors reviewed the final MS.

489

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492

493

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