Neuroligin 3 highlights sexually dimorphic circuitry in Drosophila social spacing

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Drosophila social spacing neurocircuitry

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Abstract:

In *Drosophila melanogaster*, the autism-related Neuroligin 3 (Nlg3) protein is a postsynaptic membrane protein important for synapse development and regulation, which plays a role in social spacing behaviour. Here, we report the localization of Nlg3 to the calyx of the mushroom bodies (MB), optic lobes (OL), and protocerebral bridge (PB). Using RNA interference, *nlg3* knockdown in the OL increased fly social space, especially in the males, while knocking down *nlg3* independently in the MB or the PB did not change social spacing. However, hyperactivation and silencing of these neurons in the MB, but not the PB, controls social space in males and females, while hyperactivating and silencing of all *nlg3*-expressing neurons, including within the MB, PB, and OL, regulates male and female social space. Knocking down neurotransmitter biosynthesis enzymes, which decreases the amount of neurotransmitter release, showed that reducing acetylcholine release from the MB decreased female social space, whereas knocking down any dopamine receptor in the MB increased male social space. Lastly, to investigate the sexually dimorphic effects on social spacing previously seen in *nlg3* mutants, we examined a subset of sexually dimorphic *fruitless*-expressing (*fru)P1* neurons known to regulate sexually dimorphic behaviours. Hyperactivation of those *fruP1* neurons decreased social space in both sexes, while silencing those *fruP1* neurons specifically increased male social space without affecting females. Our findings highlight a sex-specific social space neural circuitry that includes the OL, MB, and *fruP1* neurons, while uncovering the underlying basis of some of the sex differences in this behaviour.

Article Summary:

In vinegar flies (*Drosophila melanogaster*), the autism-related Neuroligin 3 protein (Nlg3) controls neuronal development and regulation, but also affects fly social behaviour. Nlg3 is localized to the mushroom bodies (MB), protocerebral bridge, and the optic lobes. Genetically reducing Nlg3 in the optic lobes made male flies more socially distant, while hyperactivating or silencing neurons in the MB affected social behavior in both sexes. Reducing acetylcholine release from the MB affects female social space, while reducing dopamine receptors of the MB only affect male behaviour. Finally, the *fruitless* sexually dimorphic neurons control social behavior differently in males and females.

Introduction:

Social behaviours are fundamental to the interaction and communication within a community and are influenced by environmental cues which are processed and integrated in the brain. Abnormal social behaviours, such as difficulties with communication or interactions with others, are clinical characteristics of individuals with autism spectrum disorders (ASD). Using *Drosophila melanogaster,* we can dissect the neural and genetic underpinnings of social interactions in a simplified system (Sokolowski 2001).

Flies exhibit social behaviours that, while simpler, can reflect the basic principles of social interaction found in more complex organisms (Sokolowski 2010). By examining how environmental cues are integrated and influence social behaviours in Drosophila, we can gain insights into the mechanisms that might be disrupted in ASD, providing a basis for understanding the molecular and neural circuitries involved in these disorders (Ferreira and Moita 2019).

An important component of Drosophila social interactions within a group context is social spacing – the distance of one individual to another in a group. We use the social space assay to assess the outcome of group behaviour displayed by flies as a measure of the average number of flies within four-body lengths (4BL) in the social space chamber (Simon et al. 2012). We and other research groups have used this assay as a metric for behavioural analysis to understand and reveal the basic underlying neural circuitry involved (Burg et al. 2013; Fernandez et al. 2017; Xie et al. 2018; Yost et al. 2020).

So far, we know that mutations in genes affecting eye function, have been found to make flies less social (Simon et al. 2012; Burg et al. 2013; Jiang et al. 2020). Both males and females have decreased social space with mutants associated to fly gustation and mechanosensation (Jiang et al. 2020). In contrast, *Orco* mutants, which affect olfactory perception, do not appear to influence social spacing in males, but result in decreased social space in females. Together, these suggest a sensory modality-specific genetic influence on social behaviour that can have sex-specific effects (Simon et al. 2012; Jiang et al. 2020).

Other genes expressed in the brain have also been shown to influence Drosophila social behaviour, such as genes important for synaptic transmission and synaptogenesis (Burg et al. 2013; Wise et al. 2015; Fernandez et al. 2017; Ueoka et al. 2018; Brenman-Suttner et al. 2019; Hope et al. 2019; Jiang et al. 2020; Kanellopoulos et al. 2020; Yost et al. 2020; Shilpa et al. 2021; Cao et al. 2022). At the synapse level, the concentration of neurotransmitters, which is tightly regulated by biosynthesis enzymes or vesicular packaging proteins, have been shown to affect social spacing. Tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and the vesicular monoamine transporter (VMAT), which packages dopamine into synaptic vesicles, both regulate dopamine levels (Simon et al. 2009; Lawal et al. 2010). Modulating the abundance or activity of these proteins influences social spacing in Drosophila, indicating that dopamine plays a role in regulating social space (Fernandez et al. 2017).

Many human homologs of neural genes have also emerged as potential candidates in neuropsychiatric disorders, bridging the gap between basic biological processes and complex

human pathologies (Grant 2012). The gene *neurobeachin*, which is implicated in ASD in humans, encodes a structural scaffolding postsynaptic density protein (Castermans et al. 2003). In Drosophila, mutations in the homolog *rugose* result in altered fly social spacing, indicating a conservation of function across species (Wise et al. 2015). Furthermore, synaptic proteins Neuroligin and Neurexin, which facilitate neurotransmission by aiding synapse formation and receptor recruitment, have been shown to influence social behaviour (Hahn et al. 2013). In humans, *Neuroligin* gene homologs are correlated with ASD (Jamain et al. 2003; Südhof 2008). Similarly, in Drosophila, mutations in these genes lead to altered social behaviours, further revealing the genetic underpinnings of social space (Corthals et al. 2017).

Drosophila Neuroligin (Nlg) is a family of post-synaptic membrane proteins with the conserved function of synapse formation and maintenance by interacting with presynaptic Neurexin to mediate cell-cell adhesion and facilitate neurotransmission (Südhof 2008). During Drosophila neural development, larval *nlg3* mutants have a higher number of synaptic connections but less GluRIIA recruitment and synaptic transmission compared to control flies (Xing et al. 2014). Furthermore, *nlg3* knockouts display a sexually dimorphic and age-associated effect on fly aggression and social space (Yost et al. 2020). Mutant *nlg3* flies had significantly less climbing in both males and females by over 30%, however, *nlg3* deletion decreases only male, but not female aggression; while increasing male social space in 3-4 day old flies, and in both sexes by 7-10 days old (Yost et al. 2020).

The mushroom bodies (MB), also known to play a role in Drosophila social spacing, are a well-studied invertebrate brain structure primarily involved in fly olfactory learning and memory. The MB are comprised of approximately 2,500 neurons and its unusually structured shape suggests a highly specialized organization and function (Erber et al. 1987). The MB have a flexible organization in response to social isolation and are the central processing structure of the Drosophila brain (Technau 1984). Consisting of densely packed neurons called Kenyon cells, the MB integrates many sources of sensory information to modulate fly behavioural responses. Social space assays have been conducted on flies with a mutation in an ion channel selectively targeted in the MB and lead to decreased social space, observing that the cholinergic signaling on its own can rescue this altered phenotype in social spacing (Burg et al. 2013).

Sexually dimorphic behaviours in Drosophila such as male courtship, female receptivity, and social interactions are known to be regulated by *fruitless* (*fru*) neurons (Pan and Baker 2014; Brenman-Suttner et al. 2018; Chowdhury et al. 2020). The *fruitless* gene encodes a transcription factor important for sex determination in Drosophila development and produces male-specific transcripts that are essential for the development and function of male-specific neural circuits (Sato and Yamamoto 2020). Regulated by multiple promoters each expressing unique transcripts, the *fruP1* transcript undergoes sex-specific splicing and contributes to the sexual differentiation of neural circuits influencing behaviours such as courtship and aggression (Wiedemann 2007; Baker et al. 2024). Promoters *p2-p5* are necessary for adult viability and fly morphology that is however not sex specific (Ito et al. 1996). Neurons expressing *fru* are widespread interneurons in the adult fly brain and ventral nerve cord, where fast acting *fru* neurons release acetylcholine, GABA, and glutamate, while other *fru* neurons also release dopamine, serotonin, tyramine, and octopamine (Palmateer et al. 2023).

While progress has been made in identifying individual genes, synaptic proteins, and neuropils like the MB that influence Drosophila social spacing, the integration of molecular players within precise circuitry or other brain structures mediating social spacing behaviour are not fully understood. This research looks deeper into the role *nlg3* has on Drosophila social space. Based on Nlg3 localization and the sex-specific differences observed, we elucidated part of the neural circuitry that directly influences social spacing behaviour.

Materials and Methods:

Fly Stocks and Husbandry

All flies were reared mixed sex in bottles containing our own fly food recipe (brown sugar, corn meal, yeast, agar, benzoic acid, methyl paraben, and propionic acid). Rearing conditions were controlled during development and aging at 50% humidity, 25ºC, and on a 12:12 hour light:dark cycle. Parents were removed after seven days of egg laying resulting in a maximum parental age of 14 days, as parental age affects social spacing of the offspring (Brenman-Suttner et al. 2018). Canton-S (Cs) control line was initially sourced from Seymour Benzer in 1998. UAS-effector lines were outcrossed five times into the Cs control line to minimize behavioural variation due to genetic background. A generalized crossing scheme used to create the appropriate experimental treatments and genetic controls can be found in the supplementary material, figure S1.

nlg3 RNAi Generation

The shRNA sequences targeting nlg3 were obtained by using the model provided by Vert et al. (2006). The length of the *nlg3* shRNA was 60 bases for the full hairpin; and was ~7.8kbp for the entire pVALIUM20 plasmid containing the shRNA was ~7.8kbp. The hairpin sequence used was:

CAGCAACTCGAACTCGAACTAtagttatattcaagcataTAGTTCGAGTTCGAGTTGCTG

Within the hairpin sequence, uppercase letters represent the nucleotides that bind to the *nlg3* mRNA, while lowercase letters represent the nucleotides that connect the two halves of the pin. The shRNA constructs were cloned into the vector pVALIUM20 (vermillion eye color maker, (Ni et al. 2011) using the protocol in Chang et al. (2014). DNA was injected into genotype *y v, nanos-integrase, attP40/attP40* embryos and were mated to the parental line after reaching adulthood. Then, offspring containing the transgene were backcrossed five times into the Cs control background (Vert et al. 2006; Chang et al. 2014).

Immunocytochemistry

Anti-Nlg3

Whole brain dissections on 3 to 4 day old, male or female brains were fixed within 20 minutes of dissection in Bouin's solution. Following fixation and washing, brains were blocked in 5% NGS in 1X PBS and stained with guinea pig anti-Nlg3 primary antibody (1:1000, Invitrogen) overnight at 4 °C, which is a polyclonal primary antibody kindly provided by Dr. Brian Mozer (Yost et al. 2020). After washing, brains were stained with goat anti-guinea pig Alexa 488

(1:500, Invitrogen) for 2 hours at room temperature. Next, all samples were left in glycerol for 1 hour then were mounted on slides using Fluoroshield™ media, stored at 4 °C and imaged the following day. Fluorescent images were visualized with a AxioImager Z1 Compound Fluorescent microscope (Zeiss). Note: we used the entirety of our polyclonal antibody stock and have been unsuccessful in generating another functioning antibody for Nlg3. We switched to genetic constructs that tag Nlg3 with a GFP reporter (*MiMIC-nlg3* and *nlg3-Gal4 > mCD8-GFP*) that both verify our findings.

Anti-GFP

Whole brain dissections on 3 to 4 day old males or females were fixed within 20 minutes of dissection in 4% paraformaldehyde in 1X PBS-T (0.3% Triton-X). Following fixation and washing, brains were blocked in 5% normal goat serum (NGS) in PBS-T and stained with rabbit anti-GFP (1:1000, Invitrogen) and mouse anti-Brp (nc82; 1:30, Developmental Studies Hybridoma Bank) for 48 hours at 4 °C. After washing, brains were stained with Alexa 660 anti-mouse (1:400, Invitrogen) and Alexa 488 anti-rabbit (1:800, Invitrogen) for 48 hours at 4°C. Samples were mounted on slides in Fluoroshield™ media, stored at 4 °C and imaged the following day. Slides were imaged on an SP8 Lightening Confocal microscopy system (Leica). All images were visualized with LAS X 2.0 software (Leica).

Social Space Assay

The assay was performed as previously described (McNeil et al. 2015; Yost et al. 2020). In short, flies were collected under CO_2 after adult eclosion and aged at 3 to 4 days old. Twenty-four hours prior to the assay, flies were sexed under cold anesthesia and separated into vials with food containing 12-17 flies for each treatment tested. Two hours before the assay, each treatment was transferred into new vials and acclimated to standard conditions (25°C and 50% humidity). Ten minutes prior to test start, if the standard acclimation temperature is different than testing condition temperature, treatments were set to the testing temperature required for neuronal hyperactivation (*TrpA1* at 27°C and 50% humidity) or silencing (Shi^{ts} at 29°C and 50% humidity). At the time of testing, flies were loaded into the triangular chamber and knocked to the bottom of the chamber to induce a startle response that forces the flies into a group at the top of the chamber. Flies are then given time to settle at their preferred social space. Each treatment was loaded into the social space apparatus and photos were taken between 20-40 minutes or as soon as flies have settled as a group (McNeil et al. 2015). Social space assay photos were processed with ImageJ (National Institute of Health, Bethesda, MA, USA) to calculate the number of flies within a four-body length (4BL or ~1cm) from macros (as described in Yost et al. 2020). Image analysis data were imported to GraphPad Prism 10 software for statistical analysis and graphical representation.

Climbing Assay

The assay was performed as previously described (McNeil et al. 2015; Yost et al. 2020). In short, flies were aged to 2 to 4 days old and 24 hours prior to conducting the assay were sexed under cold anesthesia and separated in vials with food containing 40 flies for each treatment. Two hours before the assay, each treatment was transferred into new vials and acclimated to testing conditions (25ºC and 50% humidity). Ten minutes before the assay, if the standard

acclimation temperature is different than testing condition temperature, treatments were set to testing temperature required for neuronal hyperactivation (*TrpA1* at 27°C and 50% humidity) or silencing (*Shi*^{ts} at 29°C and 50% humidity). Each treatment was transferred into a testing tube and inserted into the climbing assay. Flies were forced to the bottom of the tube and given 15 seconds to climb to the tube on top of the apparatus. This timeframe was chosen to allow approximately 80% of control flies to reach the top vial. After 15 seconds, the apparatus was closed and the number of flies in the top and bottom tubes were counted to calculate a performance index of percent flies that reached the top vial.

Statistical Analysis

Statistical analysis and graphical representation were performed in GraphPad Prism (RRID:SCR_002798, version 7.0a for Mac, GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Normality test was used to verify normality and homoscedasticity test was used to verify the homoscedasticity of the distribution before performing an ANOVA test. Two-way ANOVAs were performed on data presented on the same graph, which were collected simultaneously and had 2 variables in 2 groups. Following the ANOVAs, Šídák's post-hoc tests were performed within each sex to compare between different genotypes or transmission states, while correcting for multiple comparisons. For all graphs, an alpha level of 0.05 was used and only significant values ($p < 0.05$) were shown for the two-way ANOVAs and post hoc tests. The results of all the comparison tests for social space analysis, significant or not, can be found in **Table 1**. Supplemental figures have ANOVA results on graphs.

Drawings:

Drawings presented in figures were created on the app Procreate 5.3.5 (Savage Interactive Pty Ltd, Hobart, Australia).

245 **Results:**
246
247 *Neuroligin 3 protein is expressed at low levels in the calyx of the mushroom bodies, the*
248 *protocerebral bridge, and the optic lobes*
249
250 We investigated the expression patterns of the Nlg3 protein in the adult brain using an anti-Nlg3
251 polyclonal antibody. We found Nlg3 protein was localized within the calyx (Ca) of the
252 mushroom bodies (MB), the protocerebral bridge (PB), and the optic lobes (OL) all in low levels
253 compared to the background autofluorescence of brain lipids (**Fig. 1A,B**). For confirmation of
254 the findings done with our antibody, we used two genetic constructs (*MiMIC* p-element insertion
255 within *nlg3*, as well as an *nlg3-Gal4* insertion within the gene – see **Table 1** for specific
256 localization). The *MiMIC-nlg3* insertion which incorporates a GFP reporter into the coding
257 sequence of the host gene *nlg3* (Venken et al. 2011). From this construct we saw GFP expressed
258 at low levels to the MB and OL and at very low fluorescence we can see the PB when compared
259 to background autofluorescence of the Drosophila brain (**Fig. 1C,D**). Confirmation of expression
260 in the calyx and PB was observed with the *nlg3-Gal4* insertion driving *mCD8-GFP* at higher
261 levels than endogenous *nlg3* (**Fig. 1E,F**). Male and female localization of Nlg3 did not appear to
262 be different either qualitatively or quantitatively in 3-4 day old adult flies (representative images
263 of only the males are shown).
264
265 *Neuroligin 3 expression in the optic lobes alters fly social spacing, mostly in males*
266
267 To determine the behavioural function of Nlg3 located in these structures, we targeted RNAi
268 knock down of *nlg3* independently in each of the MB, PB, and OL and tested fly social space.
269 We observed no effect of knocking down *nlg3* in the MB (**Fig. 2A**; Two-way ANOVA – Effect
270 of genotype: $F_{1,39} = 1.005$, $p = 0.3223$) and PB (**Fig. 2B**; Two-way ANOVA – Effect of
271 genotype: $F_{1,37} = 0.068$, $p = 0.7951$) when compared to control flies. However, knocking down
272 *nlg3* in the optic lobes did reduce the number of flies within 4BL and increased fly social space
273 (**Fig. 2C**; Two-way ANOVA – Effect of genotype: $F_{1,37} = 4.837$, $p = 0.0354$), an effect mostly
274 driven by the males. Then, we used a climbing assay to assess fly locomotion and their ability to
275 respond to a startle response and climb; all to ensure the treatment flies could perform in the
276 social space assay. While there was no reduction in climbing when knocking down *nlg3* in the
277 MB (**Supp. Fig. 2A**), there was a reduction in climbing when knocking down *nlg3* in each of the
278 PB and OL (**Supp. Fig. 2B,C**). We observed that the flies were still able to climb and therefore
279 would not confound the fly decision making for social distance, but rather show a decrease in
280 their performance index on climbing. We also observed the presence or absence of defects in
281 climbing is not correlated to any changes in social space, however, we continued to check
282 climbing to ensure the flies were healthy enough to be able to climb.
283
284 *Hyperactivation and silencing nlg3-expressing neurons, independently or simultaneously,*
285 *changes fly social space in a sex-specific manner*
286
287 Next, we looked at genetically manipulating neural transmission within the brain structures
288 where Nlg3 is localized (**Fig. 1**) to examine their effect on social space. When hyperactivating
289 all neurons within the MB, we saw a lower number of flies within 4BL (increased social space)
290 in both males and females (**Fig. 3A**; Two-way ANOVA – Effect of neural transmission: $F_{1,47} =$

7.395, $p = 0.0091$). Silencing all MB neurons showed the same reduction in the number of flies within 4BL again in both males and females (**Fig. 3A**; Two-way ANOVA – Effect of neural transmission: $F_{1,30} = 14.40$, $p = 0.0007$) highlighting the importance of the MB in determining fly social space on their own. In contrast, hyperactivation of the PB neurons did not change fly social space, nor did silencing PB neurons in males but looks to have an effect in females specifically (**Fig. 3B**; Two-way ANOVA – Effect of neural transmission: $F_{1,31} = 3.443$, $p = 0.0730$). Finally, we used the *nlg3-Gal4* insertion to manipulate the transmission of all *nlg3*-expressing neurons. Hyperactivation of all *nlg3*-expressing neurons decreases the number of flies within 4BL in both males and females (**Fig. 3C**; Two-way ANOVA – Effect of neural transmission: $F_{1,28} = 12.70$, $p = 0.0013$). Silencing these neurons only increased social space in females but not males (**Fig. 3C**; Two-way ANOVA – Effect of interaction: $F_{1,22} = 6.169$, $p = 0.0211$). There was no reduction in climbing with a hyperactivation or silencing of the MB or PB neurons (**Supp. Fig. 3A,B**), but we did observe a reduction in climbing when hyperactivating *nlg3*-expressing neurons in males only (**Supp. Fig. 3C**).

Reducing acetylcholine release from the mushroom bodies decreases female social space, but knocking down dopamine receptors in the mushroom bodies increases male social space

As the MB are important for fly social space, we investigated which neurotransmitters in the MB were important for the observed effects. Using UAS-RNAi, we knocked down the rate-limiting biosynthesis enzymes within the MB (Shih et al. 2019), including *choline acetyltransferase (ChAT* for acetylcholine production) and *glutamate decarboxylase 1* (*GAD1* for GABA production; Shih et al. 2019). Acetylcholine reduction in the MB decreased fly social space but only in females (**Fig. 4A**; Two-way ANOVA – Effect of interaction: $F_{1,30} = 8.465$, $p = 0.0068$). GABA reduction did not change the number of flies within 4BL in either males or females (**Fig. 4B**; Two-way ANOVA – Effect of genotype: $F_{1,31} = 0.021$, $p = 0.8851$).

Previous research shows global dopaminergic changes in the adult fly brain will affect social space (Fernandez et al. 2017; Xie et al. 2018; Yost et al. 2020), however, the anatomical locations within the adult fly brain where alterations in dopaminergic transmission affect social behaviour is unknown. Thus, we tested the dopaminergic inputs into the MB, and found that knocking down every dopamine receptor within the MB – including Dop1R1 (**Fig. 5A**; Two-way ANOVA and Šídák's *post-hoc* test: $p < 0.0001$), Dop1R2 (**Fig. 5B**; Two-way ANOVA and Šídák's *post-hoc* test: $p = 0.0225$), Dop2R (**Fig. 5C**; Two-way ANOVA and Šídák's *post-hoc* test: $p < 0.0001$), and DopEcR (**Fig. 5D**; Two-way ANOVA and Šídák's *post-hoc* test: $p = 0.0103$) – decreased the number of male flies within 4BL but did not change female social space.

Neurons expressing the fruitless gene under the P1 promotor are involved in sexually dimorphic responses in fly social spacing

Since male and female flies appear to have differently neural circuitry underlying their social spacing preferences, we looked at a subset of sexually dimorphic *fruitless*-expressing neurons. We used the *fruP1-Gal4* driver to manipulate neural transmission. While hyperactivation of *fruP1* neurons decreased the number of flies within 4BL for both males and females (**Fig. 6**; Two-way ANOVA – Effect of neural transmission: $F_{1,33} = 22.65$, $p < 0.0001$). silencing of *fruP1* resulted in a sex specific effect. Silencing *FruP1* neurons resulted in a higher number of flies

within 4BL for males (**Fig. 6**; Two-way ANOVA and Šídák's *post-hoc* test: $F_{1,30} = 3.451$, $p = 0.0453$) but did not change female social space. Importantly, we saw a reduction in climbing in both males and females for hyperactivating and silencing *fruP1* neurons (**Supp. Fig. 5**).

To assess the effect of Nlg3 in *fruitless*-expressing neurons, we drove an RNAi against *nlg3* with the *fruP1* driver. We saw reducing levels of Nlg3 had a sexually dimorphic effect on flies. Knocking down *nlg3* in *fruitless*-expressing neurons resulted in fewer flies within 4BL for males, while females had an increased number of flies within 4BL (**Fig. 7A**; Two-way ANOVA – Effect of interaction: $F_{1,58} = 7.056$, $p = 0.0102$). Also, we observed a reduction of male climbing when knocking down *nlg3* in *fruP1* neurons (**Supp. Fig. 6A**). Next, we investigated the neurotransmitters involved in fly social space in *fruP1* neurons. Knocking down *TH*, the rate limiting-enzyme in the pathway of dopamine synthesis, in *fruP1* neurons did not change male or female social space (**Fig. 7B**; Two-way ANOVA – Effect of genotype: $F_{1,31} = 1.134$, $p = 0.2951$). However, knocking down *TH* in *fruP1* neurons decreased climbing in both males and females indicating the importance of dopamine released from *fruP1* neurons on fly climbing (**Supp. Fig. 6B**). In contrast, reduction of acetylcholine levels by knocking down *ChAT* in *fruP1* neurons increased the number of flies within 4BL in both males and females (**Fig. 7C**; Two-way ANOVA – Effect of genotype: $F_{1,48} = 38.01$, $p < 0.0001$). Acetylcholine release reduction from *fruP1* neurons also decreases fly climbing (**Supp. Fig. 6C**). While a reduction of GABA by knocking down *GAD1* in *fruP1* neurons decreased female social space but did not change male social space (**Fig. 7D**; Two-way ANOVA – Effect of interaction: $F_{1,39} = 9.327$, $p = 0.0041$), reduction of GABA resulted in a small reduction in climbing of male and female flies (**Supp. Fig. 6D**).

Discussion:

Social interactions within a group requires an integration of both attractive and repulsive social cues that lead to a behavioural output. Here, we expand the map of the neural basis for social behaviour by focusing on the sexually dimorphic role of *neuroligin 3* and its associated adult brain structures in male and female *Drosophila melanogaster*.

We show for the first time that *nlg3* is expressed at low levels in the mushroom bodies, optic lobes, and protocerebral bridge in the adult fly brain. Individually, *nlg3* in the OL is important to maintain fly social space, specifically in males, while knockdown of *nlg3* in the MB and PB did not have any effect. Next, we need to assess the effect of *nlg3* knockdown in all *nlg3* neurons simultaneously, to determine whether we see a stronger effect than what we observed only with the OL-driver. The effects of Nlg3 in the OL on social behaviour was expected as vision is known to be important for proper social spacing (Simon et al. 2012; Burg et al. 2013; Jiang et al. 2013).

Additionally, manipulating total neural transmission in these brain regions highlighted the importance of the Kenyon cells for maintaining social space. Hyperactivating and silencing MB neurons in both males and females increased fly social space. Reducing acetylcholine release from the MB decreased female social space, whereas knocking down all dopamine-type receptors increased male social space. These findings suggest that mapping the circuitry through the MB happens in a sexually dimorphic manner, where total acetylcholine release from the MB will modify female social space and dopaminergic inputs to the MB will change male social space. We still do not know the specific output from the MB leading to changes in males or the inputs to the MB leading to changes in females.

We also examined the involvement of *fru* neurons, which are known to regulate other sexually dimorphic behaviors such as male courtship or female receptivity (Chowdhury et al. 2020). Hyperactivating *fruP1* neurons decreased social space in both sexes, while silencing them showed to be sexually dimorphic with increased social space only occurring in males. Again, this demonstrates the influence *fruP1* neurons have on social space along with other fly social behaviours.

Lastly, manipulation of neurotransmitters within *fruP1* neurons revealed that the release of dopamine was not important for social space. Instead, both acetylcholine and GABA release from *fruP1* neurons play a role in fly social space, a phenocopy observed when knocking down acetylcholine in the MB. In addition, RNAi knockdown of *nlg3* in the *fruP1* neurons also showed sex-specific effects on social space.

Our antibody targeted the intracellular domain of Nlg3, which allowed us to identify protein localization specifically in the calyx (dendritic ends of the Kenyon cells) of the mushroom bodies, the medulla and lamina layers of the optic lobes, and throughout the entire protocerebral bridge (**Fig. 1**). However, there was a low signal to noise ratio when imaging with just a polyclonal anti-Nlg3 antibody (**Fig. 1A,B**) indicating a low level of expression of *nlg3* in the adult 3-4 day old brain. Attempts to create another polyclonal, or even a monoclonal antibody specific to Nlg3 were unsuccessful, so we transitioned to using two different genetic constructs

408 (*MiMIC-nlg3* and *nlg3-Gal4 > mCD8-GFP*) to confirm the results we observed with our
409 polyclonal antibody. The GFP localization in this line in the mushroom bodies and optic lobes
410 was similar to the Nlg3 protein localization (**Fig. 1C,D**). However, the GFP expression in the
411 *MiMIC-nlg3* was also low, probably due to the endogenously low expression of *nlg3* (reported in
412 Robinson, Yost et al. 2020), and we could not see any expression in the protocerebral bridge. We
413 then switched to an *nlg3-Gal4* driver to express Gal4 transcription factor in the same cells as
414 *nlg3*, allowing to activated the *UAS-GFP* at higher levels than just endogenous *nlg3* (**Fig. 1F**)
415 allowing for better visualization with immunofluorescence of the calyx and protocerebral bridge
416 (**Fig. 1E**).
417
418 After determining the localization of *nlg3* expression in the adult brain, we examined the effects
419 of targeted RNAi knockdown of *nlg3* on fly social space. Deletions of the whole *nlg3* gene leads
420 to an increase in fly social space (Yost et al. 2020), thus, we expected to see a similar phenotype
421 with RNAi knockdown. However, the knockdown of *nlg3* only increased fly social space in the
422 optic lobes with the males contributing to most of the difference between experimental and
423 control treatments (**Fig. 2C**). Knocking down *nlg3* in the optic lobes might affect fly vision but
424 we did not yet directly test this. The RNAi knockdown of *nlg3* leads to a reduction in climbing
425 (**Supp. Fig. 2C**) as the climbing assay is performed under uniform light. The reduction in
426 climbing may be influenced by impairments in vision and not solely or entirely due to
427 impairments in locomotion. Vision is known to play a role in social spacing as well (Simon et al.
428 2012; Burg et al. 2013; Jiang et al. 2020), so the role of *nlg3* on vision will need to be further
429 investigated in the future.
430
431 Notably, we observed that the knockdown of *nlg3* in all three structures individually resulted in
432 sex differences in social space with females generally settling in a group closer together than
433 males (**Fig. 2A-C**). This is in line with previously published results with sex differences seen in
434 social space (Simon et al. 2012).
435
436 As we've seen that knocking down *nlg3* individually in the protocerebral bridge or mushroom
437 bodies isn't necessary for social space, we modified the transmission of the neurons within these
438 structures to assess their potential roles in this social behaviour. Indeed, Burg et al. (2012)
439 reported the importance of the MB in fly social space. We also observed an increase to male and
440 female social space whether the entire MB neurons were hyperactivated or silenced (**Fig. 3A**).
441 This suggests that it's not *nlg3* expression that influences social space but the MB transmission
442 itself. As the MB is not only important for learning and memory but also sensory integration, it
443 would follow that manipulation of this circuitry would affect behavioural output.
444
445 There were no changes to social space when we hyperactivated the PB neurons but silencing the
446 PB neurons increased female social space specifically (**Fig. 3B**) suggesting the important of the
447 PB circuitry in regulating female social space. Next, using the *nlg3-Gal4* driver, we manipulated
448 the transmission of all *nlg3*-expressing neurons revealing their sexually dimorphic effect on
449 social space. Females will get further apart with hyperactivation or silencing of *nlg3*-containing
450 neurons, but males will only get further apart with hyperactivation and not silencing of the same
451 neurons. This might explain the sexually dimorphic effect of *nlg3* deletion on flies of the same
452 age (Yost et al. 2020) are not influenced by just one of the identified brain structures *nlg3* is

expressed in (MB, PB, OL), but instead may be due to the collective circuitry of all *nlg3*-expression neurons (**Fig. 1**).

The mushroom bodies influence on fly social space was clear with changes to neural transmission, so we then aimed to identify the neurotransmitter released from the mushroom bodies that affected social spacing. As expected, knocking down *ChAT* and reducing acetylcholine levels in the MB decreased social space, but only in females and did not affect males (**Fig. 4A**). This indicates that acetylcholine release throughout the entire MB will lead to behavioural changes in group formation for females but not males. We also found that knocking down all dopamine receptor types in the mushroom bodies will specifically increase male social space but did not significantly affect females (**Fig. 5A-D**). Of note, Dop1R1 and Dop2R knockdown did increase female social space (**Fig. 5A,C**; Two-way ANOVA and Šídák's *post-hoc* test: $p < 0.0505$; Two-way ANOVA and Šídák's *post-hoc* test: $p < 0.0276$, respectively) but this was only a small difference in the number of flies within 4BL and was at the upper end of how close flies can group together showing a ceiling effect that produced a minimal change in their behaviour. Overall, this would indicate that the transmission of the Kenyon cells themselves, specifically acetylcholine release, will influence female social space but the dopaminergic inputs into the MB modify the male decision of their social space. It was interesting to observe that all the 4 types of Drosophila dopamine receptors (Dop1R1, Dop1R2, Dop2R, and DopEcR) all had the same effect on social space while each receptor plays unique roles to fly behaviour such as sleep, courtship, feeding, and memory (Kim et al. 2003; Lebestky et al. 2009; Kuo et al. 2015).

In Drosophila social space, many genetic and neural manipulations lead to sex differences, even though both sexes respond to changes in their social environment in similar ways. For example, age, mating, and isolation all lead to decreased social space in both sexes (Simon et al. 2012; Brenman-Suttner et al. 2018; Yost et al. 2024). Due to this, we examined the influence that the sexually dimorphic *fruP1* neuron subset had on social space. Again, we saw male-female sex differences in response to modulation of *fruP1* neuronal transmission. For males, when *fruP1* neurons are hyperactivated, flies get further apart, but when these neurons are silenced, males get closer together (**Fig. 6**). Females also get further apart when *fruP1* neurons are hyperactivated, but do not change their social space when these neurons are silenced (**Fig. 6**). This indicates that *fruP1* neurons in males can increase and decrease neuronal activity resulting in a change in their social space, but for females only an increase in *fruP1* activity modifies their social space. The basal transmission of these neurons that affect female social space might not be active and therefore cannot be silenced, only hyperactivation changes this resting state and leads to females settling further in a group setting.

Knowing the importance for *fruP1* neurons in Drosophila social space, we then examined the molecular players within these neurons that are contributing to the behavioural changes. Knocking down *nlg3* in *fruP1* neurons showed a male-female sexual dimorphism with males getting further apart and females closer together (**Fig. 7A**). This confirms there are *fruitless*- and *nlg3*-expressing neurons that are contributing to fly social space behaviour. Furthermore, our results indicate that those neurons are not dopaminergic. However, the *fruP1* neurons affecting social space are cholinergic, in both males and females, a phenocopy of the effect seen on females both when reducing acetylcholine levels in the mushroom bodies and when knocking

down *nlg3* in *fruP1* neurons. Because a subset of the MB neurons is also *fruP1* neurons (Palmateer et al. 2023), we propose that the Kenyon cells *nlg3* neurons are also *fruP1* neurons, at least those that affect female social space (**Fig. 8**).

Finally, as previously reported (Jiang et al. 2020; Yost et al. 2024), we did not observe any correlation in our experiments between abnormalities in performance index leading to any changes in social space. Even when climbing was reduced, flies still were able to climb up to the upper vial of counter current apparatus and are not necessarily consistent with changes to social space.

Fly social behaviour relies heavily on an individual's ability to perceive and integrate social and environmental cues. Using the Drosophila social space assay, we show the roles of the OL and MB in regulating fly social spacing. The OL are essential for sensory perception, as they transduce visual cues into electrochemical signals that are integrated within MB, and the central complex, thereby enabling the fly to perceive its environment and alter its behaviour accordingly.

The significance of Nlg3 in the OL is reiterated here, as it is probably involved in the proper development and regulation of synaptic connections within these synaptically rich brain regions. Proper development of the OL layers is required to decode parallel circuitry pathways involved in processing color, shapes, and motion, which are crucial for interpreting environmental stimuli. Outside of the development and maintenance of synaptic connections that can affect fly behaviour, neural transmission through these brain regions reveals the MB to be important for social behaviour. The MB, which receives signals and integrates from multiple other brain regions (Aso et al. 2014), relies on proper development of synaptic connections where disruptions or improper regulations in these connections can lead to misinterpretation of signals, ultimately altering fly behavior through its descending output neurons.

By dissecting the molecular players within these neuronal subsets, we identify some of the inputs and outputs of the MB that influence fly social spacing. This behavioral data allows us to begin constructing a functional circuitry model of the neural pathways that regulate social space and where sexual dimorphic integration of cues might be taking place. We have identified that dopaminergic signalling into the MB influences social space but still need to identify which dopaminergic clusters are playing a role. We can similarly test the known dopaminergic subsets to innervate the MB (PPL1, PAM, and PPL2ab). We also identified that acetylcholine signalling from the MB will affect female social space, but we have yet to identify which mushroom body output neurons (MBONs) are associated with these behavioural changes (**Fig. 8**). We can also test the known 21 MBONs to find downstream circuits that influence this social behaviour specifically.

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Ethics Statement
Western's Animal Care Committee or Ontario Provincial and Federal regulatory bodies specifies that ethical considerations do not apply to the study of invertebrate animals including insects such as *Drosophila melanogaster*

Data availability statement:
The datasets analysed for this study will be deposited in the Dryad repository (TBD).

Conflict of Interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors contribution:
Based on Oxford Academic (Genetics journal)

JWR: Conceptualization, Supervision, Project Administration, Investigation, Formal Analysis, Methodology, Resources, Funding Acquisition, Writing - Original Draft Preparation, and Writing - Review & Editing.
ATB: Conceptualization, Investigation, Formal Analysis, Funding Acquisition, and Writing - Review & Editing.
MRE: Conceptualization, Investigation, Formal Analysis, Funding Acquisition, and Writing - Review & Editing.
JRI: Methodology
SP: Investigation and Formal Analysis.
DSL: Investigation and Formal Analysis.
AS: Methodology.
JND: Investigation, Formal Analysis and Validation.
GIR: Writing - Review & Editing.
MBS: Supervision, Resources and Funding Acquisition - Review

AFS: Conceptualization, Supervision, Project Administration, Formal Analysis, Resources, Funding Acquisition, and Writing - Review & Editing.

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776 **Figure captions**
777
778 **Figure 1. Nlg3 is expressed in the calyx of the mushroom bodies, throughout the**
779 **protocerebral bridge, and in the optic lobes. A-B**: Immunofluorescent imaging of polyclonal
780 antibody specific to the c-terminal intracellular domain with anti-Nlg3 (green) on Canton-S male
781 fly whole brains dissected at 3-4 days old. Magnification: 20X. **A.** Fluorescent imaging on
782 posterior side of adult brain shows three main structures expressing *nlg3* in low levels: calyx of
783 the MB (Ca), protocerebral bridge (PB), and the optic lobes (OL). **B.** Confocal imaging on
784 posterior side of adult brain shows definition of Nlg3 expression in the Ca, PB, and OL (medulla
785 and lamina). **C-D:** Expression patterns of MiMIC construct
786 (*Mi{y[+mDint2]=MIC}nlg3[MI08924]*) incorporating *mCD8-GFP* into *nlg3*.
787 Immunofluorescence with anti-GFP (green) and nc82 (magenta). **C.** Confocal imaging on
788 anterior side of adult brain at 20X magnification indicates low expression of Nlg3 in the MB and
789 OL. **D.** Confocal imaging at 40X magnification highlighting expression in the MB. **E-F:**
790 Expression patters of *MiMIC nlg3-Gal4* genetic construct (*Mi{Trojan-Gal4}nlg3>UAS-*
791 *mCD8::GFP*) driving exogenous expression of *mCD8-GF*P with *nlg3*. Immunofluorescence
792 with anti-GFP (green) and nc82 (magenta). **E.** Confocal imaging of posterior side of adult brain
793 at 40X magnification indicates low expression patterns in PB and Ca. **F.** Confocal imaging of
794 anterior side of adult brain at 40X magnification shows expression throughout the MB. Antennal
795 Lobes show for reference (AL). Scale bar, 50µm.
796
797 **Figure 2. Knocking down Nlg3 specifically in the optic lobes increases fly social space in**
798 **males and females but changes are seen when knocking down in the protocerebral bridge**
799 **or mushroom bodies specifically. A-C:** Average number of flies within 4 body lengths (4BL)
800 in male and female flies with RNAi knockdown of *nlg3* targeted specifically to the mushroom
801 bodies (A), protocerebral bridge (B), and the optic lobes (C). **A.** The number of flies within 4BL
802 was not different in both males and females in flies with *nlg3* knockdown targeted to the MB
803 (two-way ANOVA – Effect of genotype*:* $F_{1,39} = 1.005$, $p = 0.3223$). **B.** The average number of
804 flies within 4BL was not different in both males and females in flies with *nlg3* knockdown
805 targeted to the PB (two-way ANOVA – Effect of genotype: $F_{1,37} = 0.068$, $p = 0.7951$), but there
806 is an increase in the number of flies within 4BL in female *nlg3* knockdown flies when compared
807 to males (two-way ANOVA – Effect of sex: $F_{1,37} = 10.12$, $p = 0.0030$). **C.** Knocking down *nlg3*
808 in the OL decreases the number of flies within 4 body lengths in both males and females
809 compared to genetic control flies (two-way ANOVA – Effect of genotype: $F_{1,37} = 4.837$, $p =$
810 0.0354). Grey colour: genetic control *driver/+*; pink colour: *driver/RNAi*. The results of all two-
811 way ANOVA and *post-hoc* tests performed can be found in **Table 1**. N = 7 – 14 for all
812 treatments. Error bars are +/- s.e.m.
813
814 **Figure 3. Manipulation of neural transmission within the brain structures enriched with**
815 **Nlg3 increases fly social space. A-C:** Average number of flies within 4 body lengths (4BL) in
816 male and female flies when genetically hyperactivating or silencing neural transmission within
817 the MB (A), PB (B), and *nlg3*-containing neurons (C). **A.** Hyperactivation of MB neurons
818 decreases the number of flies within 4BL in both males and females compared to basal
819 transmission of MB neurons (two-way ANOVA – Effect of neural transmission: $F_{1,47} = 7.395$, p
820 $= 0.0091$). Silencing MB neurons also decreases the number of flies within 4BL in both males
821 and females compared to basal transmission (two-way ANOVA – Effect of neural transmission:

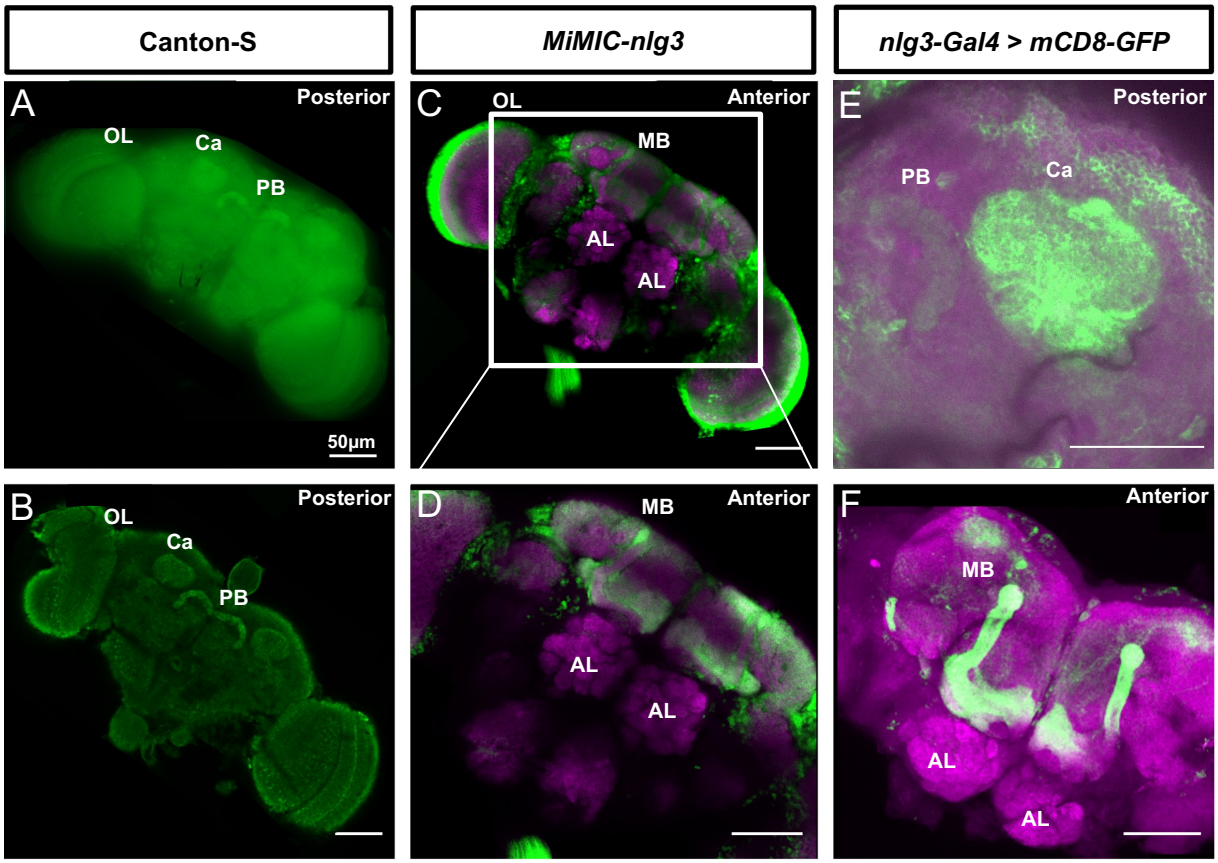
822 $F_{1,30}$ = 14.40, p = 0.0007). **B.** The number of flies within 4BL was not different in males and
823 females when the PB neurons are hyperactivated (two-way ANOVA - Effect of neural
824 transmission: $F_{1,74}$ = 17.72, p = 0.2018) or silenced (two-way ANOVA – Effect of neural
825 transmission: $F_{1,31}$ = 3.443, p = 0.0730). **C.** Hyperactivation of *nlg3*-containing neurons
826 decreases the number of flies within 4BL in both males and females compared to basal
827 transmission (two-way ANOVA – Effect of neural transmission: $F_{1,28}$ = 12.70, p = 0.0013).
828 There is an interaction between sex and silencing *nlg3*-containing neurons where females have a
829 decrease in the number of flies within 4BL (two-way ANOVA – Effect of interaction: $F_{1,22}$ =
830 6.169, p = 0.0211). Grey colour bars: inactivated genetic construct and basal transmission of
831 neurons; green colour bars: activation of genetic construct and hyperactivation of neurons with
832 *Gal4* driver; pink colour bars: activation of genetic construct and silencing of neurons with *Gal4*
833 driver. The results of all two-way ANOVA and *post-hoc* tests performed can be found in **Table**
834 **1**. N = 6 – 22 for all treatments. Error bars are +/- s.e.m.
835
836 **Figure 4. Knocking down the acetylcholine rate-limiting biosynthesis enzyme C*hAT* in the**
837 **MB decreases female fly social space. A-B:** Average number of flies within 4 body lengths
838 (4BL) in male and female flies with RNAi knockdown of *ChAT* for acetylcholine synthesis (A)
839 or *GAD1* for GABA synthesis (B) specifically in the mushroom bodies. **A.** The interaction
840 between sex and *ChAT* knockdown shows that female flies have an increased number of flies
841 within 4BL compared to female genetic control flies (two-way ANOVA – Effect of interaction:
842 $F_{1,30}$ = 8.465, p = 0.0068). **B.** Knockdown of *GAD1* in the MB does not change the number of
843 flies in 4BL in both males and females compared to genetic control flies (two-way ANOVA –
844 Effect of genotype: $F_{1,31}$ = 0.021, p = 0.8851). Grey colour bars: genetic control *driver/+*; pink
845 colour bars: *driver/RNAi*. The results of all two-way ANOVA and *post-hoc* tests performed can
846 be found in **Table 1**. N = 8 – 9 for all treatments. Error bars are +/- s.e.m.
847
848 **Figure 5. RNAi knockdown of all dopamine receptors in the MB increases male social**
849 **space. A-D:** Average number of flies within 4 body lengths (4BL) in male and female flies
850 knocking down dopamine receptors *Dop1R1* (A), *Dop1R2* (B), *Dop2R* (C), and *DopEcR* (D)
851 specifically in the mushroom bodies. **A.** *Dop1R1* knockdown in the MB of males has a decreased
852 number of flies within 4BL compared to genetic controls (two-way ANOVA with Šídák's *post-*
853 *hoc* test: p < 0.0001). **B.** *Dop1R2* knockdown in the MB of males has a decreased number of
854 flies within 4BL compared to genetic controls (two-way ANOVA and Šídák's *post-hoc* test: p =
855 0.0225). **C.** *Dop2R* knockdown in the MB of males has a decreased number of flies within 4BL
856 compared to genetic controls (two-way ANOVA and Šídák's *post-hoc* test: p < 0.0001). **D.**
857 *DopEcR* knockdown in the MB of males has a decreased number of flies within 4BL compared
858 to genetic controls (two-way ANOVA and Šídák's *post-hoc* test: p = 0.0103). Grey colour bars:
859 genetic control *driver/+*; pink colour bars: *driver/RNAi*. The results of all two-way ANOVA and
860 *post-hoc* tests performed can be found in **Table 1**. N = 9 for all treatments. Error bars are +/-
861 s.e.m.
862
863 **Figure 6. Hyperactivation of *FruP1* neurons increases social space in males and females**
864 **while silencing *FruP1* neurons only decreases social space in males.** Average number of flies
865 within 4 body lengths (4BL) in males and females when genetically hyperactivating neural
866 transmission in *FruP1* neurons. Hyperactivation of *FruP1* neurons decreases the number of flies
867 within 4BL in both males and females compared their basal transmission (two-way ANOVA –

Effect of neural transmission: $F_{1,33} = 22.65$, $p < 0.0001$). Silencing *FruP1* neurons did not alter change female social space but increased the number of flies within 4BL in males (two-way ANOVA with Šídák's *post-hoc* test: $p = 0.0453$). Grey colour bars: inactivated genetic construct and basal transmission of neurons; green colour bars: activation of genetic construct and hyperactivation of neurons with Gal4 driver; pink colour bars: activation of genetic construct and silencing of neurons with Gal4 driver. The results of all two-way ANOVA and *post-hoc* tests performed can be found in **Table 1**. N = 7 – 11 for all treatments. Error bars are +/- s.e.m.

Figure 7. Knocking down *nlg3* in *FruP1* neurons decreases female social space, while a reduction of acetylcholine in *FruP1* neurons decreases social space in male and female flies. **A-D:** Average number of flies within 4 body lengths (4BL) in male and female flies with RNAi knockdown of *nlg3* (A), *TH* for dopamine synthesis (B), *ChAT* for acetylcholine synthesis (C) or *GAD1* for GABA synthesis (D) specifically in *FruP1* neurons. **A.** Knocking down *nlg3* in *FruP1* has an interaction between sex for number of flies within 4BL (two-way ANOVA – Effect of interaction: $F_{1,58} = 7.056$, $p = 0.0102$) **B.** *TH* knockdown in *FruP1* neurons does not change social space in males or females (two-way ANOVA – Effect of genotype: $F_{1,31} = 1.134$, $p = 0.2951$). **C.** *ChAT* knockdown in *FruP1* neurons increases the number of flies within 4BL in both males and females compared to the genetic control flies (two-way ANOVA – Effect of genotype: $F_{1,48} = 38.01$, $p < 0.0001$). **D.** *GAD1* knockdown in *FruP1* neurons increases the number of flies within 4BL in only females (two-way ANOVA – Effect of interaction: $F_{1,39} = 9.327$, $p = 0.0041$). Grey colour bars: genetic control *driver/+*; pink colour bars: *driver/RNAi*. The results of all two-way ANOVA and *post-hoc* tests performed can be found in **Table 1**. N = 9 - 13 for all treatments. Error bars are +/- s.e.m.

Figure 8. Working model of functional neural influences on Drosophila social space: Dopaminergic inputs into the mushroom body neurons (dark blue line) influence male social space while cholinergic outputs from the mushroom bodies influence female social space. Cholinergic outputs from *fruP1* neurons (light green line) also influence male and female social space while some mushroom body neurons also express *fru*. Neuroligin protein (red dots) enrichment is also found to be located in the optic lobes, calyx of the mushroom bodies, and protocerebral bridge.

901 **Figure 1**
902



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907 **Figure 2**
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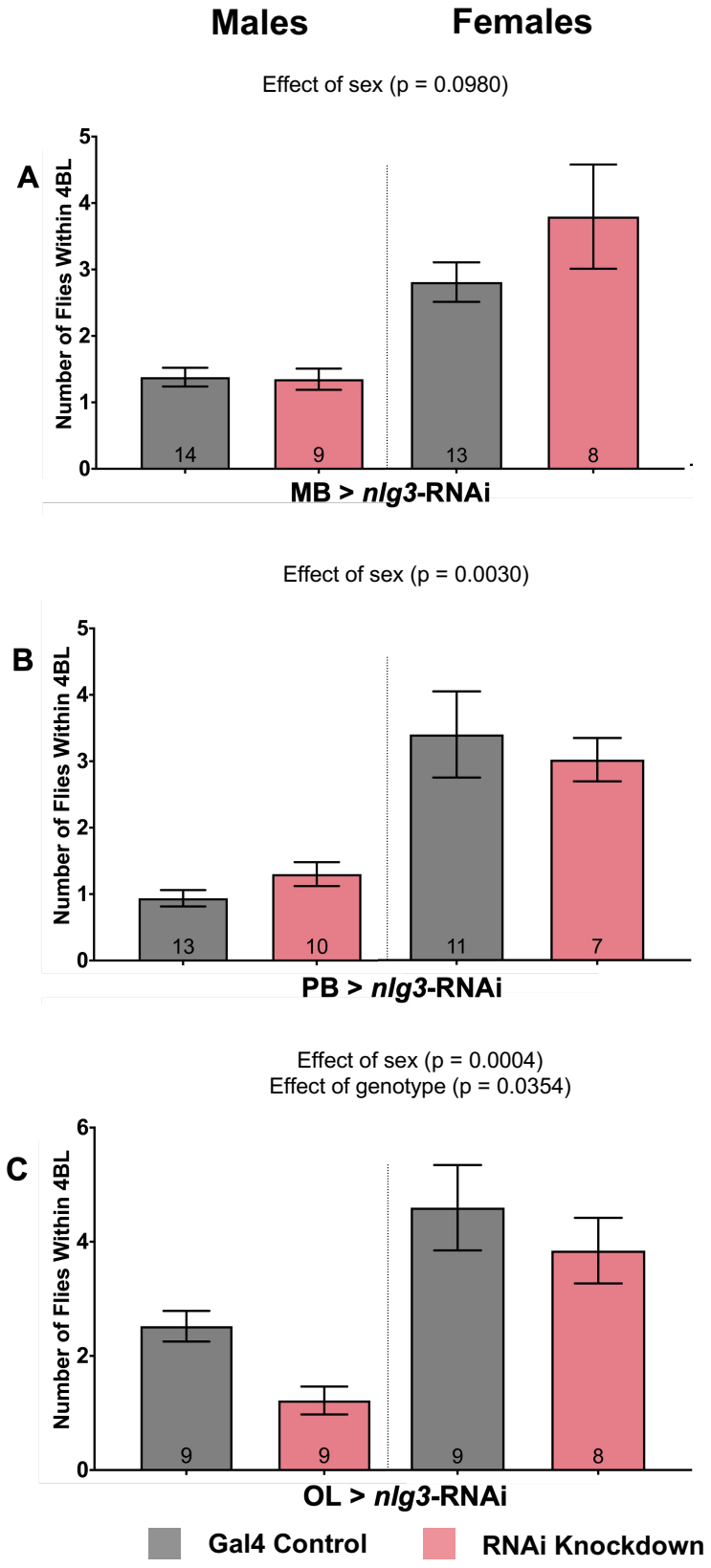

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910 **Figure 3**
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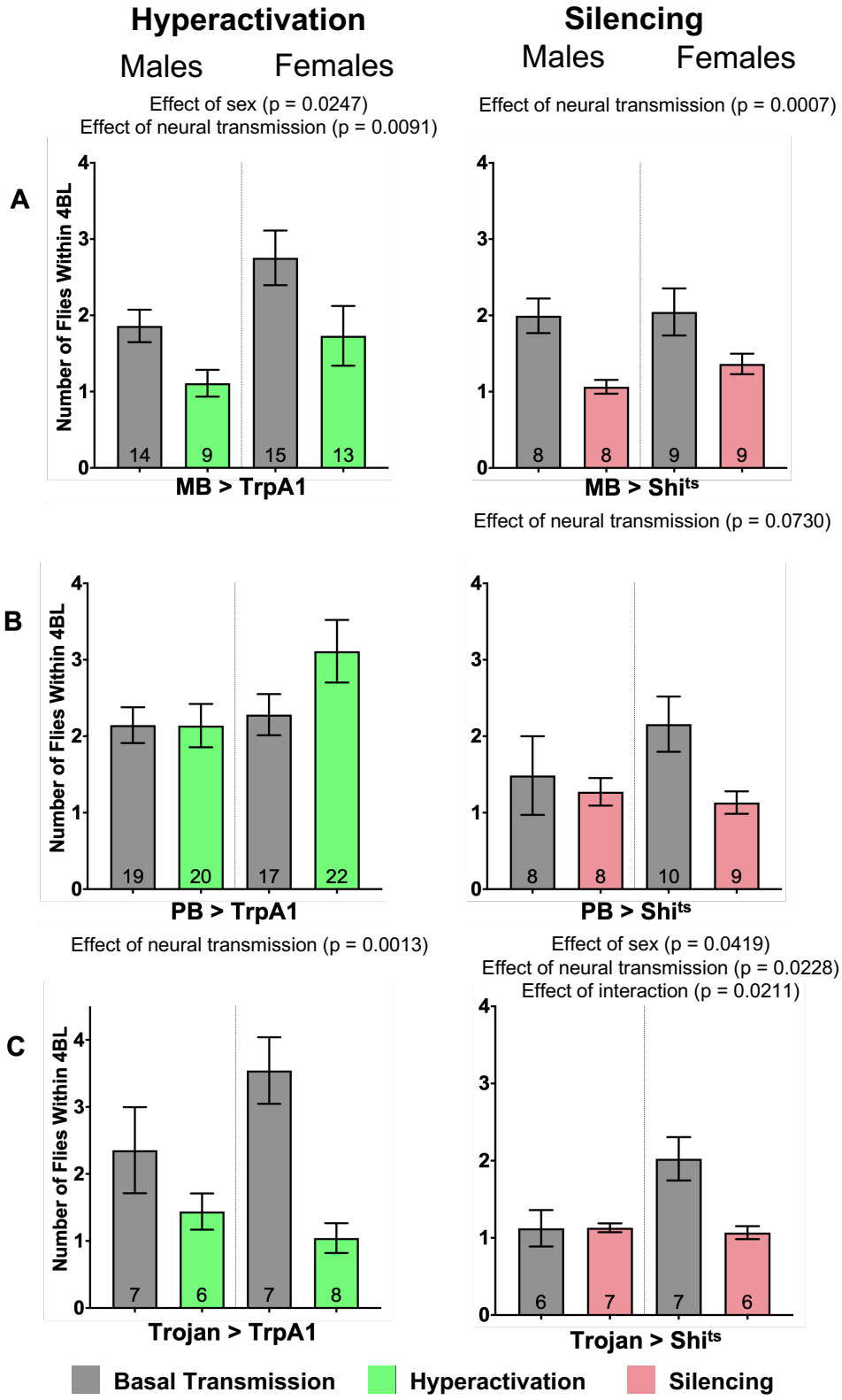

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914 **Figure 4**
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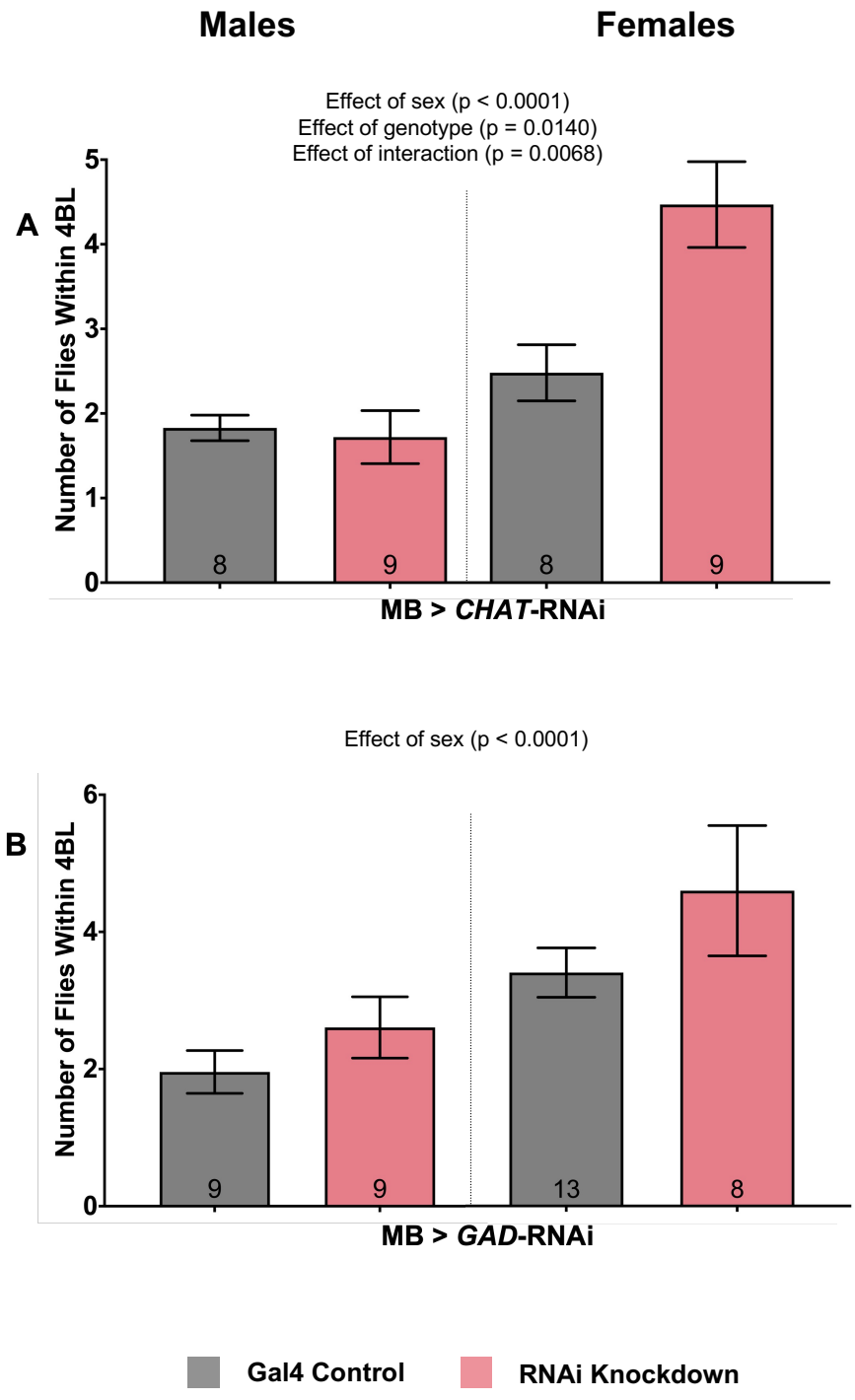

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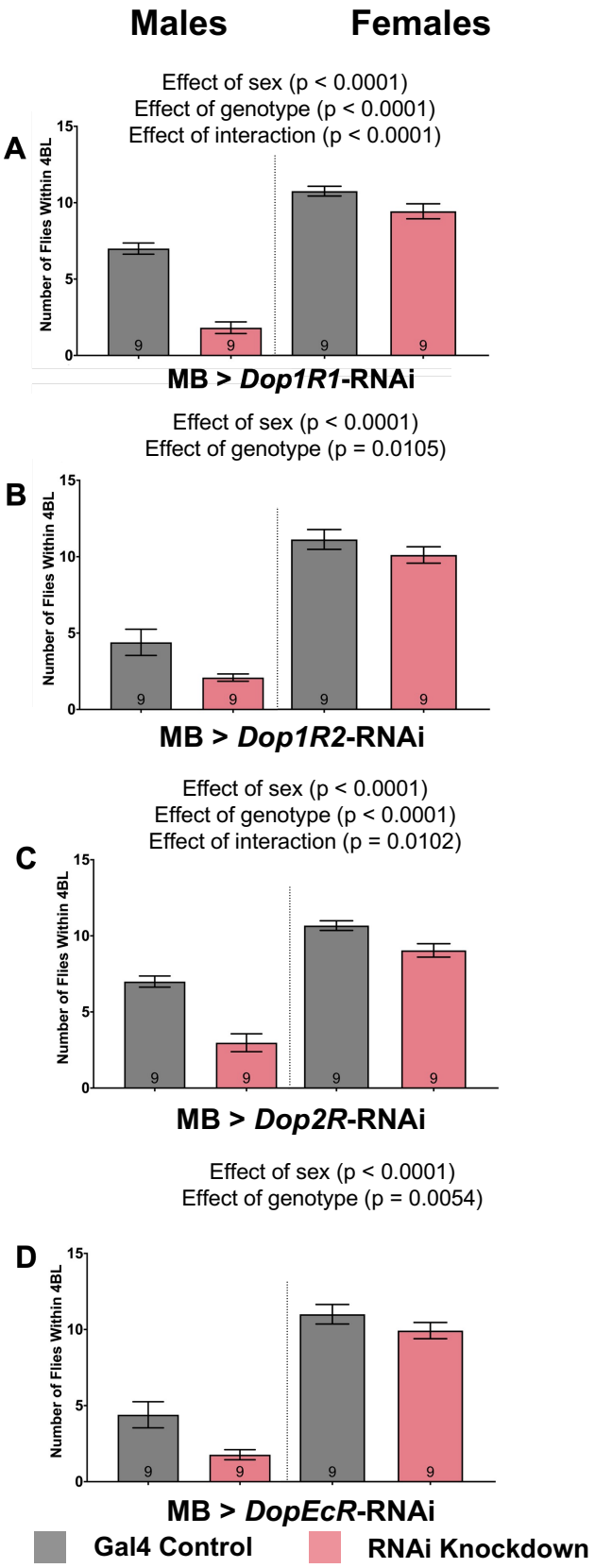
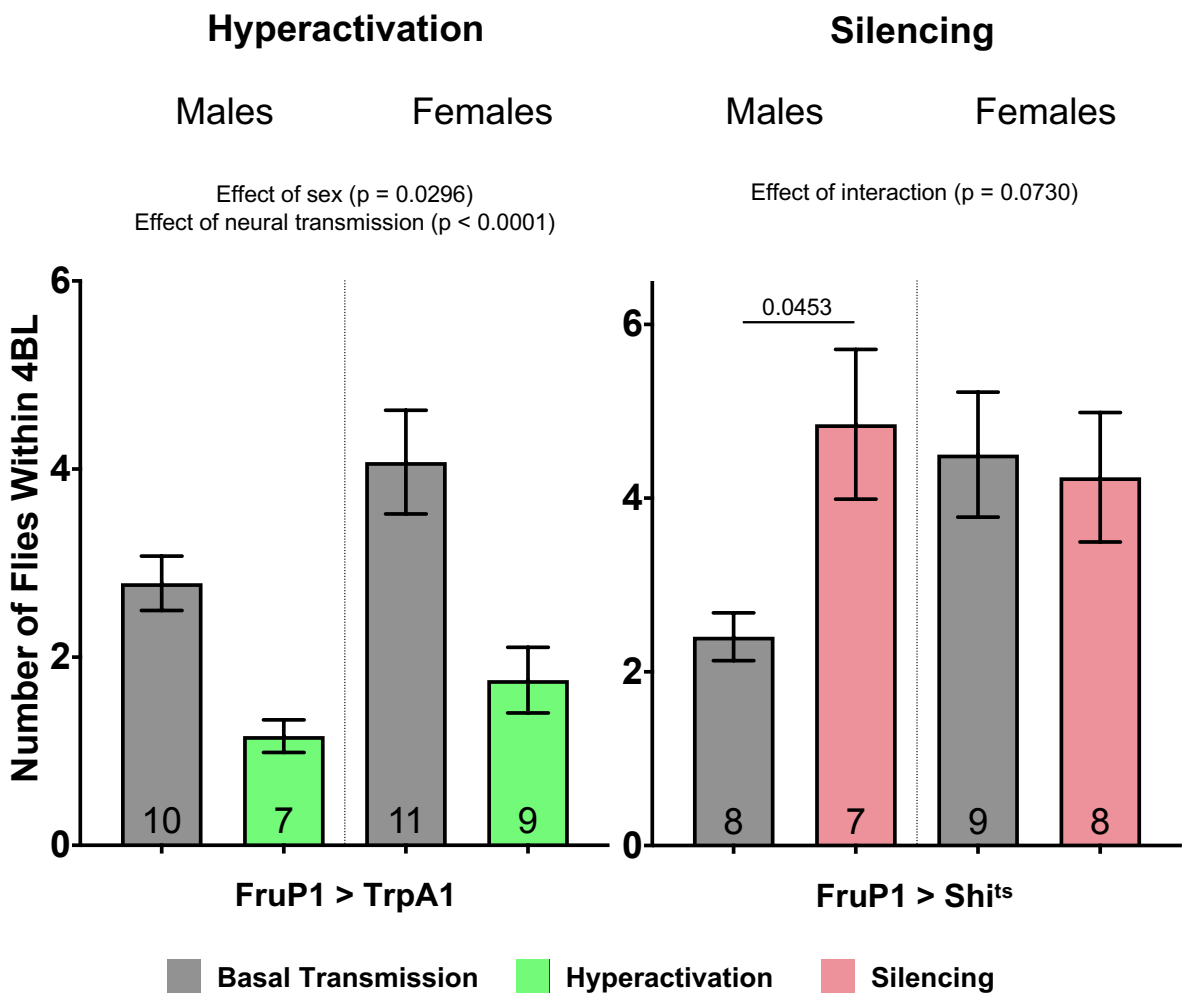
Figure 5


Males
Females
Effect of sex (p < 0.0001)
Effect of genotype (p < 0.0001)
Effect of interaction (p < 0.0001)
A
Number of Flies Within 4BL
MB > Dop1R1-RNAi
Effect of sex (p < 0.0001)
Effect of genotype (p = 0.0105)
B
Number of Flies Within 4BL
MB > Dop1R2-RNAi
Effect of sex (p < 0.0001)
Effect of genotype (p < 0.0001)
Effect of interaction (p = 0.0102)
C
Number of Flies Within 4BL
MB > Dop2R-RNAi
Effect of sex (p < 0.0001)
Effect of genotype (p = 0.0054)
D
Number of Flies Within 4BL
MB > DopEcR-RNAi
Gal4 Control
RNAi Knockdown

921 **Figure 6**
922



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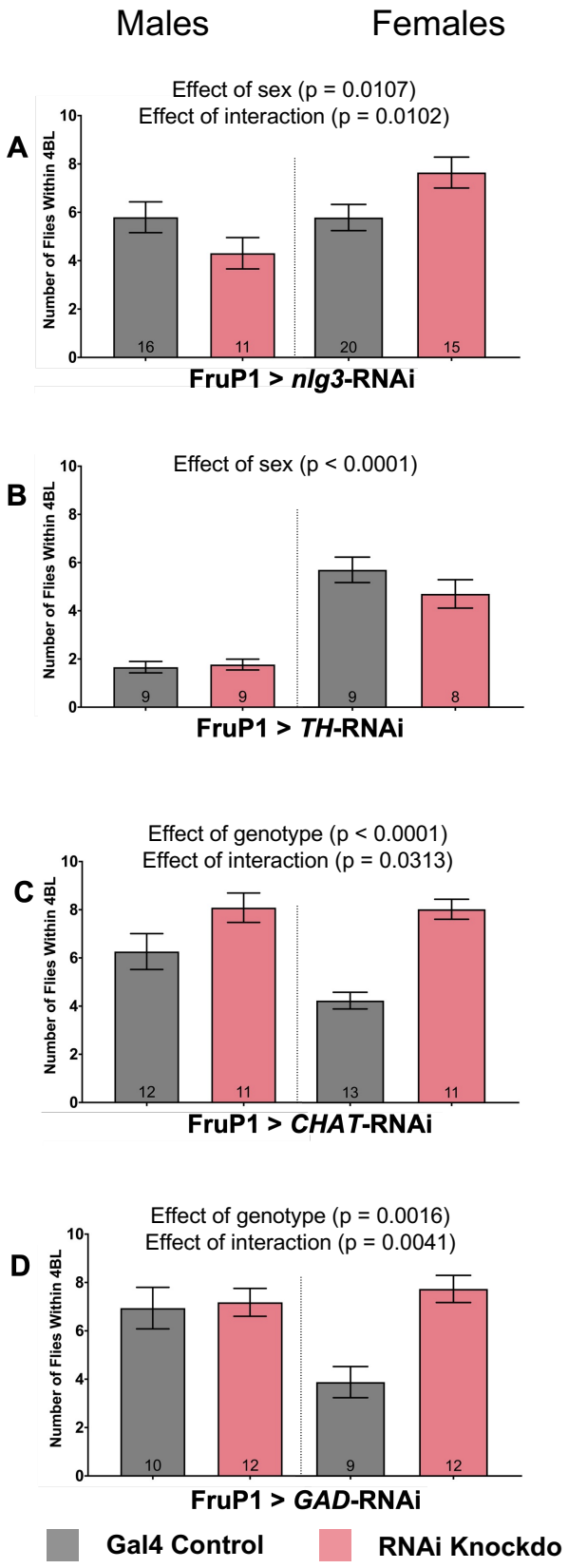
925 **Figure 7**



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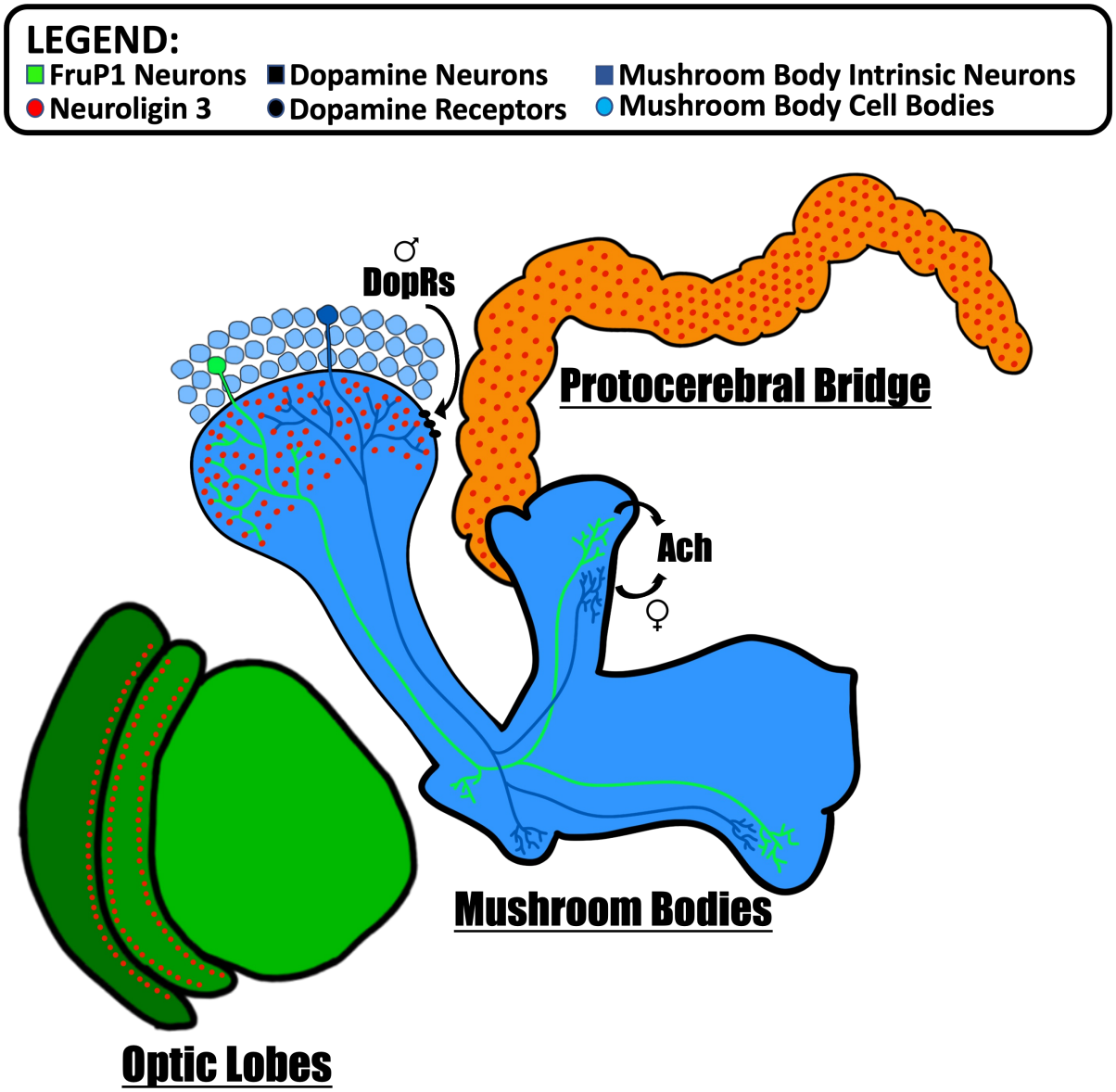
927 **Figure 8**



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929

930 **Supplemental Figure Captions**
931
932 **Supplemental Table 1. ANOVA table for all Two-Way ANOVAs performed for social**
933 **space experiments presented in Figures 2-7.** Statistically significant results are bolded.
934
935 **Supplemental Figure 1: Schematic of crossing scheme used to generate genotype controls**
936 **for all RNAi knockdown experiments.** Bolded genotypes are presenting in main figures.
937 Results for all genotypes are in the supplemental data below. Main conclusions are unchanged
938 when adding or removing the *UAS/+* genetic control.
939
940 **Supplemental Figure 2: Knocking down Nlg3 specifically in the Protocerebral bridge and**
941 **optic lobes decreases fly climbing in males and females but not when knocking down in the**
942 **mushroom bodies specifically. A-C:** Startle-induced climbing represented by the mean
943 percentage of flies reaching the top vial (performance index). Genetic constructs include RNAi
944 knockdown of *nlg3* targeted specifically to the mushroom bodies (A), protocerebral bridge (B),
945 and the optic lobes (C). Grey colour: genetic control *driver/+*; pink colour: *driver/RNAi*. The
946 results of all two-way ANOVA performed are shown on the figure. N = 5 – 10 for all treatments.
947 Errors bar are +/- s.e.m.
948
949 **Supplemental Figure 3: Hyperactivation of all the brain structures enriched with Nlg3**
950 **decreases fly climbing. A-C:** Startle-induced climbing represented by the mean percentage of
951 flies reaching the top vial (performance index). Hyperactivation or silencing neurons specifically
952 to the mushroom bodies (A), protocerebral bridge (B), and all *nlg3-expressing* neurons (C). Grey
953 colour bars: inactivated genetic construct and basal transmission of neurons; green colour bars:
954 activation of genetic construct and hyperactivation of neurons with *Gal4* driver; pink colour
955 bars: activation of genetic construct and silencing of neurons with *Gal4* driver. The results of all
956 two-way ANOVA performed are shown on the figure. N = 4 – 7 for all treatments. Error bars are
957 +/- s.e.m.
958
959 **Supplemental Figure 4: Hyperactivation and silencing of *FruP1* neurons decreases**
960 **climbing in males and females.** Startle-induced climbing represented by the mean percentage of
961 flies reaching the top vial (performance index). Hyperactivation or silencing neurons specifically
962 in *FruP1* neuron subset. Grey colour bars: inactivated genetic construct and basal transmission
963 of neurons; green colour bars: activation of genetic construct and hyperactivation of neurons
964 with *Gal4* driver; pink colour bars: activation of genetic construct and silencing of neurons with
965 *Gal4* driver. The results of all two-way ANOVA performed are shown on the figure. N = 9 for
966 all treatments. Error bars are +/- s.e.m.
967
968 **Supplemental Figure 5: Knocking down *nlg3* in *FruP1* neurons decreases male climbing,**
969 **while a reduction of dopamine, acetylcholine, and GABA in *FruP1* neurons all decrease**
970 **climbing in male and female flies. A-D:** Startle-induced climbing represented by the mean
971 percentage of flies reaching the top vial (performance index). Genetic constructs include RNAi
972 knockdown targeted specifically to the *FruP1* subset of neurons of *nlg3* (A), knocking down
973 dopamine (B), acetylcholine (C), and GABA (D). Grey colour: genetic control *driver/+*; pink
974 colour: *driver/RNAi*. The results of all two-way ANOVA performed are shown on the figure. N
975 = 6 – 15 for all treatments. Errors bar are +/- s.e.m.

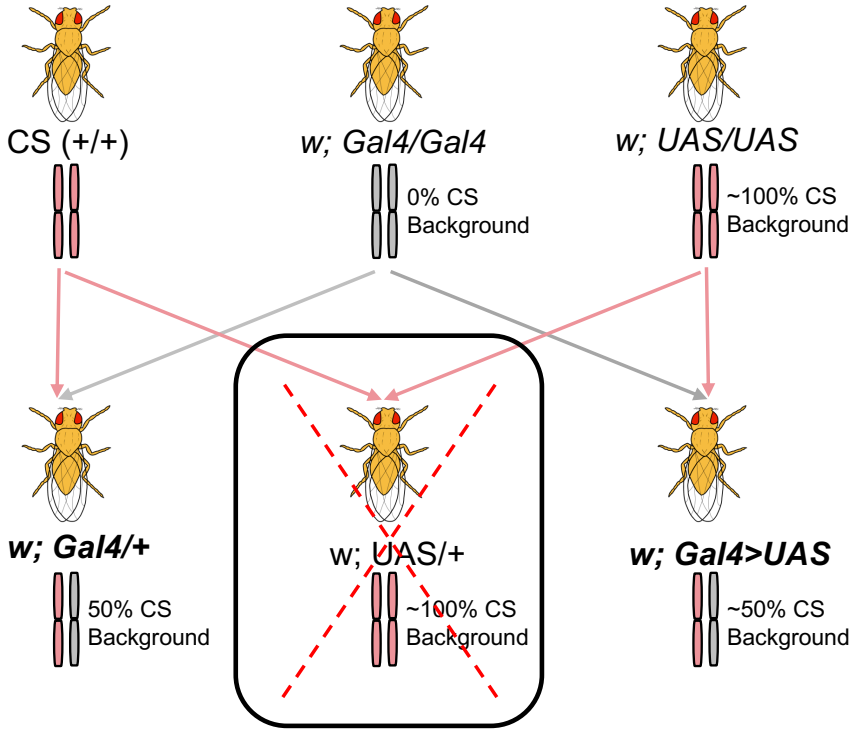
976 **Supplemental Figure 6: Elevated temperature on Cs, *Trp1A/+*, and *Shi^{ts}/+* does not affect**
977 **social space or climbing.**
978
979 **Supplemental Figure 7: Knocking down Nlg3 specifically in the optic lobes increases fly**
980 **social space in males and females but changes are seen when knocking down in the**
981 **protocerebral bridge or mushroom bodies specifically (both genetic controls shown).**
982
983 **Supplemental Figure 8. Knocking down the acetylcholine rate-limiting biosynthesis enzyme**
984 **C*hAT* in the MB decreases female fly social space (both genetic controls shown).**
985
986 **Supplemental Figure 9: RNAi knockdown of all dopamine receptors in the MB increases**
987 **male social space (both genetic controls shown).**
988
989 **Supplemental Figure 10: Knocking down *nlg3* in *FruP1* neurons decreases female social**
990 **space, while a reduction of acetylcholine in *FruP1* neurons decreases social space in male**
991 **and female flies (both genetic controls shown).**
992

Supp. Table 1

Experiment	Figure	Effect	*Df*	*F*	*p*	*Šídák's post hoc*		
Social space						**Comparison Group**	**Compared with**	***p***
MB>nlg3-RNAi	2A	Sex	(1,39)	2.873	0.0980	*Male*		
		Genotype	(1,39)	1.005	0.3223	MB>RNAi	MB/+	0.9602
		Sex * Genotype	(1,39)	1.849	0.1817	*Female*		
						MB>RNAi	MB/+	0.1992
PB>nlg3-RNAi	2B	**Sex**	(1,37)	10.12	**0.0030**	*Male*		
		Genotype	(1,37)	0.068	0.7951	PB>RNAi	PB/+	0.4937
		Sex * Genotype	(1,37)	1.328	0.2566	*Female*		
						PB>RNAi	PB/+	0.8051
OL>nlg3-RNAi	2C	**Sex**	(1,31)	15.93	**0.0004**	*Male*		
		Genotype	(1,31)	4.837	**0.0354**	OL>RNAi	OL/+	0.0845
		Sex * Genotype	(1,31)	0.542	0.4670	*Female*		
						OL>RNAi	OL/+	0.5322
MB>TrpA1	3A	**Sex**	(1,47)	5.382	**0.0247**	*Male MB>Trp*		
		Neural Transmission	(1,47)	7.395	**0.0091**	Activated	Inactivated	0.2437
		Sex * Neural	(1,47)	0.172	0.6796	*Female MB>Trp*		
		Transmission				**Activated**	**Inactivated**	**0.0442**
MB>Shi^{ts}	3B	Sex	(1,30)	0.680	0.4161	*Male MB>Shi*		
		Neural Transmission	(1,30)	14.40	**0.0007**	**Activated**	**Inactivated**	**0.0105**
		Sex * Neural	(1,30)	0.344	0.5617	*Female MB>Shi*		
		Transmission				Activated	Inactivated	0.0518
PB>TrpA1	3C	Sex	(1,74)	39.94	0.0868	*Male PB>Trp*		
		Neural Transmission	(1,74)	17.72	0.2018	Activated	Inactivated	0.9999
		Sex * Neural	(1,74)	13.62	0.1953	*Female PB>Trp*		
		Transmission				Activated	Inactivated	0.1382
PB>Shi^{ts}	3D	Sex	(1,31)	0.6335	0.4321	*Male PB>Shi*		
		Neural Transmission	(1,31)	3.443	0.0730	Activated	Inactivated	0.8897
		Sex * Neural	(1,31)	1.483	0.2325	*Female PB>Shi*		
		Transmission				Activated	Inactivated	0.0596
Trojan>TrpA1	3E	Sex	(1,28)	2.380	0.1341	*Male Trojan>Trp*		
		Neural Transmission	(1,28)	12.70	**0.0013**	Activated	Inactivated	0.2214
		Sex * Neural	(1,28)	1.366	0.2523	*Female Trojan>Trp*		
		Transmission				**Activated**	**Inactivated**	**0.0029**
Trojan>Shi^{ts}	3F	**Sex**	(1,22)	4.666	**0.0419**	*Male MB>Trp*		
		Neural Transmission	(1,22)	5.998	**0.0228**	Activated	Inactivated	0.9996
		Sex * Neural	(1,22)	6.169	**0.0211**	*Female MB>Trp*		
		Transmission				**Activated**	**Inactivated**	**0.0042**
MB>CHAT25-RNAi	4A	**Sex**	(1,30)	22.26	**<0.0001**	*Male*		
		Genotype	(1,30)	6.805	**0.0140**	MB>RNAi	MB/+	0.9721
		Sex * Genotype	(1,30)	8.465	**0.0068**	*Female*		
						MB>RNAi	**MB/+**	**0.0010**
MB>GAD51-RNAi	4B	**Sex**	(1,31)	42.89	**<0.0001**	*Male*		
		Genotype	(1,31)	0.021	0.8851	MB>RNAi	MB/+	0.4656
		Sex * Genotype	(1,31)	2.012	0.1651	*Female*		
						MB>RNAi	MB/+	0.6167
MB>Dop1R1-RNAi	5A	**Sex**	(1,32)	65.51	**<0.0001**	*Male*		
		Genotype	(1,32)	19.61	**<0.0001**	**MB>RNAi**	**MB/+**	**<0.0001**
		Sex * Genotype	(1,32)	27.24	**<0.0001**	*Female*		
						MB>RNAi	MB/+	0.0505
MB>Dop1R2-RNAi	5B	**Sex**	(1,32)	139.4	**<0.0001**	*Male*		
		Genotype	(1,32)	7.401	**0.0105**	**MB>RNAi**	**MB/+**	**0.0225**
		Sex * Genotype	(1,32)	1.168	0.2880	*Female*		
						MB>RNAi	MB/+	0.4447
MB>Dop2R-RNAi	5C	**Sex**	(1,32)	120.7	**<0.0001**	*Male*		
		Genotype	(1,32)	41.10	**<0.0001**	**MB>RNAi**	**MB/+**	**<0.0001**
		Sex * Genotype	(1,32)	7.448	**0.0102**	*Female*		
						MB>RNAi	**MB/+**	**0.0276**
MB>DopEcR-RNAi	5D	**Sex**	(1,32)	139.3	**<0.0001**	*Male*		
		Genotype	(1,32)	8.905	**0.0054**	**MB>RNAi**	**MB/+**	**0.0103**
		Sex * Genotype	(1,32)	1.586	0.2167	*Female*		
						MB>RNAi	MB/+	0.4098
FruP1>TrpA1	6A	**Sex**	(1,33)	5.168	**0.0296**	*Male FruP1>Trp*		
		Neural Transmission	(1,33)	22.65	**<0.0001**	**Activated**	**Inactivated**	**0.0241**
		Sex * Neural	(1,33)	0.6987	0.4092	*Female FruP1>Trp*		
		Transmission				**Activated**	**Inactivated**	**0.0004**
FruP1>Shi^{ts}	6B	Sex	(1,30)	0.5671	0.4573	*Male FruP1>Trp*		
		Neural Transmission	(1,30)	2.350	0.1358	**Activated**	**Inactivated**	**0.0453**
		Sex * Neural	(1,30)	3.451	0.0730	*Female FruP1>Trp*		
		Transmission				Activated	Inactivated	0.9676
FruP1>nlg3-RNAi	7A	**Sex**	(1,58)	6.969	**0.0107**	*Male*		
		Genotype	(1,58)	0.087	0.7690	FruP1>RNAi	FruP1/+	0.2303
		Sex * Genotype	(1,58)	7.056	**0.0102**	*Female*		
						FruP1>RNAi	FruP1/+	0.0563
FruP1>TH-RNAi	7B	**Sex**	(1,31)	69.88	**<0.0001**	*Male*		
		Genotype	(1,31)	1.134	0.2951	FruP1>RNAi	FruP1/+	0.9788
		Sex * Genotype	(1,31)	1.750	0.1956	*Female*		
						FruP1>RNAi	FruP1/+	0.2013
FruP1>CHAT25-RNAi	7C	Sex	(1,48)	0.777	0.3824	*Male*		
		Genotype	(1,48)	38.01	**<0.0001**	**FruP1>RNAi**	**FruP1/+**	**0.0150**
		Sex * Genotype	(1,48)	4.919	**0.0313**	*Female*		
						FruP1>RNAi	**FruP1/+**	**<0.0001**
FruP1>GAD51-RNAi	7D	Sex	(1,39)	0.008	0.9279	*Male*		
		Genotype	(1,39)	11.47	**0.0016**	FruP1>RNAi	FruP1/+	0.9649
		Sex * Genotype	(1,39)	9.327	**0.0041**	*Female*		
						FruP1>RNAi	**FruP1/+**	**0.0001**

Table 1. ANOVA table for all Two-way ANOVAs performed. Statistically significant results are bolded

995 **Supp. Figure 1**
996



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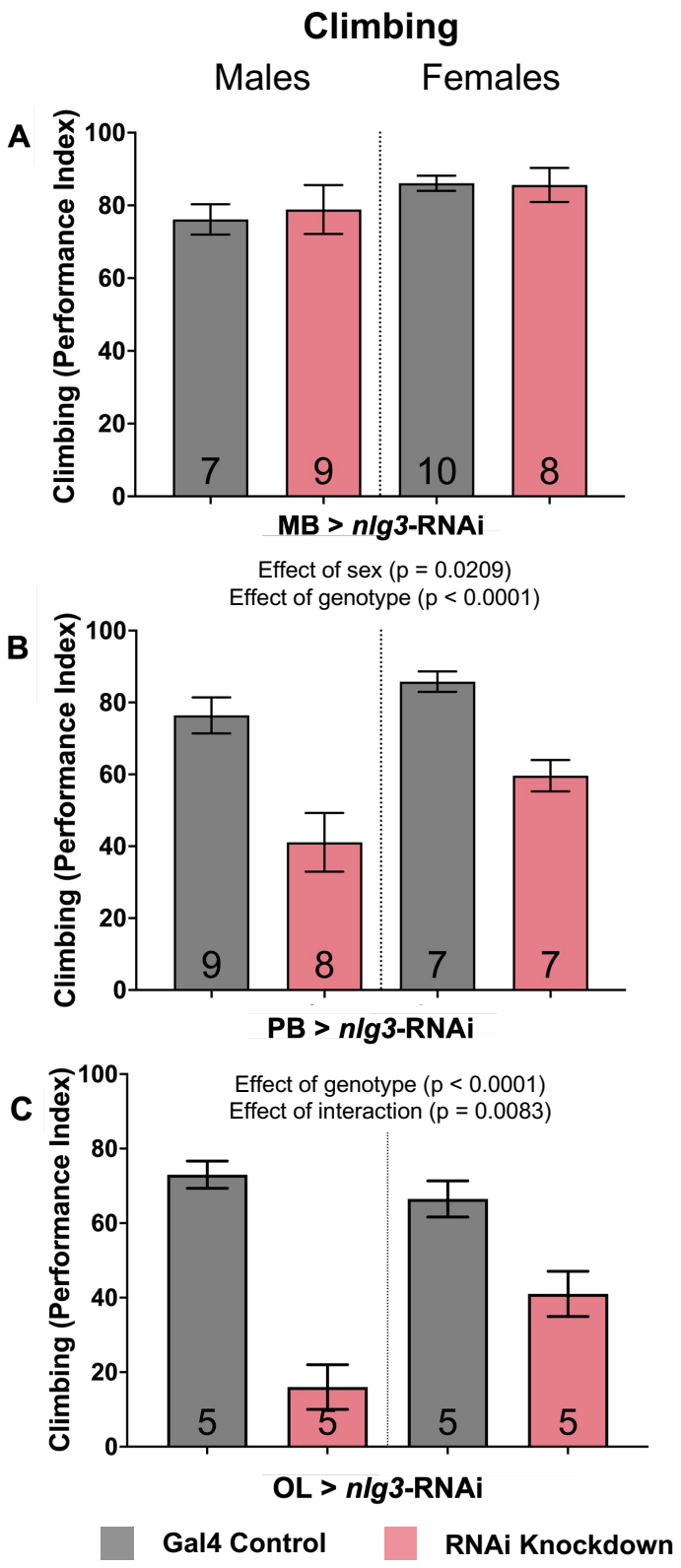

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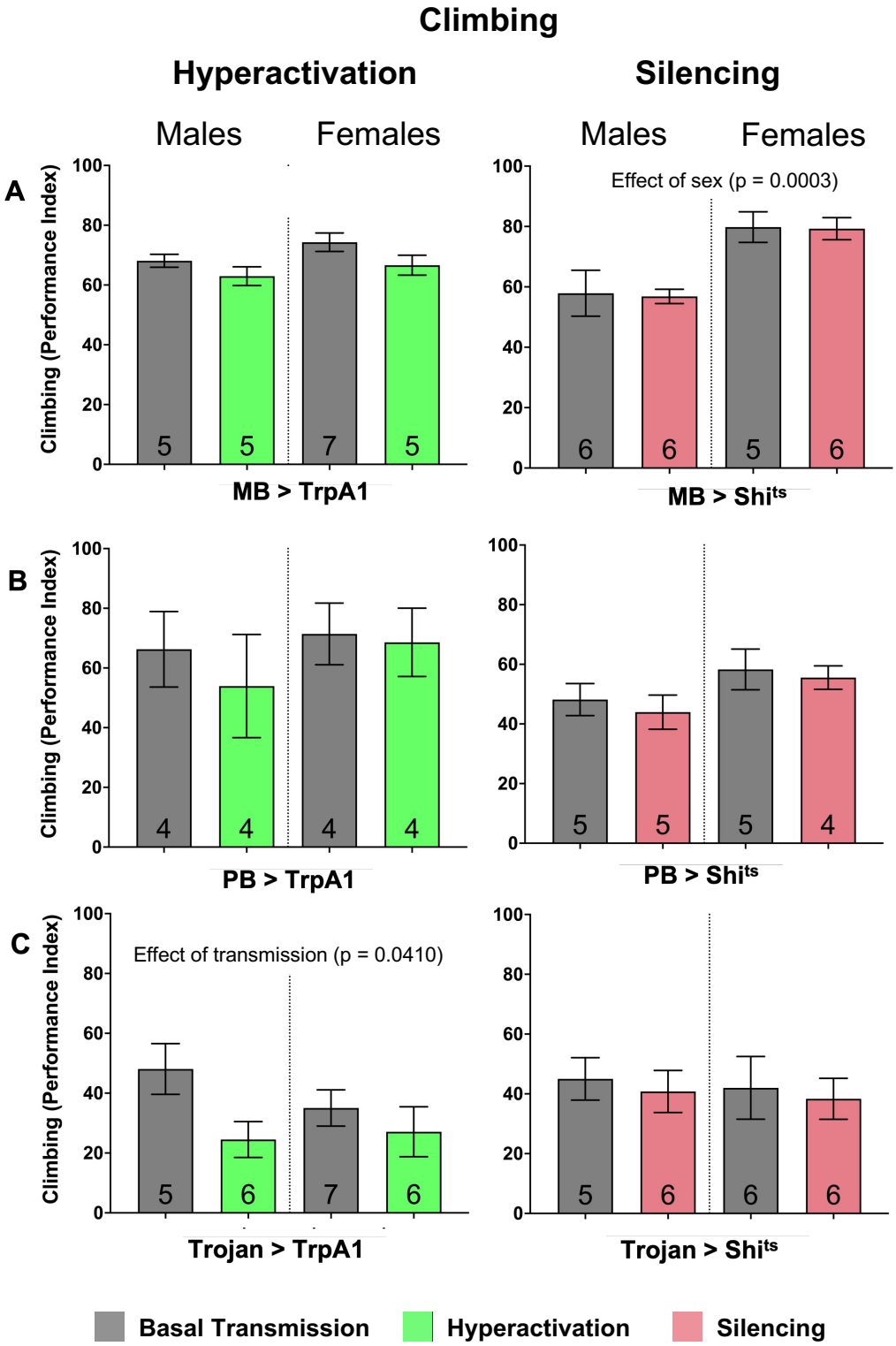

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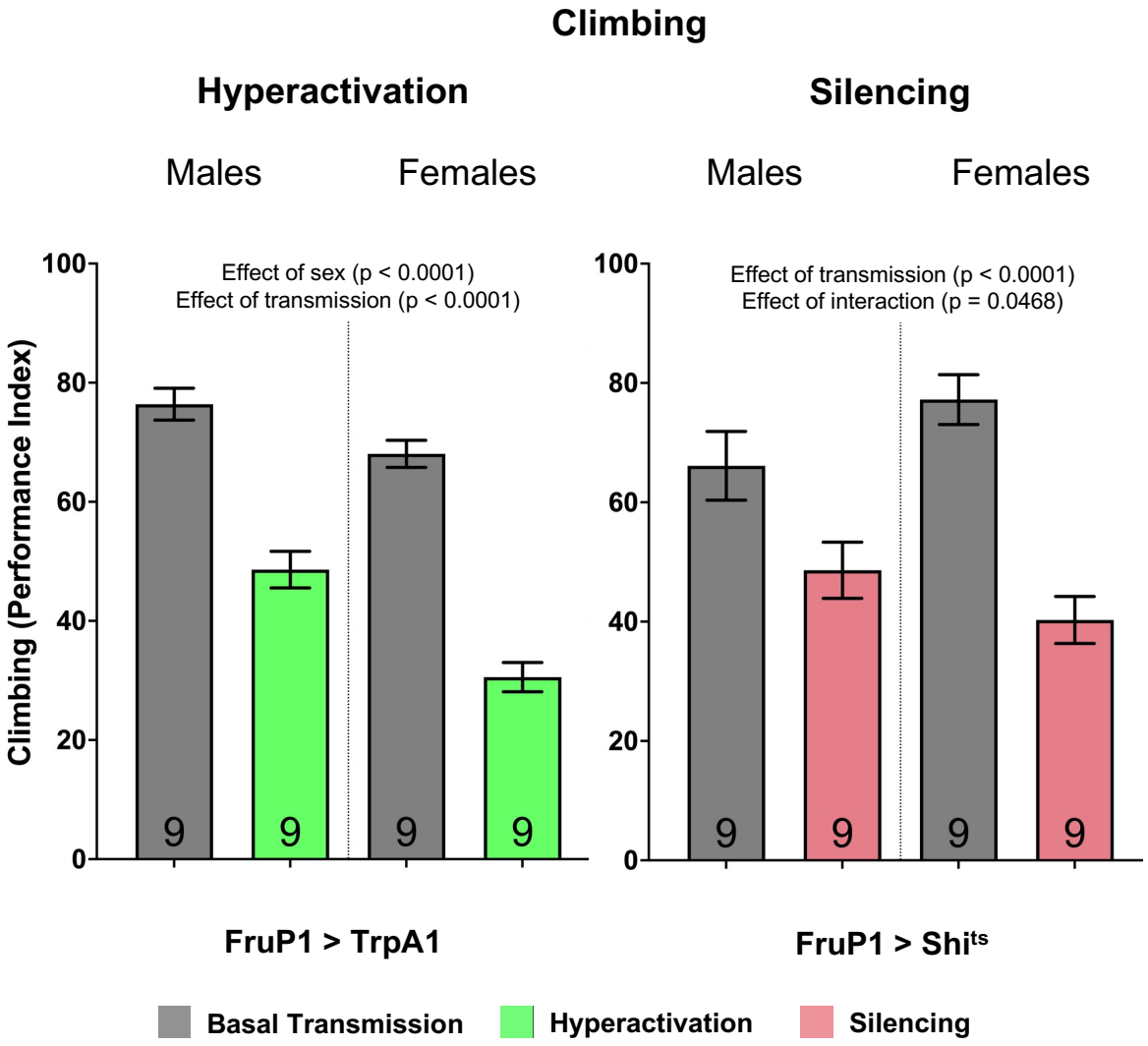

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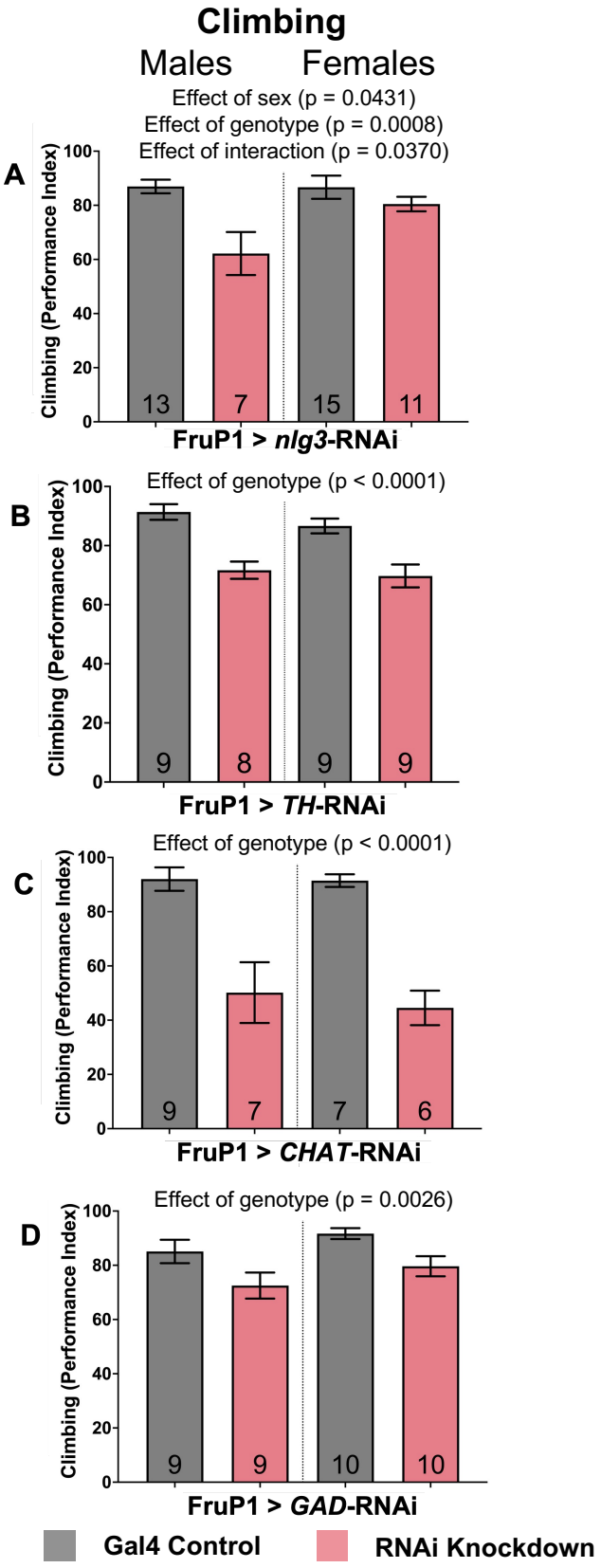

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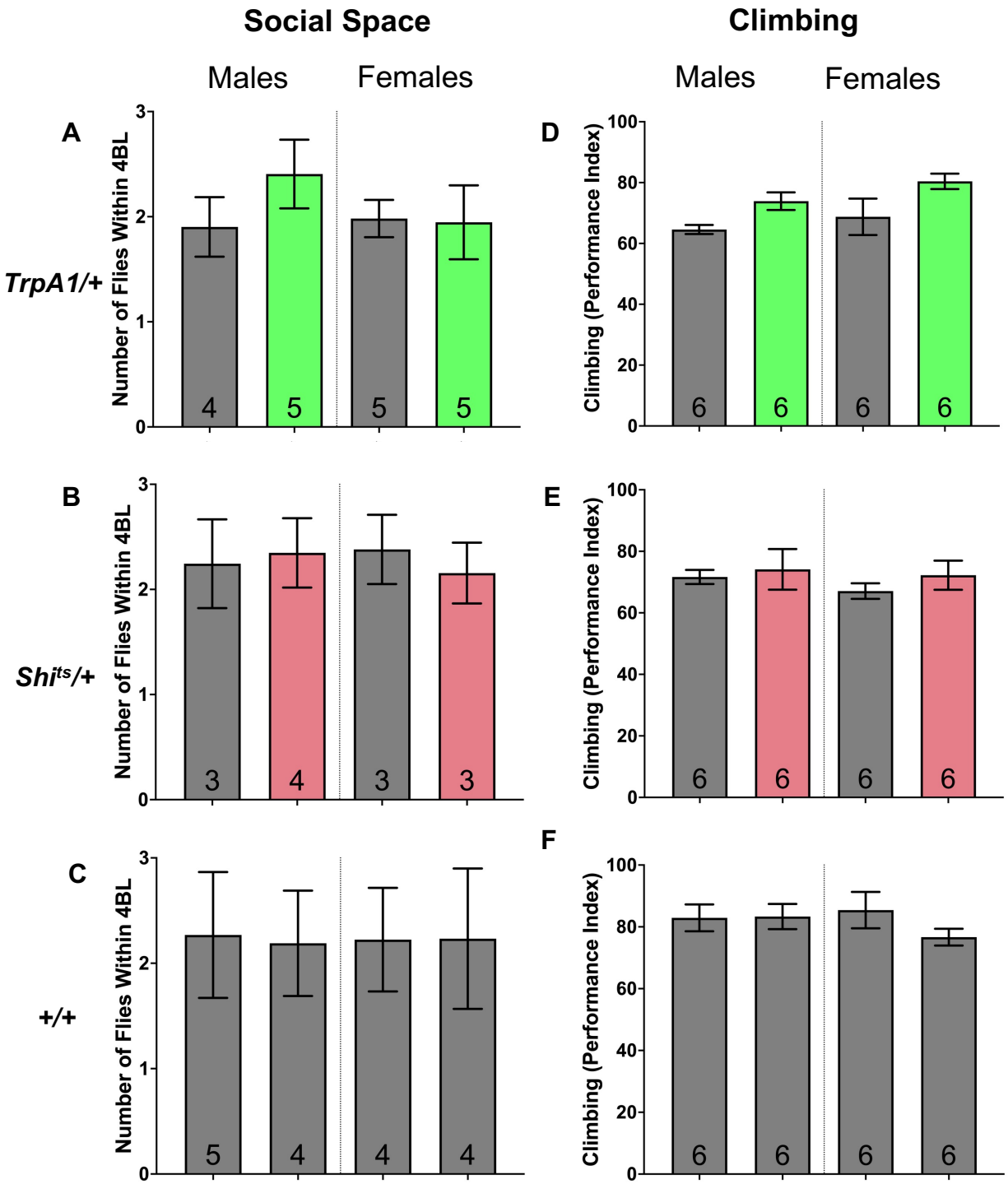

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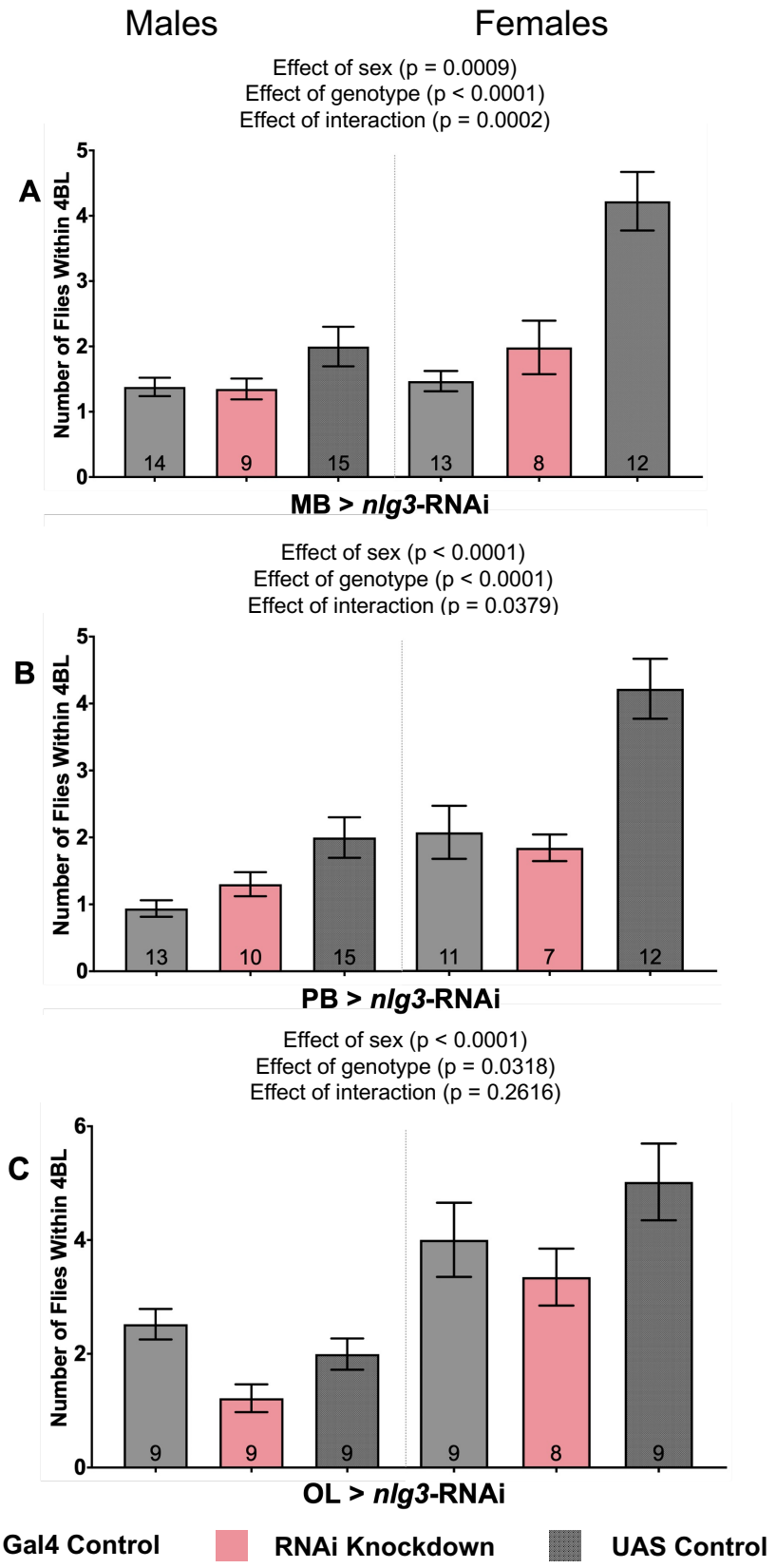

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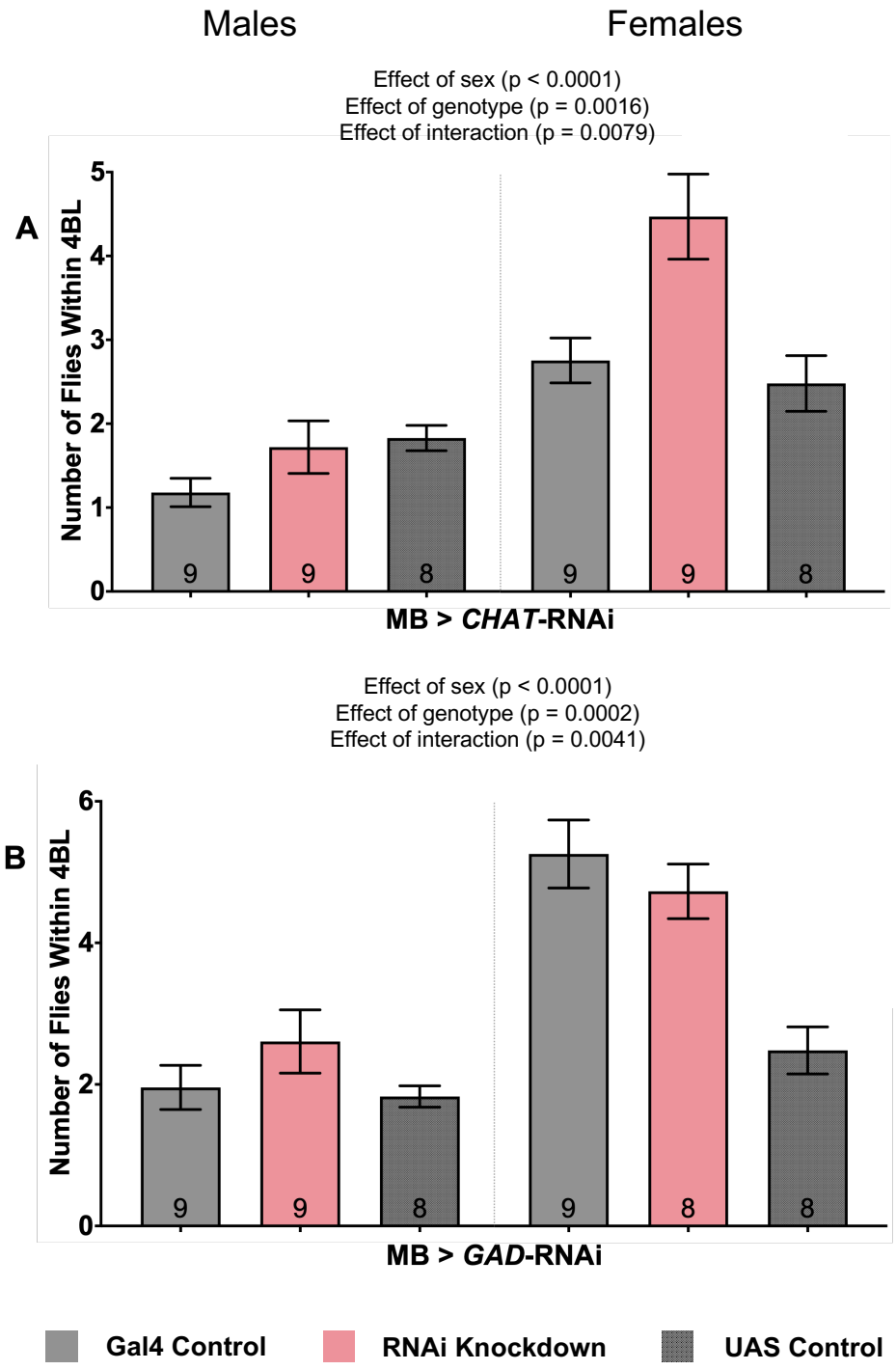

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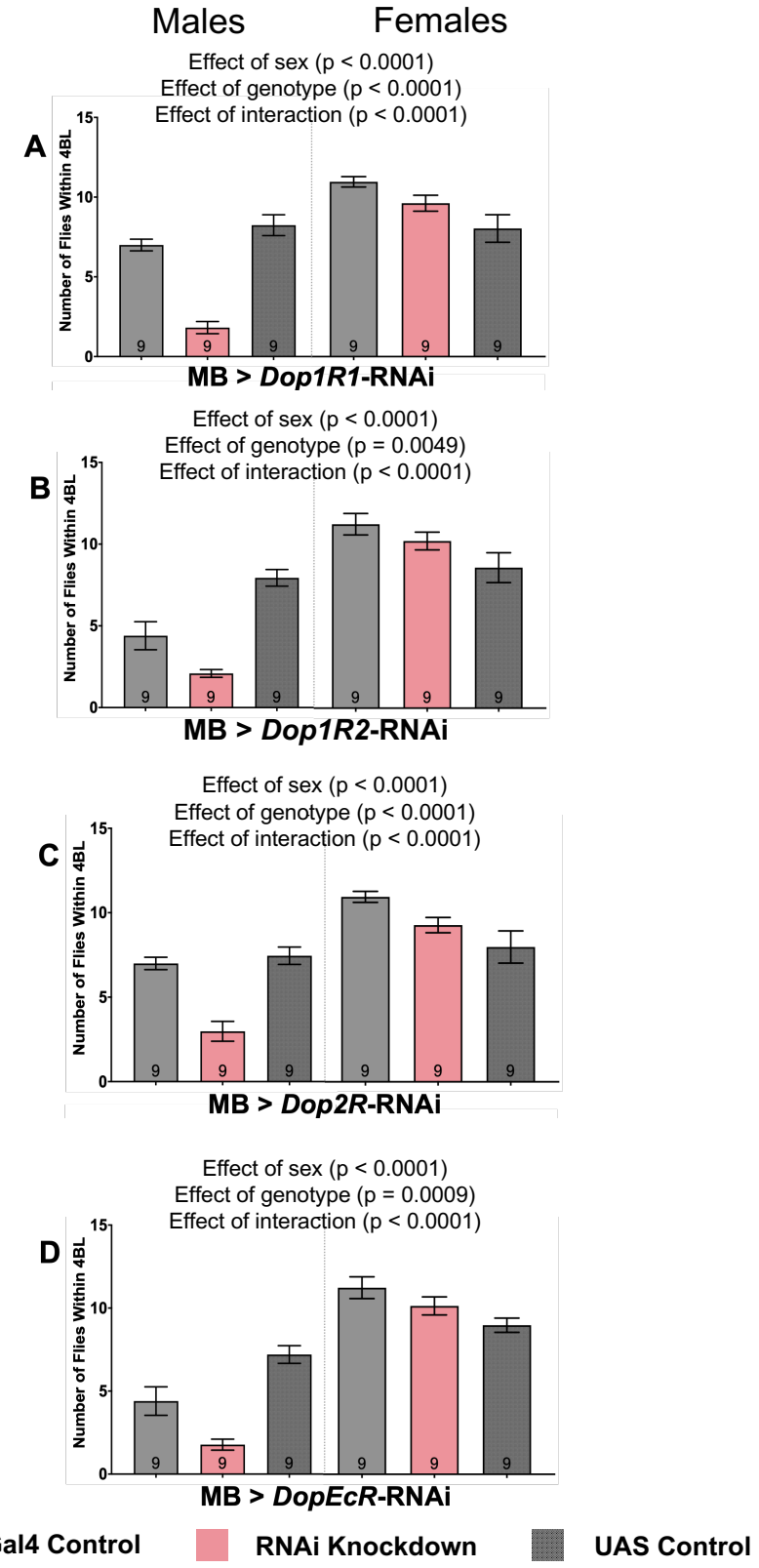

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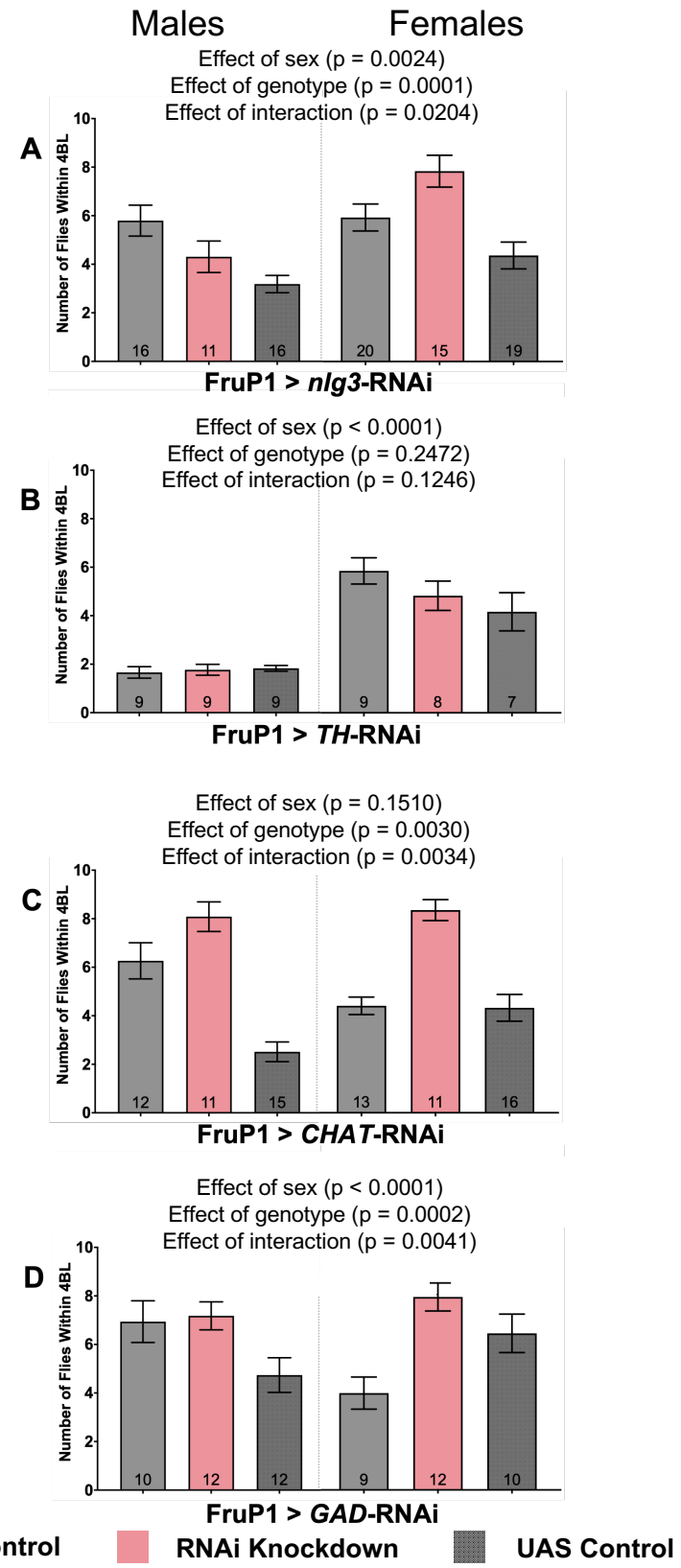

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