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Supplemental Data

Two Different Forms of Arousal in Drosophila Are Oppositely Regulated by the Dopamine D1 Receptor Ortholog DopR via Distinct Neural Circuits

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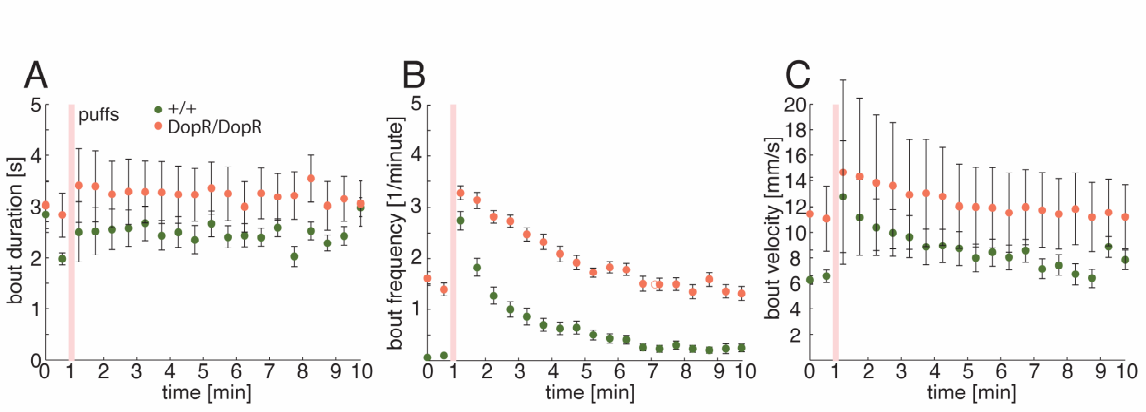
Supplemental Figure S1

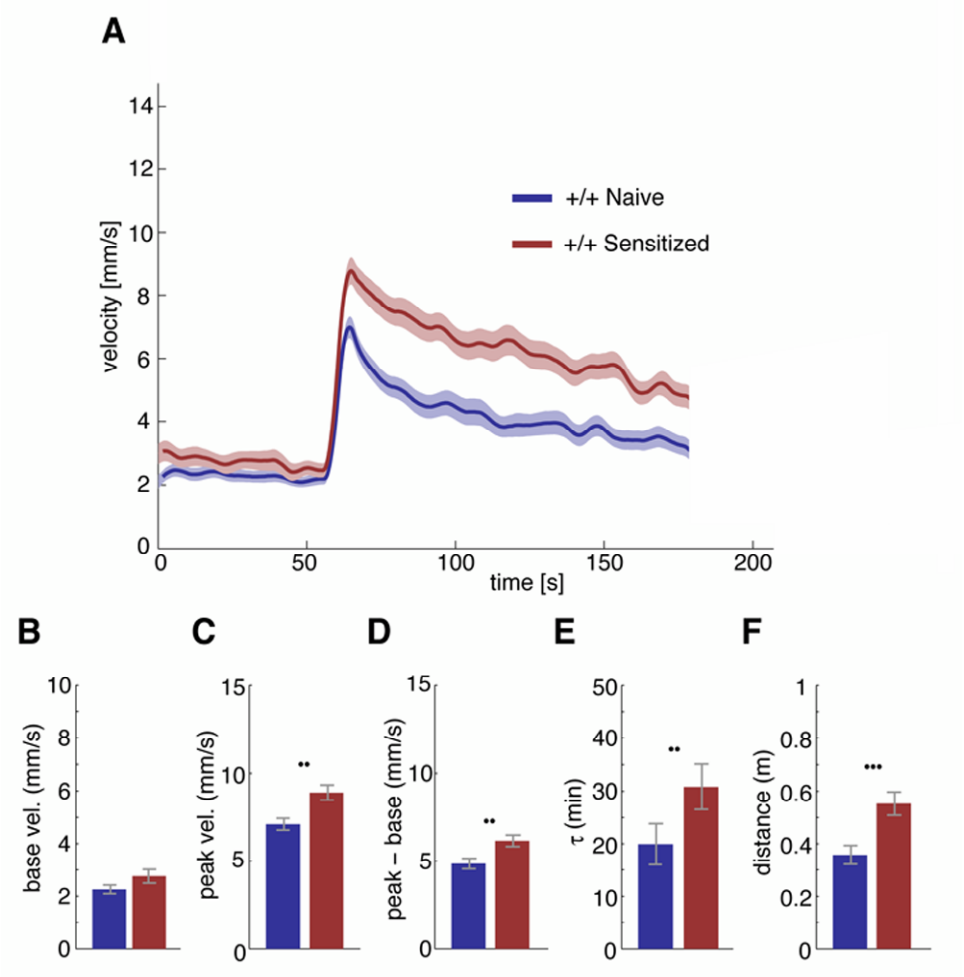
Bout structure analysis of wild-type and DopR/DopR flies



2-puff trials were analyzed as in Figure 1D. Vertical bar indicates period when puffs were delivered. (A) bout duration. (B) bout frequency. (C) bout velocity. Error bars indicate S.E.M.

Supplemental Figure S2

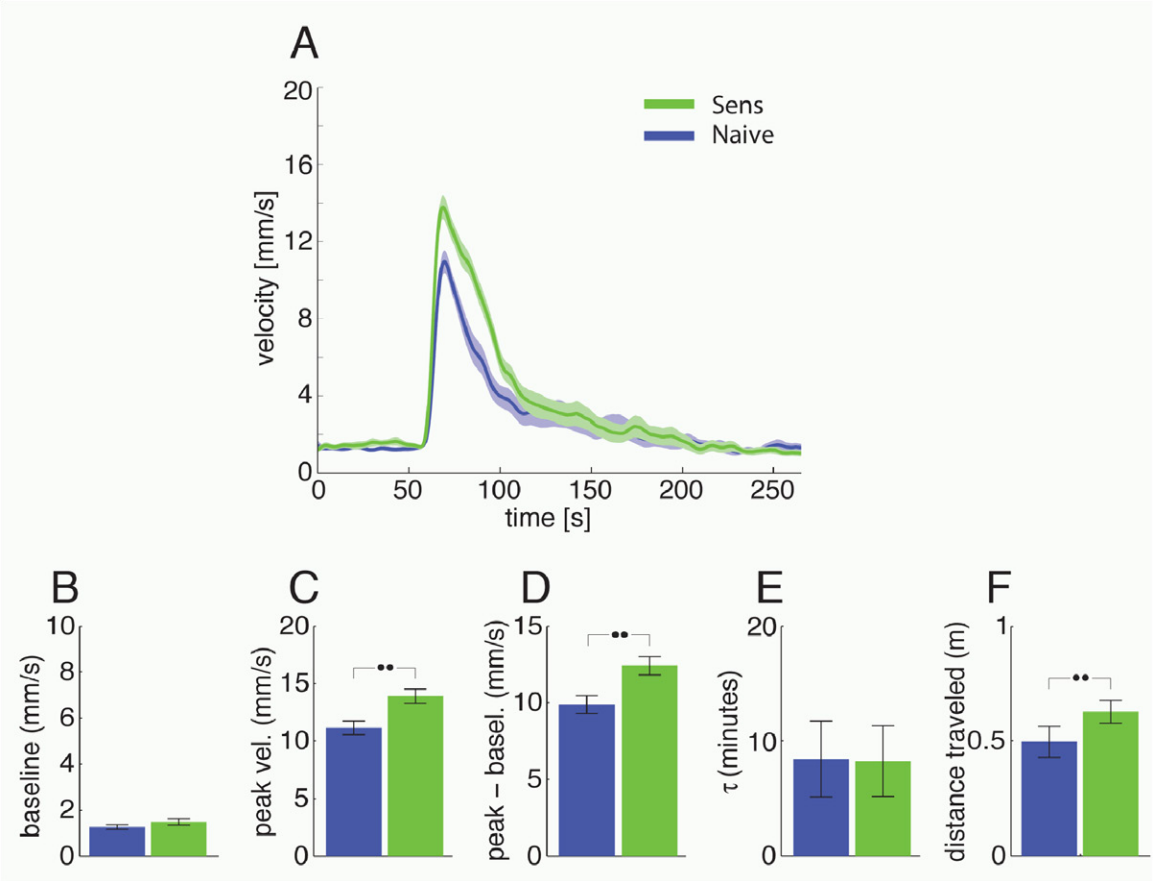
Sensitization of stress-induced hyperactivity



(A) Response to single-puffs of naïve flies, or flies previously exposed to 6 successive puffs and allowed to settle for 10 minutes prior to a single-puff trial ("Sens"). (B-F) Parameters from experiment in (A). •••, p<.001.

Supplemental Figure S3

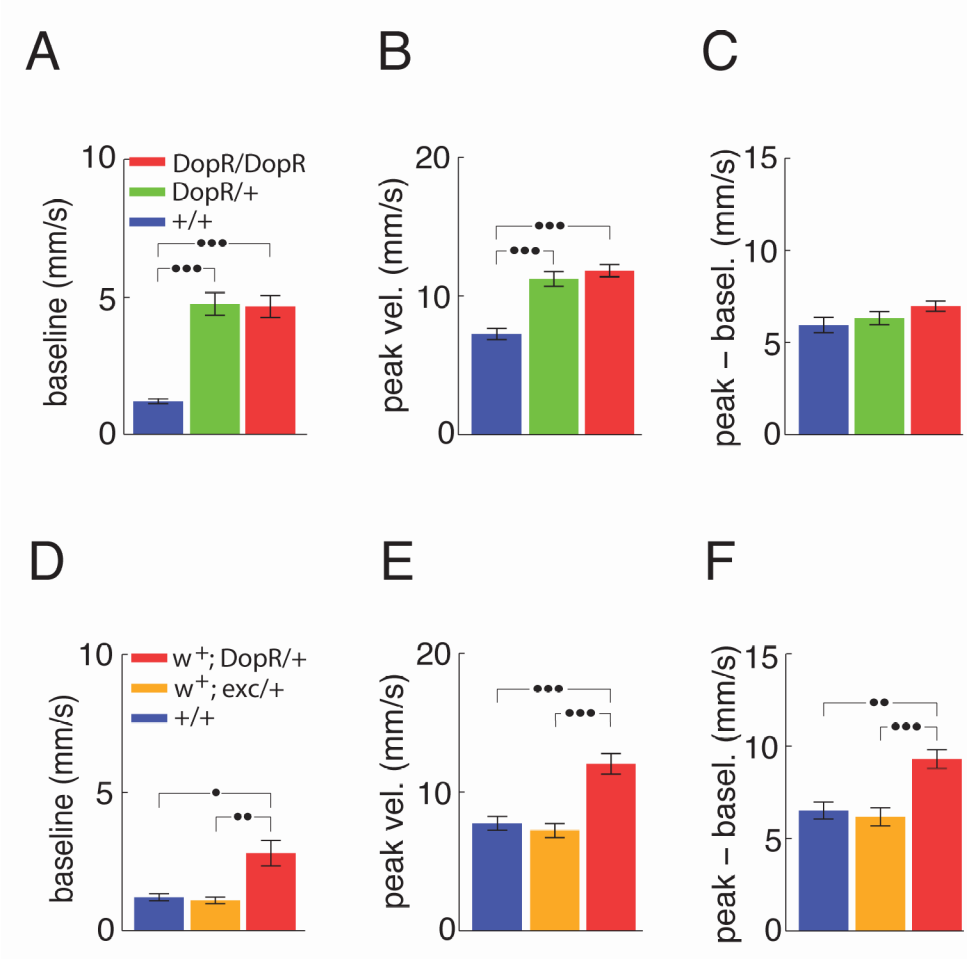
Mechanical startle facilitates sensitization of olfactory startle responses



(A) Olfactory startle curves for either naïve CS flies presented with MCH for 5s (blue), or CS flies that had been sensitized by presentation of 6 air puffs, followed by a 10 min. recovery period, prior to presentation of the MCH stimulus (green). (B-F) Parameter plots from data in (A). •, p<.051, ••, p<.01. Note that baseline velocity is not statistically different, and sensitized animals are not hyperactive prior to MCH presentation. Sensitized animals display significant differences in peak velocity (B) and distance travelled (F) in behavioral response to MCH presentation. MCH odorant is made as a 50% dilution in paraffin oil, delivered through the tubing of the Puff-o-Mat at 1 psi.

Supplemental Figure S4

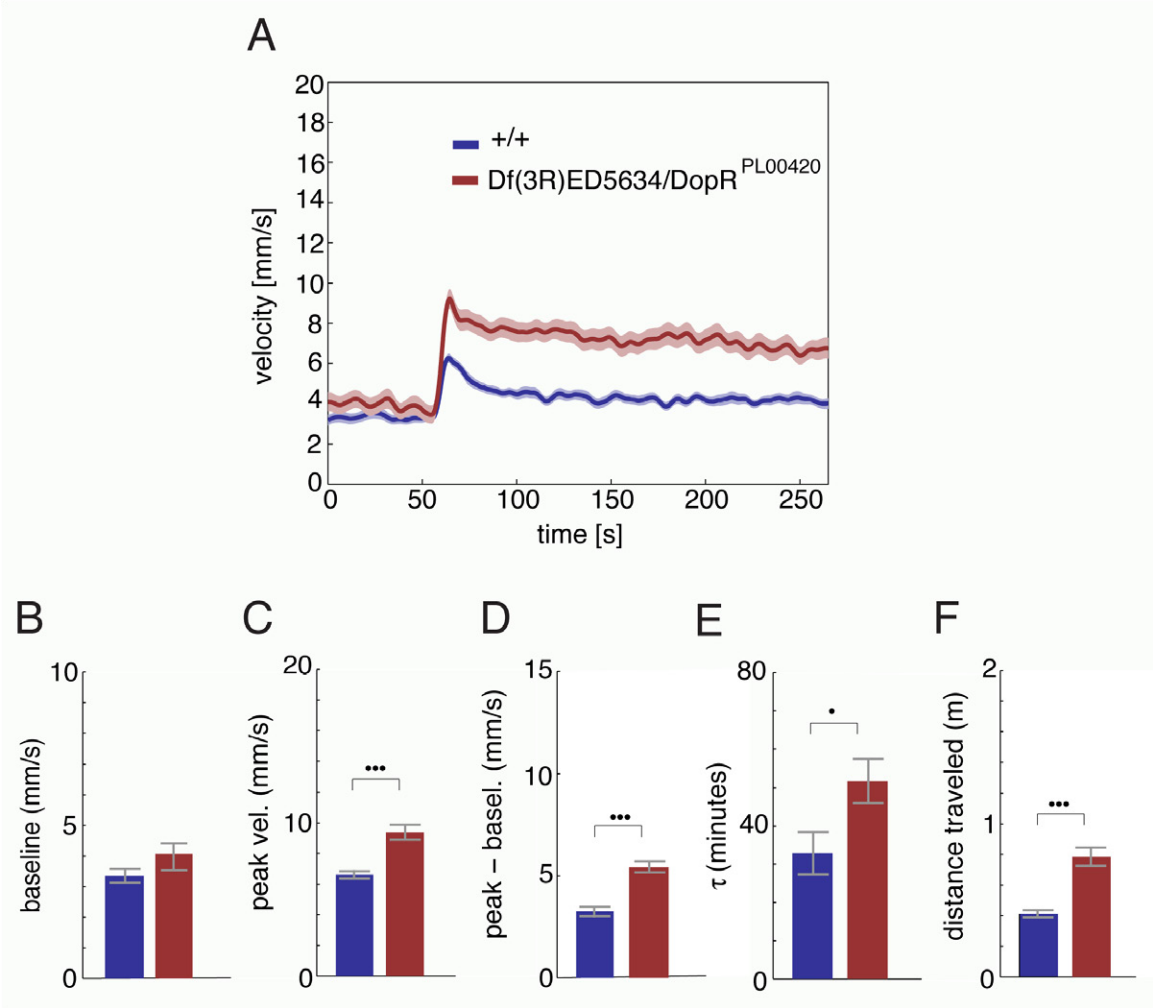
Individual Behavioral Parameters for DopR mutants and excision lines



(A-C) Behavioral parameters calculated from the data in Figure 2B. (A) Baseline velocity. (B) Peak velocity. (C) Peak velocity minus baseline velocity. (D-F) Behavioral parameters from the data in Figure 2E. (D) Baseline velocity. (E) Peak velocity. (F) Peak velocity minus baseline velocity. •, p<.017; ••, p<.003; •••, p<.0003; Kruskal-Wallis ANOVA followed by Mann-Whitney U-test with Bonferroni correction for multiple comparisons.

Supplemental Figure S5

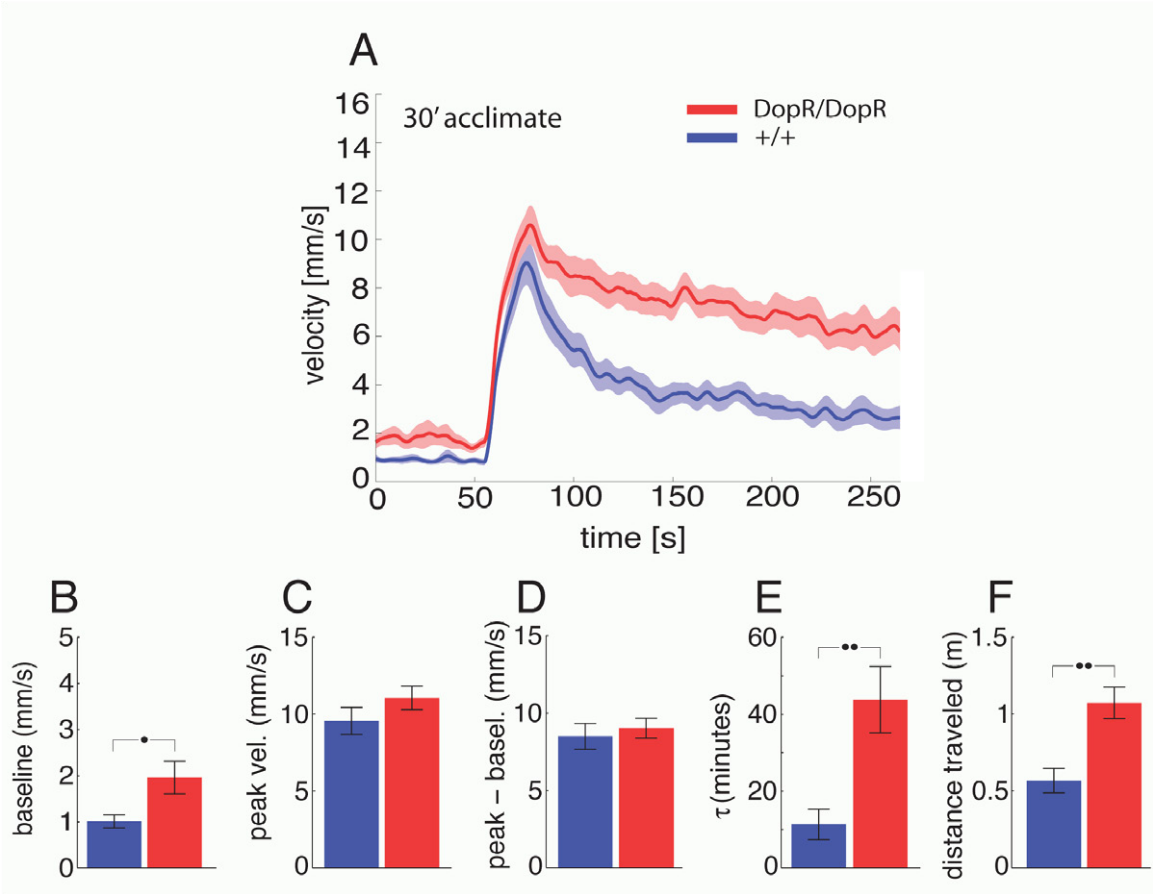
***Df(3R)ED5634/DopR^{PL00420}* flies display exaggerated post-puff responses after increased acclimation time in the puff-o-mat**



(A) 1-puff trials of flies of the indicated genotypes. Puff intensity reduced to 10 psi to highlight the difference between mutant and wildtype genotypes. *Df(3R)ED5634* removes the entire DopR locus. *DopR^{PL00420}* is an insertional piggyBac allele in the first intron. Q-RTPCR analysis indicated that *DopR^{PL00420}/Df(3R)ED5634* flies exhibit an 80% reduction in *DopR* transcript levels. (B-F) Parameter plots from the data in (A). ˙, p<.05 ; ¨, p<.01 ; ˙˙˙, p<.001. After 40 minutes of acclimation in the puff-o-mat tubes, the baseline velocities are statistically identical (B), but the post-puff behavior of the *Df/DopR^{PL00420}* mutant flies is significantly different from wildtype controls for all other parameters (C-F). n = 36 tubes per genotype. *DopR^{PL00420}*/+ showed no phenotypic difference from +/+ in any of the parameters, while Df/+ showed a significant increase in peak-baseline velocity and integrated velocity, and a trend to an increase in tau.

Supplemental Figure S6

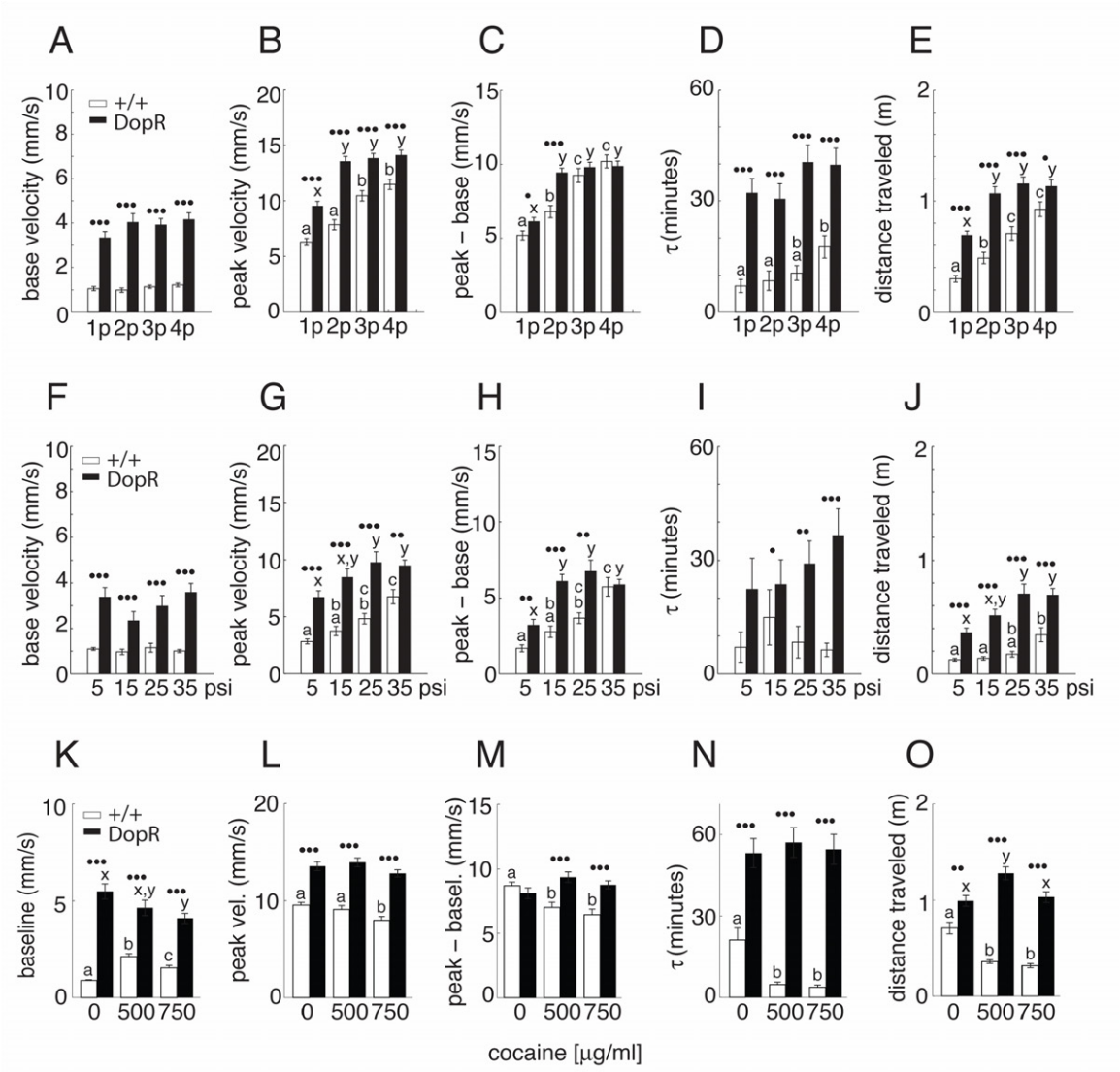
***DopR/DopR* flies still show exaggerated post-puff responses after increased acclimatization time in the puff-o-mat**



(A) Puff-o-mat response curves (2-puff trials) from wild-type and *DopR/DopR* flies allowed to acclimatize for 30 minutes in the puff-o-mat. (B-F) Parameter plots from data in (A). •, p<.01, ••, p<.01.

Supplemental Figure S7

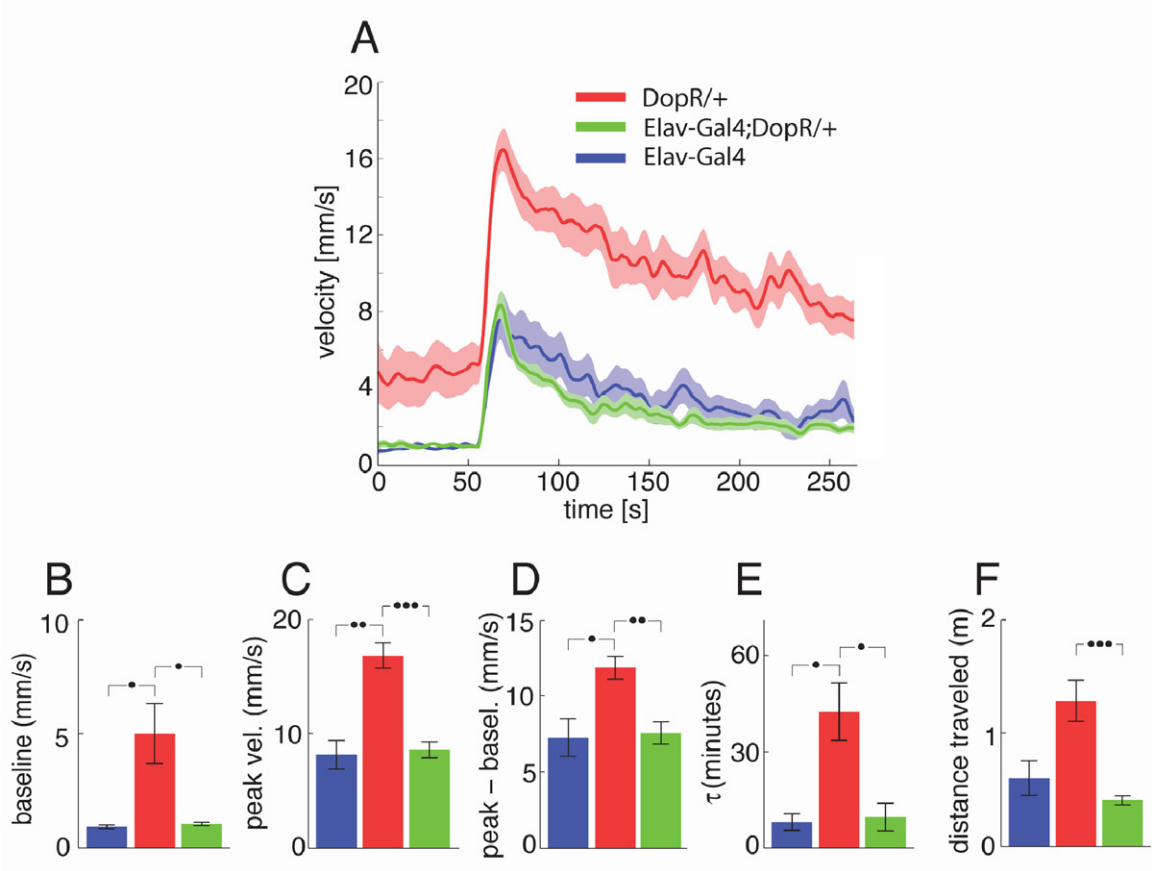
The *DopR* mutation increases sensitivity to puff number and puff intensity



(A-E) Analysis of behavioral parameters from the data in Figure 3A, B. White bars, CS (+/+) flies; black bars, *DopR/DopR* flies. (F-J) Analysis of behavioral parameters from the data in Figure 3C, D. (K-O) Analysis of behavioral parameters from the data in Figure 3E, F. Note the suppression of τ (ν) and distance traveled (O) by cocaine in CS flies (white bars), and the lack of an effect of cocaine in *DopR/DopR* flies (black bars).Letters (a-c or x-z) denote significant differences ($p<.017$) within genotypes, •'s indicate significant differences between genotypes (black vs. white bars). •••, $p<.001$, ••, $p<.01$, •, $p<.05$

Supplemental Figure S8

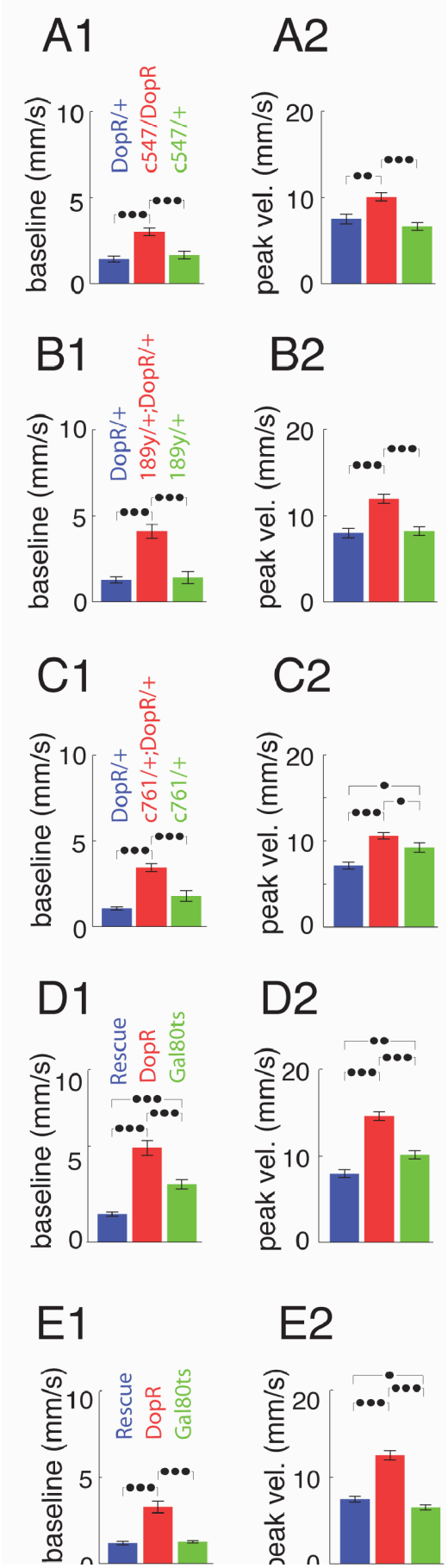
Rescue of *DopR/+* phenotype by Elav-Gal4



(A) 2-puff trials of flies of the indicated genotype. Elav-Gal4 was backcrossed 6 generations into the same CS background as the *DopR*^{f02676} allele. (B-F) parameter plots from the data in (A). •, p<.05; ••, p<.01; •••, p<.001. Note that *Elav-Gal4; DopR/+* flies are statistically indistinguishable from control (*Elav-Gal4; +/+*) flies.

Supplemental Figure S9

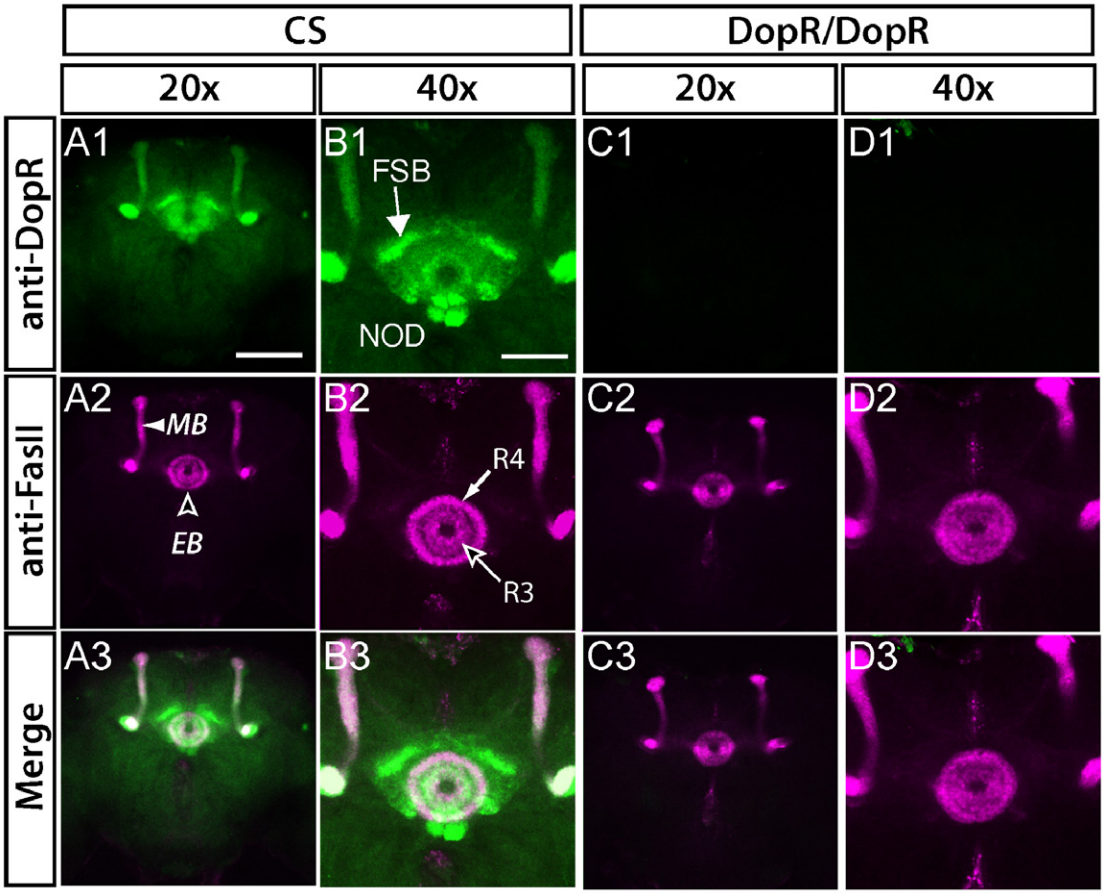
Neuroanatomical mapping of DopR function



(A1-E2) Additional Parametric analysis of the data in (Figure 5A-E), respectively. "•••," p<.0003, "••," p<.003, "•," p<.017. (A1-C2) Selective rescue of the ReSH phenotype by restoration of DopR function in the Ellipsoid Body by different Gal4 lines. 2-puff trials applied to *DopR/+* heterozygotes (red, "*DopR"*), *Gal4*/+ controls (blue), and *Gal4*/+; DopR/+ double heterozygotes (green). (D1-E2) Conditional rescue of *DopR* in adult flies using *c547-Gal4* and *tubulin-Gal80^{ts}*. "*DopR," DopR/DopR* mutants; "Rescue," *c547-Gal4, DopR/DopR*; "*Gal80ts*,"*tub-Gal80ts; c547-Gal4, DopR/DopR*. Flies were raised at 18°C and tested at either 18° (D) or 30° (E). (A1, B1, C1, D1, E1) Comparison of baseline velocity. (A2, B2, C2, D2, E2) Comparison of peak velocity.

Supplemental Figure S10

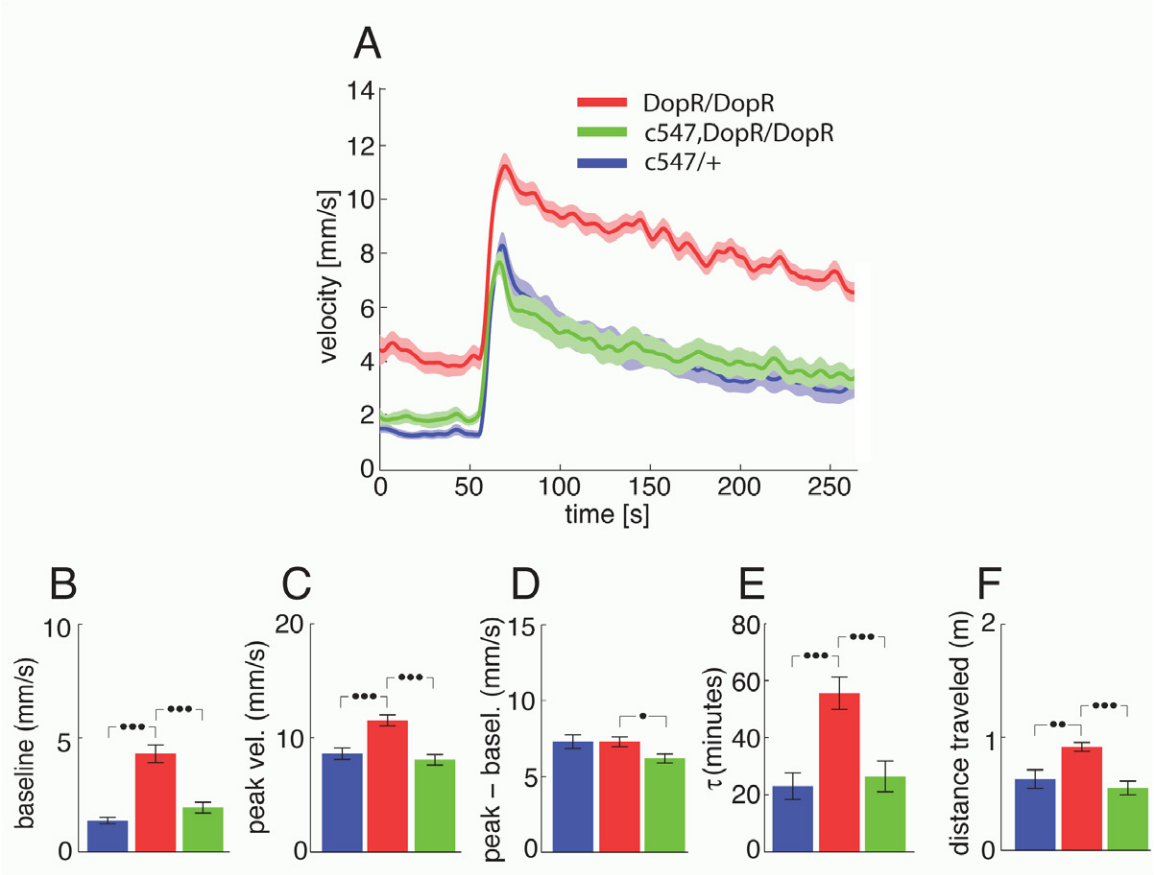
Specificity of anti-DopR antibody staining in the Central Complex



Single confocal sections of whole mount brains double-labeled with antibody to DopR (green) and FasII (magenta). MB, Mushroom Body; FSB, fan-shaped body; NOD, noduli; EB, ellipsoid body. Panels A1 and C1 were imaged at the same gain setting, as were panels B1 and D1. (A3, B3, C3 and D3) represent merged images of the single channels shown in (A1-2), (B1-2), (C1-2) and (D1-2), respectively. Scale bar in all 20X panels, 80 μm; in 40X panels, 40 μm.

Supplemental Figure S11

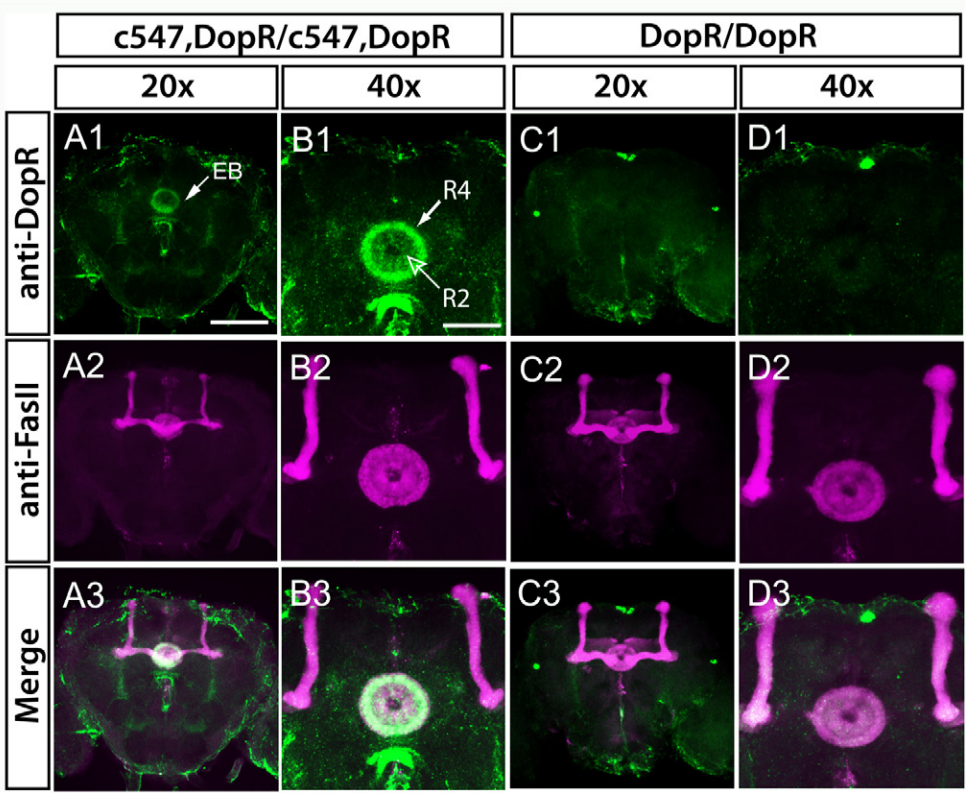
c547 rescue of *DopR/DopR* homozygous flies



The enhancer trap line c547 was recombined onto the *DopR*^{f02676} chromosome, to generate *c547,DopR/TM6* flies, from which the parental stock the indicated genotypes were generated. (A) 2-puff response curves of the indicated genotypes. (B-F) parameter plots from curves in (A). p values are defined as in Supplemental Figure S5.

Supplemental Figure S12

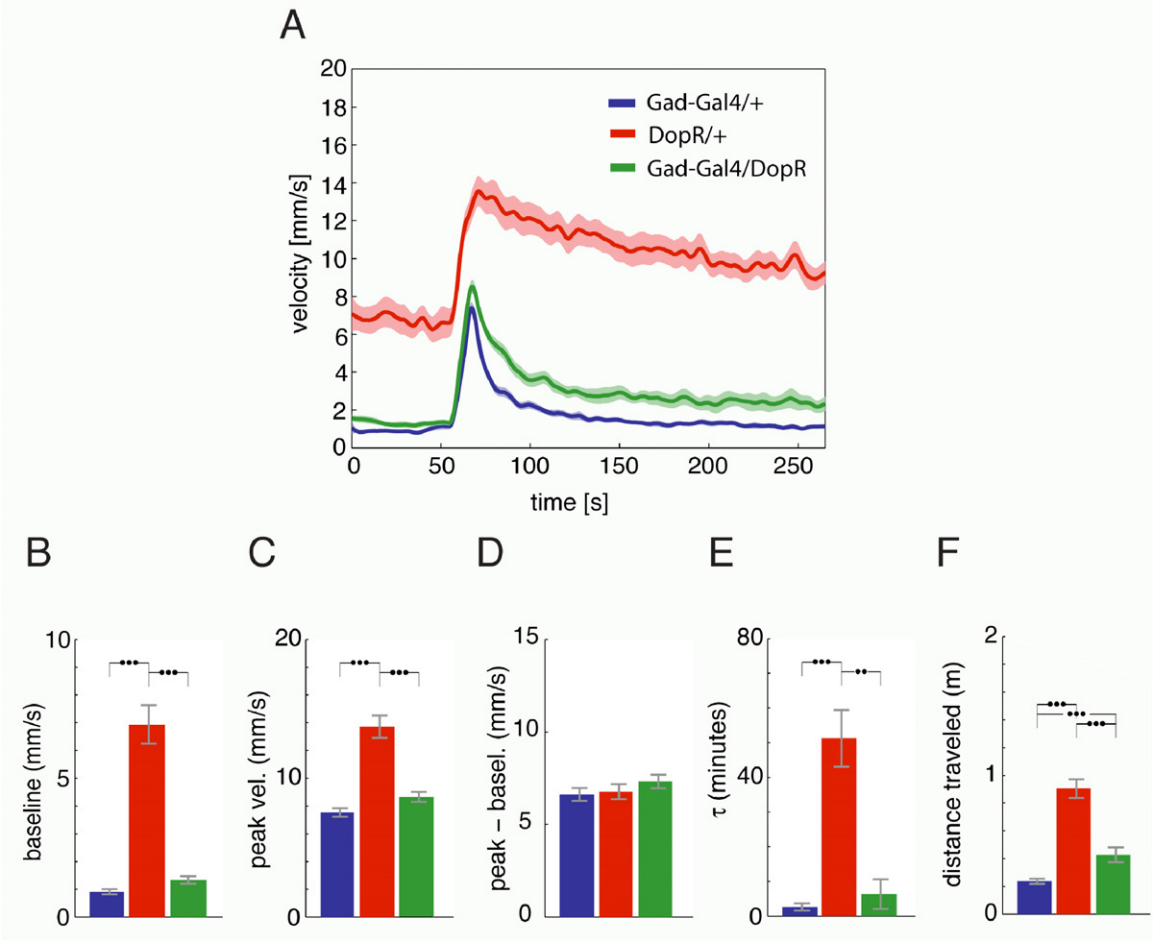
Restoration of DopR expression to the EB by line c547



(A-D) The panels illustrate z-stacks from brains of the indicated 20x and 40x images of *c547,DopR* homozygotes. Consistent with observed in the R2 and R4m regions of the EB (arrows B1) when DopR is driven from the UAS element in the *DopR*^{f02676} allele in the strong hypomorph background. (C1-D3) 20x and 40x images of *DopR* homozygotes. Scale bars in A1 and B1 correspond to 40um and 80um respectively. (A3, B3, C3 and D3) represent merged images of the single channels shown in (A1-2), (B1-2), (C1-2) and (D1-2), respectively. Scale bar in all 20X panels, 80 μm; in 40X panels, 40 μm.

Supplemental Figure S13

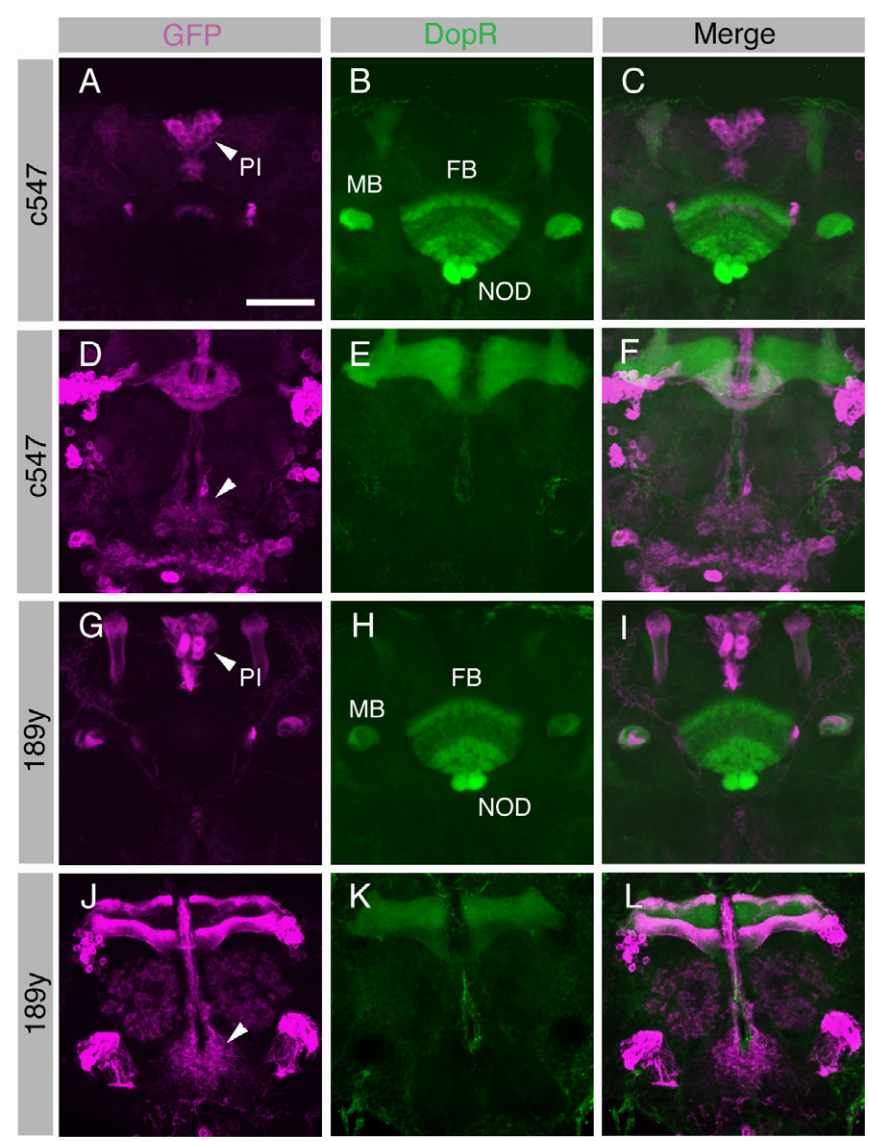
Rescue of DopR phenotype by GAD-Gal4



Expression of DopR in GABAergic neurons results in significant rescue of all parameters. (A) 2-puff trials of flies of the indicated genotypes. (B-F) Parameter plots from the data in (A).¨, p<.01 ; ¨¨, p<.001. n = 20 tubes per genotype.

Supplemental Figure S14

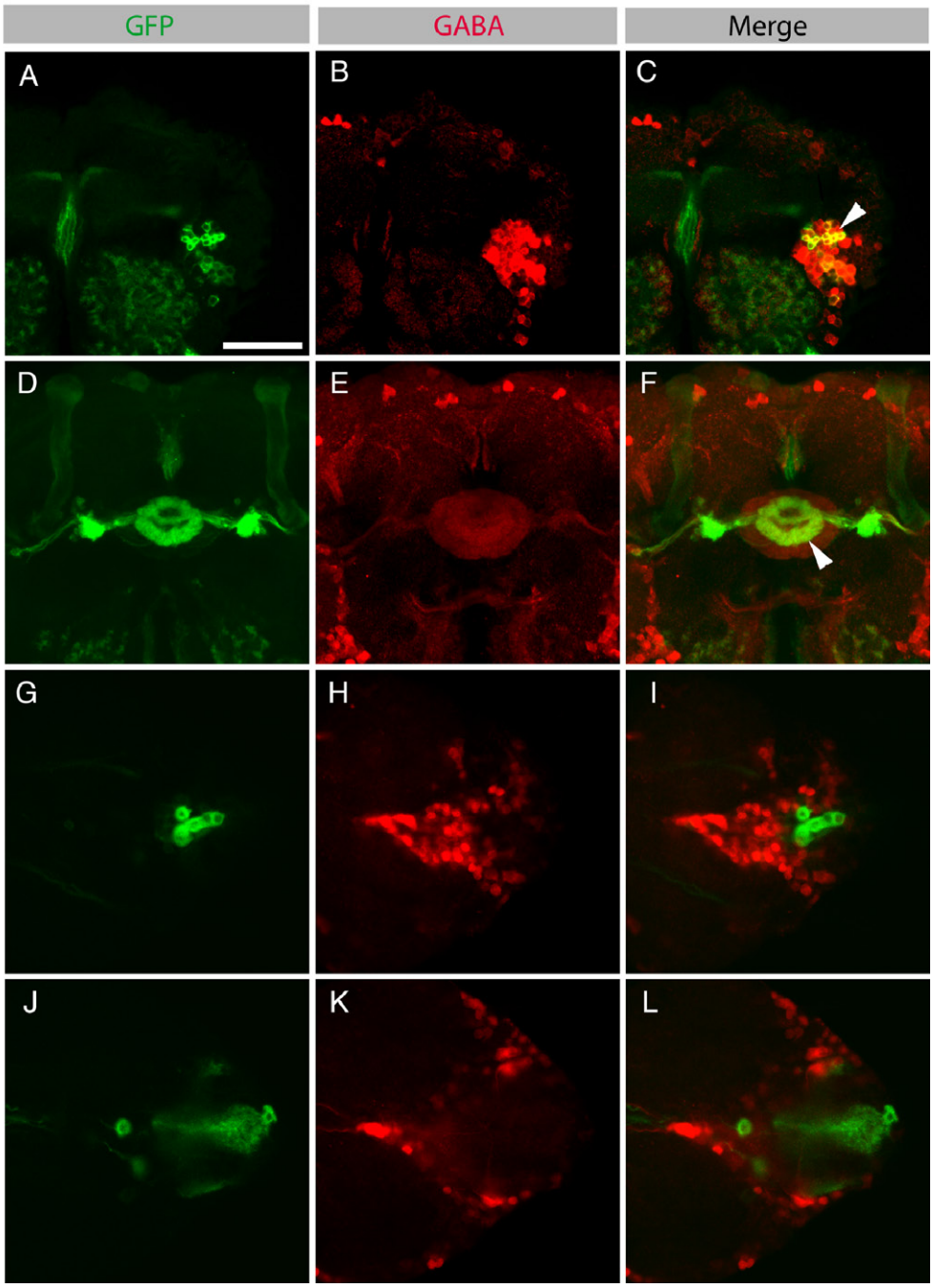
DopR is not expressed in the PI fibers



(A-F) UAS-mCD8-GFP/+ ; c547-gal4/+ wholemount brains double-labeled with antibodies to GFP (magenta) and DopR (green). (A) arrowhead indicates GFP expression in Pars Intercerebralis cell bodies. (B) Expression of DopR protein observed in the Mushroom Bodies and Central Complex. (C) Merged image shows absence of DopR expression in PI cells. (D) Arrowhead indicates GFP^{+} neuronal fibers from the PI cell bodies in (A). (E) Expression of DopR observed in Mushroom Bodies. (F) Merged image shows absence of DopR expression in PI fibers. (G-L) 189y-gal4/UAS-mCD8-GFP wholemount brains double-labeled with antibodies to GFP (magenta) and DopR (green). (G) arrowhead indicates GFP expression in Pars Intercerebralis cell bodies. (H) Expression of DopR protein observed in the Mushroom Bodies and Central Complex. (I) Merged image shows absence of DopR expression in PI cells. (J) Arrowhead indicates GFP^{+} neuronal fibers from the PI cell bodies in (G). (K) Expression of DopR observed in Mushroom Bodies. (L) Merged image shows absence of DopR expression in PI fibers. Scale Bar = 40um.

Supplemental Figure S15

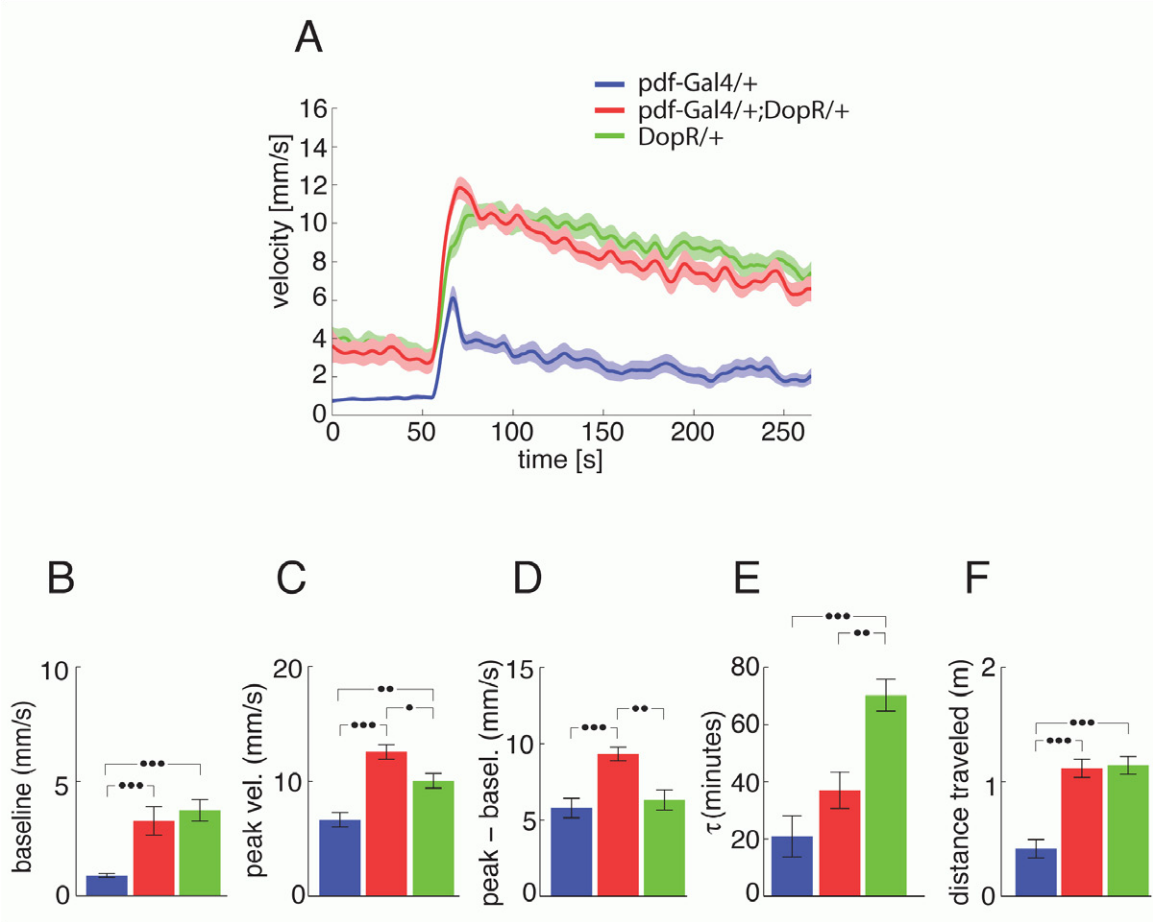
189y ellipsoid body neurons are GABAergic



(A-L) 40x Confocal images of wholemount 189y-Gal4/UAS-mCD8-GFP brains double-labeled with antibodies to mCD8 (green) and GABA (red). (A-C) Single 2um optical section of the wholemount brain. Immunolocalization of 189y-gal4^{+} cell bodies in the anterior brain innervating the ellipsoid body. Arrowhead in C indicates overlap of GFP cells with GABA. (D-F) Compressed 22um confocal z-stack of the ellipsoid body. GABAergic innervation of the ellipsoid body (red) is observed throughout all layers. Arrowhead indicates region of overlap between 189y and GABA in the R3 region. (G-L) Single 2um optical sections of the abdominal ganglion. Anterior is left. 189y-Gal4 displays limited expression in the thoracic/abdominal ganglion (green), and no overlap with GABA (red). (G-I) Ventral layer. (J-L) Dorsal layer. Scale Bar = 40um.

Supplemental Figure S16

Phenotype of PDF-Gal4; DopR/+ flies



(A) *pdf-Gal4* does not rescue the stress-induced hyperactivity phenotype of *DopR/+* flies (n=20 tubes per genotype). (B-F) represent the parameter plots for the curves represented in (A). •, p<.017; ••, p<.003; •••, p<.0003.

Supplemental Methods

Molecular Biology

The Applied Biosystems 7300 Real Time PCR System was used to quantify DopR mRNA levels, according to established guidelines (Pfaffl, 2001). Taqman TAMRA probes were used for DopR (TGCCTGGCATCTACACG-exon3) and a control reference probe from the b-Catenin/Armadillo gene (ACGTGGTCACCTGTGCCGC). 25 adult Drosophila heads of each genotype were used per replicate. Data represents the average values of 3 replicate experiments/genotype. mRNA was extracted and made into cDNA using the Absolutely RNA miniprep kit (Stratagene) and iScript cDNA synthesis kit (BioRad).

Behavioral Assays

Our observation chamber for mechanical startle assays (Puff-o-Mat) was a gift from the Heberlein lab and is described in detail in Wolf et al. (2002). A Sony handycam DCR-TRV18 was used for all film acquisition in concert with iMovie (Apple). The chamber is raised approximately 8" above the benchtop to allow for backlighting (Dynalume Halogen lamps-Scientific Instruments Inc.) through a white opaque plastic diffuser plate (McMaster-Carr) underneath the Puff-o-Mat. Medical grade breathable air was used for all air puffs. An Ingersoll-Rand air regulator was utilized for precise control of PSI for experiments which modulated puff intensity (5psi - 35psi). Solenoid valves (Parker/Hannifin) under Matlab control program from a triggering computer controlled the timing and duration of all air puffs.

Cocaine (Sigma-C5776) was solubilized and added to agar food for both mechanical startle and circadian monitor experiments. The solid agar food recipe is a modified recipe from the Benzer lab at Caltech (Ja et al., 2007): 1% Bacto-Agar, 5% Sucrose, 1% Yeast + either blank, 500ug/ml or 750ug/ml final concentration of cocaine. For mechanical startle assays, 10 flies were held in 10ml Falcon tubes (DNA miniprep tubes with a cotton plug) with 1ml of cocaine food at the bottom for the 48 hr incubation, eating ad libitum. For circadian trials, individual flies ate cocaine food ad libitum from solid agar food plugs (approximately 10mm at one end) in the standard Trikinetics individual glass tubes.

For all circadian monitor experiments, males (1-2 days old) were collected and the flies were acclimated post collection for 2 days on a 12-12 cycle prior to transfer into monitors. Activity data was collected in 5 minute bins. All measures reported were taken from the third day/night after acclimation within monitors. The longest consecutive sleep bout over the 12 hr subjective night phase was calculated per individual and averaged across all individuals for the average longest bout. Average sleep bout duration was calculated as the average total sleep for a given genotype divided by average bout number.

Learning and memory experiments were performed using 1% MCH or OCT in mineral oil as associative odorants. Approximately 60-80 flies were trained in a T-maze apparatus by receiving electric shocks applied across a copper grid at 60 V (direct current) for 1 min 30 sec in the presence of an

associative odor (Similar shock conditions to Suh GS et al. 2004). Three training sessions were performed prior to testing. A training session consisted of 30 s blank air, 1min30s shock-associative odor, 30s blank air, 1min30s non-associative odor. Animals were left to recover for 5 minutes after the last training session prior to moving them into the T-maze elevator and then held for 30s before allowing the animals to choose between the tubes containing the associative and non-associative odor (odorants were filtered into the choice tubes by positive pressure that is equalized for both tubes and passes through the t-maze). Flies were allowed 1 min to choose and then the animals were collected and counted. Performance Index was calculated as the difference between animals making the correct and incorrect choices, divided by the total number of animals. A single experimental n is the average of PI values for two counterbalanced odorant experiments for the same genotype (n=4 or 8 trials for each genotype reported).

Genetics

Excisions of the *DopR*^{f02676} piggyBac allele were generated by crossing the homozygous *DopR*^{f02676} flies to *w*^{1118} *; CyO, [pTub-Pbac]/wg*^{Sp}. Virgin females were collected and crossed to *w*^{1118}*; TM6* males. White eyed, excision males balanced over TM6 were then backcrossed in the CS background for 5 generations. *DopR*^{f0267} , c547-Gal4 recombinants were generated by recombination crosses and screened/verified by PCR, as the dark eye color of c547/+ flies made recombinants difficult to score by this marker.

Immunohistochemistry

Adult Brains were dissected from 3-4 day old flies and fixed in 2% Paraformadehyde in PBS for 2 hours on ice. Brains were washed in PBT (0.05% Triton X-100 in PBS). Primary antibodies were incubated overnight at 4^{o} for single label GFP experiments and for 36-48 hours for anti-DopR double-labeling experiments. Brains were washed in PBT (10 exchanges) in one hour prior to secondary incubations. Secondary incubations were performed overnight at 4 degrees, followed by extensive washing and mounting in Vectashield (Vector laboratories). The following primary antibodies were used: affinity purified anti-DopR 1:100 (Guinea Pig-Pocono Rabbit Farm, Canadensis PA), anti-FasII ID4 1:50(Mouse-DSHB Iowa Hybridoma Bank), anti-GFP 1:500 (Rabbit-Molecular Probes), anti-TH (Mouse-Immunostar) 1:1000. Alexa-Fluor secondary antibodies from Molecular Probes (wavelengths 488, 568, 633) were used.

Automated Tracker Velocity/Decay Analysis

The software tracks flies viewed by a top-view video camera. Using a Matlab image analysis program, flies are first detected/identified in each frame and then tracked to obtain trajectories. A detection is defined as an image region that differs from the background, and whose size matches that of a fly. Tracking is performed by position-based nearest-neighbor matching of a detected fly across consecutive frames. See Supplemental Methods for description of curve-fitting and data analysis.

All parameters analyzed were first calculated as an average across the 10 individual flies within each tube, and then averaged across tubes to generate the final curves. For each frame the average velocity of the fly population was

calculated. The velocity curve was smoothed from acquired video at 10 frames/sec (or 100ms) to 5s by applying a moving average window. The baseline velocity n_{base} was determined by taking the median of all velocity values prior to the first puff. The peak velocity ν_{peak} was defined to be the maximum fly velocity during the puff period, with the periods of the actual airpuff time period (200ms) deleted to ensure accurate tracking of behavioral responses in the post-puff periods. The exponential decay function fit to the part of the velocity curve beginning at ν_{peak} is given in Equation 1.

Eq. 1:

$$\nu(t) = M * e^{-(t/\tau)^{\alpha}} + \nu_{baseline}$$

where $M = (\nu_{peak}-\nu_{baseline})$

with time t, free decay parameter t, and α= 0.5. The fitting was numerically solved to retrieve τ. The distance traveled was determined by normalizing the curves by subtracting the median base velocity, and then integrating the velocity curve beginning at ν_{peak}. This statistical normalization was performed to ensure that post-puff parameters were measured independent of basal velocity.

The measurements of n tubes of the same genotype were taken to calculate mean and standard error of the mean (SEM) for each parameter ($\nu_{baseline}$, ν_{peak}, $[\nu_{peak}-\nu_{baseline}]$, τ, distance traveled).

Automated Tracker Acceleration Analysis

Zero velocity is assumed for the flies at puff time t_{puff}. The acceleration at a time point t of the flies due to a puff stimulus within the inter-puff interval is therefore: (velocity(t) - 0) / (t - t_{puff}). The maximum acceleration was chosen from the calculated inter-puff accelerations.

Automated Walking Bout Analysis

We analyzed walking bout parameters over time for each individual fly (Wolf et al., 2002). The minimum walking velocity was defined to be 2 mm/s. Period with velocities below 2 mm/s were defined as 'stop bouts'. Stop bouts with gaps of no more than 5 frames were merged. The periods between stop bouts were defined as 'walking bouts'. For each detected walking bout we measured the velocity (mean), duration and start time. Bout start times were binned into 30 seconds bins. For each time bin, histograms of walking bout duration, occurrence frequency (1/minute) and velocity were determined and a mean and SEM value calculated.

Statistics

A nonparametric one-way ANOVA (Kruskal-Wallis test) was applied to each group of genotypes followed by a pairwise Mann-Whitney U-test with Bonferroni correction for multiple comparisons. All statistically significant differences are also observed using standard one-way ANOVA.

Supplemental References

Ja, W.W., Carvalho, G.B., Mak, E.M., de la Rosa, N.N., Fang, A.Y., Liong, J.C., Brummel, T., and Benzer, S. (2007). Prandiology of Drosophila and the CAFE

assay. Proceedings of the National Academy of Sciences of the United States of America *104*, 8253-8256.

Wolf, F.W., Rodan, A.R., Tsai, L.T., and Heberlein, U. (2002). High-resolution analysis of ethanol-induced locomotor stimulation in Drosophila. J Neurosci *22*, 11035-11044.

SUPPLEMENTAL FOOTNOTES

Supplemental Footnote S1:

The higher pre-puff baseline activity of *DopR* mutants (Fig. 2C) may be due to the fact that the flies are subjected to mechanical stress when they are transferred into the puff apparatus, and the ensuing hyperactivity response decays more slowly in these flies than in controls. Two lines of evidence support this hypothesis. In one experiment, flies were allowed to acclimate for 30 minutes following their introduction into the apparatus, rather than for the standard 10-minute period. Following this extended acclimation period, the pre-puff baseline of *DopR/DopR* flies was only ~2-fold greater than that of wild-type flies (Supplemental Figure S6A, B), rather than ~5-fold greater as observed after 10 minutes (Supplemental Figure S4A). Nevertheless, when flies were subjected to air puffs after this 30 minute acclimation period, the value of t in *DopR/DopR* flies was still > 4-fold higher than in wild-type flies (Fig. S6E), similar to the result obtained after 10 minutes of acclimatization (Fig. 2D). In a second experiment, flies were anaesthetized (using either cold or nitrogen) prior to introduction into the puff-o-mat, to avoid mechanical stress altogether, and were subjected to puff stimulation following recovery from anaesthesia. Under these conditions, the pre-puff baseline velocities of *DopR* and wild-type flies were indistinguishable, but *DopR* flies nevertheless showed an increased ReSH response, in comparison to CS flies, following the air-puff stimulus (data not shown). Taken together, these data support the idea that the ReSH phenotype of *DopR* mutant flies is not simply due to a generalized increase in basal locomotor activity, but rather reflects an exaggerated and persistent response to repetitive mechanical startle-induced stress, a conclusion supported by our circadian analysis of spontaneous locomotor activity.