Supplemental Material for:

Modulation of social space by dopamine in *Drosophila melanogaster,* but no effect on the avoidance of the *Drosophila* stress odorant

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Supplemental results:

Males flies with a heterozygote insertion of a P-element in the *VMAT* gene (p/+) [1] show a significant increase in social spacing compared to CS (**Supplemental Figure 1A)**. However, these flies are closer than CS flies in a non-social condition [2], indicating that they still interact together**.** In contrast, all of these mutants were able to avoid the social signal emitted by stress flies (dSO), regardless of their sex (**Supplemental Figure 2A-D)**.

The p/+ mutants have been shown to only have decrease levels of dopamine and not serotonin [17]. To confirm a dopaminergic specific effect, we drove the expression of VMAT RNAi lines in dopaminergic cells, using a TH-gal 4 driver. Both VMAT RNAi lines expression driven by TH-Gal4 led to an increase in social spacing, which was more prominent with DoppelX-VMAT RNAi. In fact, the social spacing of the mutant was statistically similar to that of the non-social CS males (**Supplemental Figure 1B,C**, and **Figure 1A**).

Note: All data were obtained from experiments performed in the Simon lab, apart for the results reported in Supplemental **Figure 3G** and used for comparison compiled in **Figure 2** .

Supplemental MATERIAL AND METHODS

***Drosophila* stocks and husbandry**: *Drosophila* stocks were raised in standard food cornmeal/molasses/agar bottles or vials at 25°C with a relative humidity of 50% in a 12-hour day-night cycle. Canton-S (CS, FlybaseID: FBst0000001) and $w^{1118}CS_{10}$ (w^{1118} FlybaseID*:* FBal0018186, outcrossed 10 times to Canton-S) were from our laboratory stocks [3].

Mutant lines of the *VMAT* gene (Flybase ID: FBgn0260964): The following lines were outcrossed 6 times with $w^{1118}CS_{10}$: *UAS-cDNA-VMAT-A(II)* [4]; *Da-Gal4*, and *TH-Gal4* [5], and a P-element insertion l(2)SHO459, previously shown to be inserted in the last coding exon of *dVMAT*, obtained from [6]. Stocks of heterozygotes l(2)SH0459 (thereafter $dVMAT^{P}$) were kept over CyO balancer ($dVMAT^{P}$/CyO). The flies show a reduction in VMAT protein expression, a decrease in dopamine but not in serotonin levels, and are perfectly viable and fertile [7]. Their locomotion, phototaxis and negative geotaxis performances are slightly better than that their genetic control, CS [7, 8]. We crossed males $dVMAT^{P}$ with females CS, to generate males flies with a wild-type X chromosome and wild-type eye color (w+;p/+); and crossed males $dVMAT^{P}$ with females $w^{1118}CS_{10}$ to generate males with w^{1118} on the X, and orange eye color, due the presence of mini-white in the P-element (w-;p/+).

We also used *UAS-VMAT RNAi* (thereafter *DoppelX RNAi*); generously provided by Dr. Hovemann contains four *UAS-VMAT RNAi* insertions: one against *VMAT-A*, two against one against a common region [9].

The driver lines were mated to the expression lines and to the control strain CS to generate the heterozygous lines described in the text and figure legends. TH-Gal4 (III) described initially in [19], was gifted to our laboratories from Dr. Hirsh, University of Virginia, School of Medicine.

Tyrosine hydroxylase (FlybaseID: FBgn0005626) **mutants:** Two UAS transgenes carrying respectively the "wild-type" (*DTHg)* and a frameshift mutation (*DTHgFS±)* of the *pale* gene that does not allow for expression in the central nervous system, were used to rescue homozygous *pale* mutants, through a cross with a combined TH-Gal4 and Ddc-Gal driver line, also in *pale* mutant background (Ddc-Gal4; TH-Gal4, ple2/TM6b). *DTHgFS±; ple* and and its control *DTHg; ple* [10], were generously provided by Dr. Hirsh, University of Virginia, School of Medicine. We also used *Ple2/TM3, Sb* (thereafter *ple2*) a loss of function mutant of *pale* [11].

***Catecholamines up* (*catsup*) (**FlybaseID: FBgn0005626) **mutants:** The 600 bp deletion immediately upstream of start codon in yw^{1118}*; Catsup26/CyO* was generated by P(lacW) incision [12]. *UAS-Catsup-RNAi(III)* [12] was a gift from Dr. Larry Reiter, University of Tennessee Health Sciences.

L-DOPA and 3-iodotyrosine feeding: 3-4 days old male flies were fed for 24 hours on the following mixture: 10 ml volume of standard food was melted and either an equal amount of distilled water and red food dye (vehicle with no drug present) was added; or an equal amount of freshly made solutions containing red food dye and 3-iodotyrosine (3-IT; Sigma-Aldrich Product Number: I8250-1G) or L-DOPA (Sigma-Aldrich Product Number: D9628-5G) to reach a final concentration of 30 mM and 1 mM, respectively; and a final volume of 2ml in each vial. These concentrations have been shown to alter DA levels [13].

Behavior assays

Handling: All behavioral experiments were performed in a genotype-balanced manner. To avoid interference with courtship or aggressive behavior, flies were kept well-fed on standard food as mixed sexes to allow mating, and separated by sex only prior to the experiment.

All experiments used young (3-7 days old) and naïve flies, i.e. not previously tested in the assay. All flies were reared on a 12 hour day-night cycle, at 25°C, 50% relative humidity, in an environmental chamber. Each behavioral assay had 6 to 9 trials, obtained from 2-3 independent experiments, between Zeitgeber time ZT4 (hours after the onset of light) and ZT7, unless specified. Experiments were performed under ambient light, and the flies were allowed to habituate to the testing room for 2 hours before each experiment.

Locomotion assay: 10 flies were habituated for 1min. in a circular chamber of 9 cm in diameter, and 0.8 cm deep (the top cover of a Petri dish) over a $5mm^2$ paper grid. The number of grid lines that were crossed over periods of 1 min was scored from video-recordings.

Social Spacing assay: Flies were raised in bottles, collected on ice 3-4 days old after eclosion, and placed in vials (40 flies/vial) one day prior to the experiment. Two hours prior each experiment, the vials were habituated to the test room (25°C). We performed

the experiment as described by McNeil et. [2, 14]. In short, flies were collected from vials, and introduced a two-dimensional, vertical arena composed of a series of glass and acrylic pieces that form a hollow triangle. In this set up, the flies were forced into a tight group, as they all reached the top of the triangle through their phototaxis tendencies, and then they were let to explore the arena to finally settle at their preferred social distance. Digital images were collected after the flies reached a stable position (~ 30 minutes). The digital images were imported in the free NIH image analysis software, ImageJ (NIH, http://imagej.nih.gov/ij/), to determine the distance to the closest neighbour, which is their preferred social spacing. As indicated in the main text, we used either large chambers, with 40 flies (inner dimensions of 16.5 cm by 16.5 cm by 14.5 cm), or small chambers with 15 flies (inner dimensions of 10.2 cm by 8.9 cm by 8.9 cm), both types of chamber have the same density of flies (2.16 cm2per fly) [14].

dSO avoidance assay: dSO avoidance was performed as described in [15, 16]. In short, 70 control flies of mixed sex (CS) served as emitters of a stressed signal (dSO) by mechanical agitation on a vortex in a vial. Forty (40) flies of same sex were then tested in a T-maze (a two-arm choice apparatus) for their response to the dSO signal emitted by the agitated flies. The flies tested were given various amount of time (between 15 to 60 sec, as indicated in the figure legends) to choose whether to go into the vial that previously contained stressed (mechanically agitated) flies, or a fresh vial with no dSO present. In this assay, we quantified the efficiency at which responder flies are able to avoid the marking left by other flies that were stressed. A performance index of stress signal avoidance was thus calculated (the number of flies in the fresh vial minus the number of flies in the stressed vial divided by the summation of all the flies).

Statistical analyses

Analyses were performed using GraphPad Prism 7 as described below.

Social space data: Social space data have a skewed distribution, because although in most cases all the flies form one group, some flies in some experiments tend to be located away from that main group [2, 17]. The data are shown as box and whiskers, with the box representing the 1st quartile (25th percent) and the 3rd quartile (75th percent), the line in the box representing the median, and 90-10% whiskers (Figures 1, and supplemental Figures 1 and 3). We thus analyzed the whole distribution of the data, using non-parametric statistical tools for that purpose. When comparing two data set we used Mann-Whitney, when comparing more than two, we used Kruskall-Wallis, followed by a post-test (Dunn's multiple comparison – tables 1 and 2).

However, to compare the different treatments when obtained in different experiments we normalized the data by pooling the means of the means in each replicates of the experiments (Figure 2). To obtain reproducible means, we first aimed at limiting the skew in the distribution, by removing these few outlier flies when present, using a gentle using robust regression and outlier removal (ROUT) analysis, with the lowest rate of false discovery (Q of 0.1%). We then averaged the means of biological replicates. Those means were then normalized to the mean of the controls genotypes for each experiment (TH-Gal4/+ or Canton-S, as indicated in Table1). We plotted these normalized means and ordered the data based on expected changes in dopamine levels.

Locomotion and dSO avoidance: Locomotion is estimated by counting the number of lines crossed in 1min. These results are also represented using a box plot (with 90-10% whiskers) in Supplemental Figure 3. But the data distribution passed the D'Agostino and Pearson normality test, so they were analyzed using t-test to compare the groups (table 2). dSO avoidance data are presented with bar graphs of mean performance index +/- s.e.m. We used t-tests when comparing two data sets and one-way ANOVA when comparing three or more.

Supplemental Table 1:

Sup Experiments	Replicates and conditions	Statistical test performed
Sup Fig. 1 A Social spacing P in VMAT	Canton-S (Cs) n= 2, w-: p/+ n=2; w+: p/+ n=3, repeats of 35-40 males in large chambers. For Canton-S non-social 40 individual flies were pictured independently and the image flattened (Simon et al. 2012).	Kruskal-Wallis p<0.0001 Dunnet post-test multiple comparison a#b p<0.003, a#c p<0.03, a#d p<0.007
Sup Fig. 1 B VMAT-RNAi	Cs n=4; TH-Gal4/+ n=4; VMAT RNAi/+ n= 3; TH>VMAT RNAi n= 4 Independent repeats of 35-40 males in large chambers	Kruskal-Wallis p<0. 0.0001 Dunnet post-test multiple comparison a#b p<0.003, a#c p<0.0001, b#c p<0.009
Sup Fig 1 C VMAT- Dopplex RNAi	TH-Gal4/+ n=4; Doppelx VMAT RNAi/+ n=2; TH>Doppelx VMAT RNAi; TH-Gal4/+ n= 3 Independent repeats of 35-40 flies in large chambers	Kruskal-Wallis p<0.0001 Dunnet post-test multiple comparison a#b p<0.00001
Sup Fig 1 D UAS-VMAT	TH>VMAT cDNA /+ n=6; VMAT cDNA/+ n=2, TH-Gal4/+ n=2 Independent repeats of 35-40 males in large chambers	Kruskal-Wallis p<0.02 Dunnet post-test multiple comparison a#b p<0.02
Sup Fig. 2 A dSO avoidance	n=6 for all conditions, apart for Cs n=5	One way ANOVA p= 0.9
Sup Fig. 2 B	n=6 for all conditions	One way ANOVA p= 0.54
Sup Fig 2 C	n=6 for all conditions	One way ANOVA p= 0.88
Sup Fig 2 D	n=6 for all conditions, apart for Cs n=5	One way ANOVA p= 0.59
Sup Fig 3 A Social spacing day	n= 6 DTHg $FS^{\pm}$ and n=5 DTH-S; ple repeats of 30 females placed in large chambers	Mann-Whitney test p<0.02
Sup Fig 3 B Social spacing eve	n= 6 DTHg $FS^{\pm}$ and n=5 DTH-S; ple repeats of 30 females placed in large chambers	Mann-Whitney test p<0.69 F test of variances p<0.0001.
Sup Fig 3 C locomotion	n=2X30 females	t-test p<0.05
Sup Fig 3 D Social spacing	Canton-S Males n= 5, Females n=5; TH>catsup-RNAi Males n=3, Females n=3. Independent repeats of 20-40 flies in large chambers	Males: Kolmogorov-Smirnov test p<0.0001 Females: Kolmogorov-Smirnov test n.s.
Sup Fig 3 E Social spacing	Canton-S Males n=4, females n= 4; cat26/+ Males n=4, Females n=3; ple2/+ Males n=3, Females n=5 Independent repeats of 35-40 flies in large chambers	Kruskal-Wallis test for males, n.s. Kruskal-Wallis test for females, p <0.0001, Dunnet post-test multiple comparison to Cs, p <0.0001 for cat 26 and pale
Sup Fig. 3 F Social spacing	All conditions n=6 3 independent repeats with 2 internal replicates of 40 males	Kruskal-Wallis p<0.04 Dunnet post-test multiple comparison a#b p<0.03
Sup Fig. 3 G dSO avoidance	All conditions n=8 4 independent repeats with 2 internal replicates of 40 males	Kruskal-Wallis p<0.0001 Statistically different from Veh in a Wilcoxon-rank a#b: p<0.00015, a#c: p<0.0001.
Sup Fig 3 H locomotion	Veh n=13, 3-IT n=8 and L-DOPA n=9 males	t-test, each letter indicate groups statistically different to L-DOPA a: p<0.002, b: p<0.018).

Supplemental Figure legends:

Supplemental Figure 1: Increased social spacing in loss of function of VMAT males in large chambers. In these experiments, social spacing was performed on groups of 40 flies in large chambers (see text).
A: A heterozygous P element insertion in VMAT - L(2)SH0459 - tested both in red eyed (w+;p/+) and orange eyed (w-;p/+) background (both in doted box) shows the same decrease in social space, significantly different from that of the red eyed wild-type background control Canton-S (Cs – in dark grey box), and from the non-social situation (in open box – see text; n= 2 to 3 repeats of 40 males, $p < 0.0001$; a#b $p<0.003$, a#c $p<0.03$, a#d $p<0.007$).
B. Males with one UAS-VMAT RNAi under a TH-Gal 4 driver (n=4X40) show a trend to have increased social spacing compared to controls (CS n=4X40, TH-Gal/+ n=4X40, VMAT RNAi/+ n=3X40; $p < 0.0001$; letters indicate statically different groups a#b $p<0.003$, a#c $p<0.0001$, b#c $p<0.009$).
C. Males with 2 copies of UAS-RNAi VMAT-A (Dopplex VMAT RNAi) under TH-Gal4 driver (doted box, n=3X40) displays the same trend as the P-element loss of function: increased social spacing, compared to its controls (TH-Gal/+ n=2X40, Dopplex VMAT RNAi/+ n=2X40, all in dark grey boxes). But it is not different from non-social flies (open box). $p < 0.0001$; a#b $p<0.00001$.
D: Males overexpressing UAS-VMAT under a TH-Gal4 driver (n=6X40) have a slight but statistically significant decrease in social space compared to their controls (UAS-VMAT/+ and TH-Gal4/+, each n=2X40) in dark grey ($p<0.02$, a#b $p<0.02$).

Supplemental Figure 2: Normal dSO avoidance for mutants with a p-element insertion in *VMAT*, regardless of sex or decision time. Social avoidance of Cs emitter flies by mixed gender responders of the indicated sexes and genotypes was tested under different decision times, at 60 sec (**A-C**) and 15 sec (**D**). **A:** Females, **B:** males, **C:** mixed sex at 60 sec. **D:** 15 sec decision time (Cs, dark grey; VMAT p-element insertion (L(2)SH0459) mutant in red eyed w+;p/+, and orange eyed w-;p/+, background - both in dotted grey. n=6 for all conditions, apart for males Cs at 15 and 60 sec. n=5).

Supplemental Figure 3: Altered social spacing in biosynthesis genetic and pharmacologic manipulation of Dopamine synthesis. Social spacing during the day **(A)** and evening **(B)**, and locomotion **(C)** of dopamine-deficient flies DTHg FS±; ple. **D-E:** Social spacing of Males (M) and Females (F) overexpressing catsup-RNAi under a TH-Gal4 driver (**D**), or mutant for catsup (Cat^{26}/+) and pale (ple^{2}/+) (E). **F:** Social spacing, **G:** dSO avoidance and **H:** locomotion, of male flies fed 3-IT and L-DOPA. Letters indicate groups that are statistically different in multiple comparisons (see **supplemental table**).

Reference for supplemental material:

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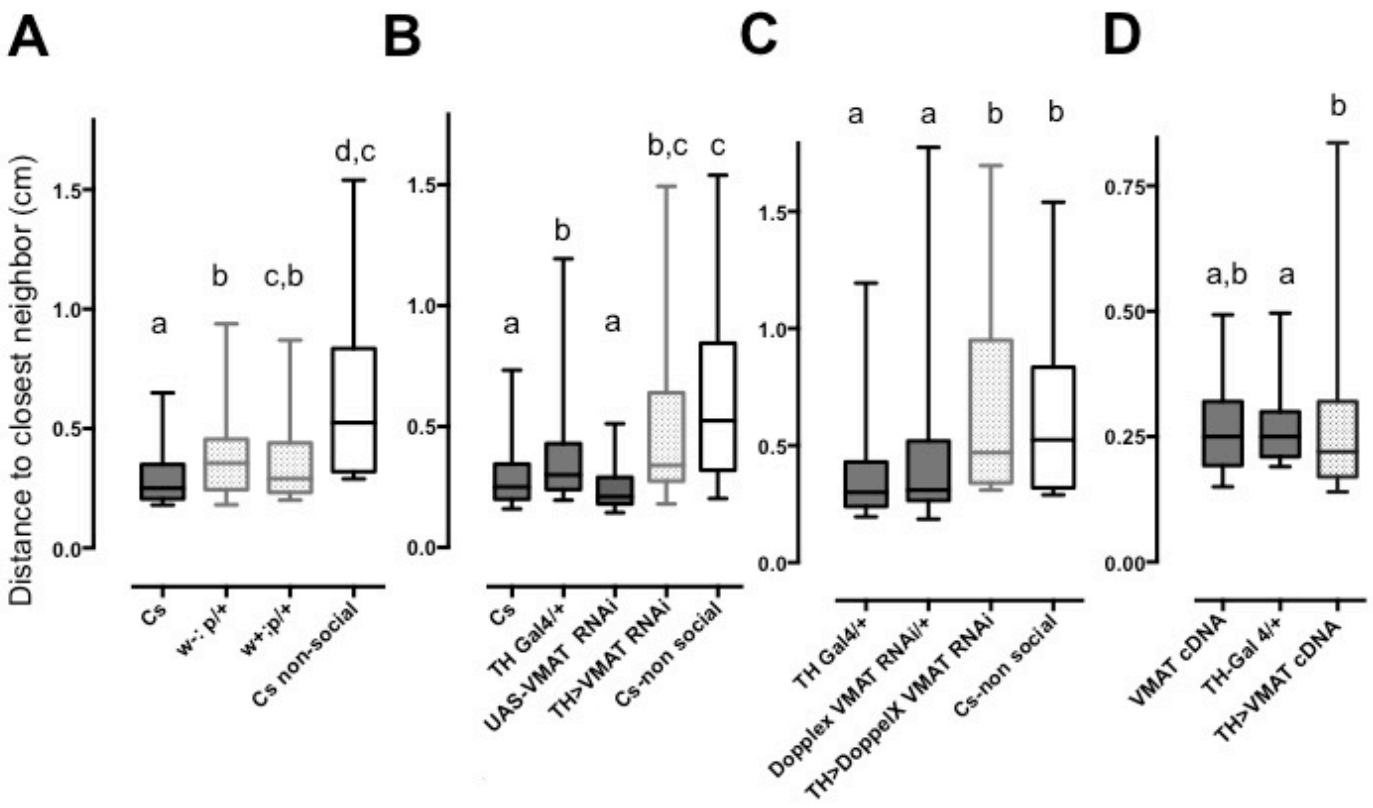
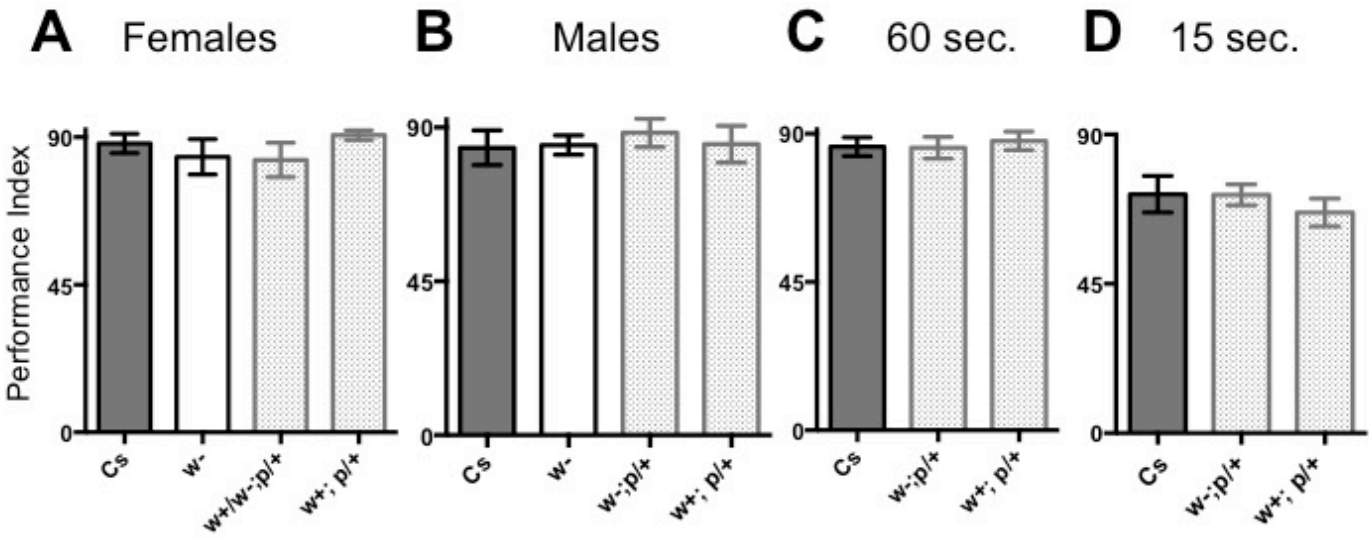
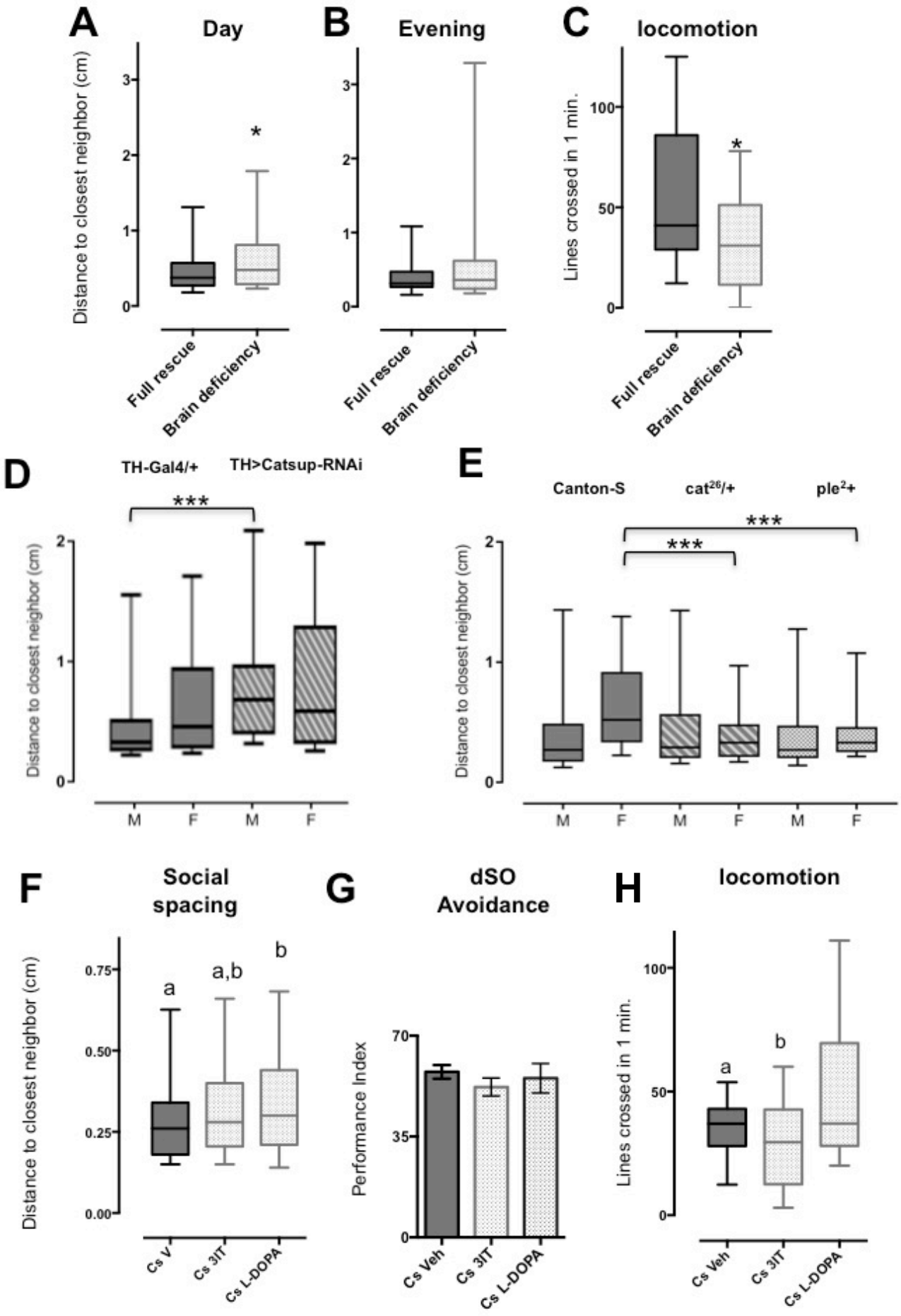


Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3