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The D1 family dopamine receptor, *DopR*, potentiates hind leg grooming behavior in *Drosophila*

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Drosophila **groom away debris and pathogens from the body using their legs in a stereotyped sequence of innate motor behaviors. Here, we investigated one aspect of the grooming repertoire by characterizing the D1 family dopamine receptor, DopR. Removal of *DopR* results in decreased hind leg grooming, as substantiated by quantitation of dye remaining on mutant and RNAi animals vs. controls and direct scoring of behavioral events. These data are also supported by pharmacological results that D1 receptor agonists fail to potentiate grooming behaviors in headless *DopR* flies. DopR protein is broadly expressed in the neuropil of the thoracic ganglion and overlaps with TH-positive dopaminergic neurons. Broad neuronal expression of dopamine receptor in mutant animals restored normal grooming behaviors. These data provide evidence for the role of *DopR* in potentiating hind leg grooming behaviors in the thoracic ganglion of adult *Drosophila*. This is a remarkable juxtaposition to the considerable role of D1 family dopamine receptors in rodent grooming, and future investigations of evolutionary relationships of circuitry may be warranted.**

Keywords: Behavior, dfmr1, dopamine, dopamine receptor, *Drosophila*, fragile X, grooming, thoracic ganglion, vMAT

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Animals are likely to engage in grooming behaviors to remove potential immune threats from their body surface (reviewed in Spruijt *et al.* 1992). This behavior also prevents dust and detritus from coating essential body structures in a way that interferes with essential physiological function, such as vision, reproductive behavior or flight. Grooming behavior in *Drosophila melanogaster* (Dawkins & Dawkins 1976; Szebenyi 1969) interprets a mechanical stimulus or a microbial signal that triggers a swift central regulation of the grooming behavior (Corfas & Dudai 1989; Vandervorst & Ghysen 1980; Yanagawa *et al.* 2014). A dusting stimulus that coats the entire body initiates grooming in a hierarchical, stepwise progression that acknowledges successful completion of one program and subsequently relieves motor suppression of the next tier in grooming behavior, allowing efficient progress along the hierarchy (Seeds *et al.* 2014). In *Drosophila*, this robust innate behavior involves a coordinated locomotor program that uses the foreleg and hind leg pairs to groom the head, wings, thorax, abdomen and the legs themselves (Dawkins & Dawkins 1976; Seeds *et al.* 2014; Szebenyi 1969).

Monoamine neurotransmitter systems have been implicated in the regulation of grooming behaviors in *Drosophila*, and overexpression of the *Drosophila* vesicular monoamine transporter, *dVMAT*, results in a 10-fold increase in grooming events (Chang *et al.* 2006). Furthermore, mutants for the *Drosophila* homologue of the human fragile X mental retardation 1 gene, *dfmr1*, display both increased grooming and an increase in the expression of DVMAT in adult animals (Tauber *et al.* 2011). These results suggest that phenotypes are the result of changes on the presynaptic side of a monoaminergic neural circuit, where neurotransmitters are packaged by VMAT for release.

Postsynaptically, no mutant studies have been performed to examine the role of aminergic G-protein-coupled receptors (GPCRs) in grooming behavior. However, pharmacological characterization of grooming behavior in headless flies, which are capable of executing stereotyped grooming behaviors for an extended period after removing the head, had prospectively identified selective agonists and antagonists for type I family dopamine receptors that mediate grooming behavior (Yellman *et al.* 1997). Four dopamine receptors have been identified and characterized in *Drosophila*, including two type I (D1) family receptors, *DopR* (also known as *dDA1 or dumb*) and *damb* (Feng *et al.* 1996; Gotzes *et al.* 1994; Han *et al.* 1996; Sugamori *et al.* 1995). A single type II (D2) family receptor, DD2R, has been identified and characterized (Brody & Cravchik 2000; Hearn *et al.* 2002). Furthermore, a hybrid receptor with molecular homologies of both GPCR and steroid receptors, DopEcR, has shown to be responsive to dopamine (Brody & Cravchik 2000; Srivastava *et al.* 2005).

We determined the role of *DopR* in grooming behaviors. This receptor has been shown to have a number of functions in adult *Drosophila* behavior, mediating startle-based arousal, sleep, ethanol sensitivity, aversive learning and memory, male-male courtship, courtship learning and persistence of copulation (Chen *et al.* 2012; Crickmore & Vosshall 2013; Lebestky *et al.* 2009; Keleman *et al.* 2012; Kim *et al.* 2007; Kong *et al.* 2010; Liu *et al.* 2012). Differences in grooming

behavior are observed in both intact and headless *DopR* mutant animals, which remain active and responsive to mechanosensory response and evoked grooming behaviors when coated with powdered dye, even in the absence of an intact central brain. These results may suggest a role for DopR in mediating behavioral changes within the thoracic ganglion.

Materials and methods

Genetics

The $DopR^{f02676}$ allele was backcrossed for six generations into a Canton S (CS) background. Wild-type +/+ control is the CS background and is used for all genetic and pharmacological experiments. The *UAS-DopR-IR* RNAi line, $DopR^{attp}$ null allele generated by ends-out targeting, and the $DopR^{attp}$ null background containing the genomic rescue of the DopR locus were donated from the Dickson and Keleman Laboratories. $DopR^{attp}$ stocks were backcrossed for four to five generations in the CS background prior to use in experiments (Keleman *et al.* 2012). The *Elav-Gal4* line (second chromosome) and rut^1 mutation were obtained from the Bloomington Stock Center.

Behavior

All flies used in assays were 3- to 5-day-old males and reared on a 12:12 day:night cycle (lights on at 0800 h and off at 2000 h) at 25°C. Flies were kept in vials of 10 animals prior to use in the individual grooming assays. Assays were performed between 1300 and 1700 h. Flies for behavior experiments were anesthetized once for collection, and then allowed to recover for a minimum of 1 day for behavior assays.

Grooming assay

Grooming chamber design is available as a blueprint file and protocol for production using a laser cutter (Appendix S2) to shape transparent acrylic plastic, nylon mesh, and then adding simple screws (supplier of all raw materials: McMaster Carr).

About 5 mg of brilliant yellow dye (Sigma 201375) was deposited in each well of the grooming chamber (Fig. 1a). An individual conscious fly was aspirated into each well (15 wells per grooming chamber). The chamber was secured and then vortexed by holding the chamber to the vortex cup for 2 seconds while rotating the chamber. The chamber was then knocked twice against the bench to deliver dust into the lower chamber away from the flies. The chamber was then left for 30 min before anesthetizing the flies and placing each individual fly in an eppendorf tube containing 1 ml of EtOH. After 3 h, each tube was vortexed to ensure mixing and clearance of the remainder of the dye off the individual fly. About 50 µl of sample was added to 200 µl of EtOH on a microplate and the absorbance of sample was measured at 397 nm on a BioTek Synergy HT microplate reader, subtracting blank EtOH reading from each sample. Resultant values were multiplied by the dilution factor (×5) and averaged across flies for a given condition. Acute, non-groomed conditions were performed exactly as described above without giving the animals the 30 min of grooming time. Statistical comparisons were performed using GRAPHPAD PRISM 6 software.

Calculation of grooming percentage difference was calculated by the following equation: (dye accumulation mean value at time 0′ – dye accumulation mean value at time 30′/dye accumulation mean value at time 0) × 100. This percentage is expressed for each genotype in the bar plots and compared between genotypes to assess an internally controlled percentage for differences in relative grooming behavior.

Pharmacology

All dopamine receptor agonists (10 mM SKF81297 and 10 mM SKF38393) and antagonist (5 mM SCH22390) were purchased from Tocris Bioscience. Dilutions were performed in sterile water to achieve the final active concentrations described above.

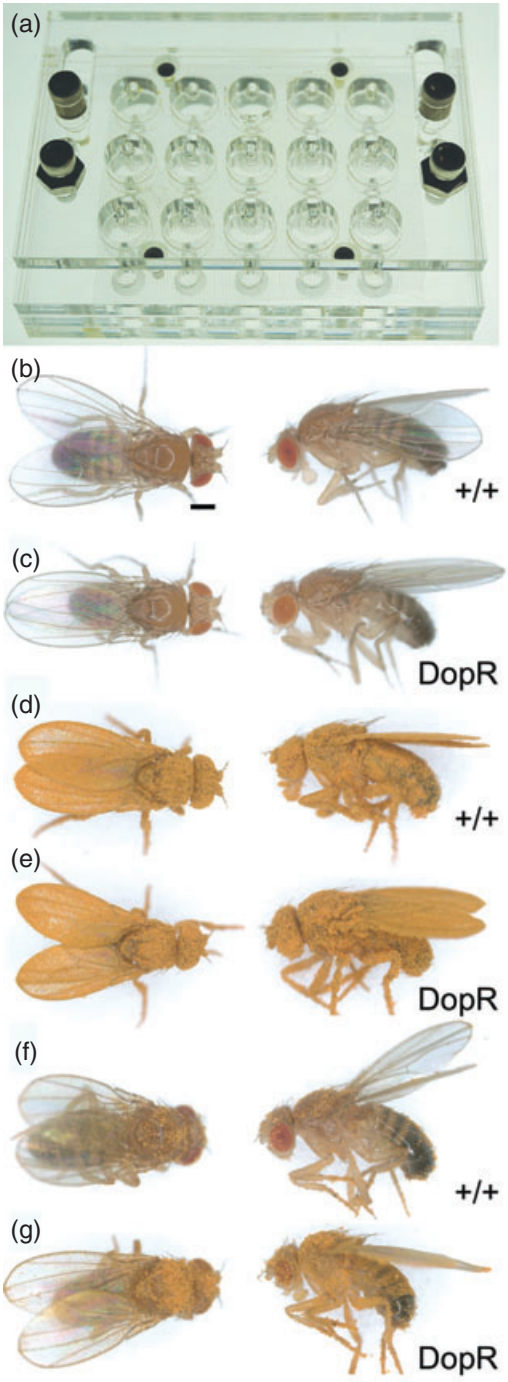

Figure 1: Quantification of grooming based on dye accumulation. (a) Grooming chamber. Individual flies and brilliant yellow dye are placed in individual wells (one fly:one well). Dimensions and blueprints for production in Appendix S2. (b, d, f**)** Wild-type adult male *Drosophila* (+/+). (c, e, g) $DopR^{f02676}/DopR^{f02676}$ adult male *Drosophila*. (b, c) Flies before dusting. (d, e) Flies immediately after dusting by vortexing chamber, prior to grooming (time: 0 min). (f, g) Flies after grooming (time: 30 min). Scale bar = 400 µm.

Pharmacology experiments followed the protocol outlined in Yellman *et al*. (1997). Flies were decapitated using a razor blade and left for 30 min in a humidified petri plate, containing a kimwipe saturated with water lined around the inner rim of the plate. Flies that retained postural control (stand on all six legs) and responded to light touch of a brush to the rear abdomen were used in the assay. One microlitre of blank (dH20) or drug is touched to the neck connective of the decapitated fly for 2 seconds. Flies were then monitored for behavioral changes for 2 min following application of drug. Fly behaviors were recorded by a Canon Vixia digital camcorder and scored for total grooming events during that time, including limb, head, wing and body/abdomen grooming events.

Behavioral observation

Characterization of hind leg and foreleg grooming events was performed using the standard grooming assay described above and eight individual chambers were recorded simultaneously using a Canon Vixia digital camcorder. Grooming events were scored for 20 min/fly/chamber. A multichannel cell counter was used in scoring foreleg vs. hind leg events.

Immunohistochemistry

Thoracic ganglia were dissected in 1× phosphate-buffered saline containing 0.05% Triton-X-100 (PBT) and then fixed overnight at 4°C in 2% paraformaldehyde (EM Sciences) in PBT. They were washed five times for 10 min in 0.1% PBT, blocked for 1 h in 0.1% PBT with 0.5% bovine serum albumin and 5% normal goat serum and then incubated with primary antibodies overnight at 4°C. They were then washed, blocked and incubated with secondary antibodies overnight at 4°C. Washed tissues were mounted on glass slides in Vectashield (Vector Laboratories), small pieces of broken coverslips served as posts, covered with a coverslip and sealed. Primary antibodies were rabbit anti-DopR (1:1250; Kong *et al*. 2010) and mouse anti-TH (1:100, Immunostar), and secondary antibodies were goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 (Life Technologies). The tissues were imaged on a Nikon Eclipse Ti C1 Confocal System using 1.15 μm steps and ×40 oil-immersion lens. Quantification of signal intensity was performed using Image J (version IJ 1.46r) (http://imagej.nih.gov/ij/).

Results

This behavioral study was facilitated by mass-producing a simple grooming chamber made of stacks of transparent acrylic plastic with a nylon mesh in the middle to separate the flies from excess dye that falls to the bottom of the chamber (Fig. 1a). Both the ceiling and the floor of the chamber were designed to slide laterally to allow delivery of conscious flies using a mouth aspirator into individual wells containing the dye. One fly was delivered to each well, but this design may also be adapted to population-based assays if the experimenter so desires. When occupied with a fly, the chamber ceiling slides back to a locked position, preventing exit of the fly. Screws that are accessible from the top of the chamber can regulate open or closed positions. After a grooming period, the floor can be shifted to remove dust, and flies were anesthetized and removed from the chamber by removing the ceiling. Detailed production designs and instructions for producing this chamber on a laser cutter are available (Appendix S2).

Adult *Drosophila* immediately begin grooming upon mechanosensory stimulation by coating the animals with dust or dye (Phillis *et al*. 1993; Seeds *et al*. 2014). Initial observations comparing wild-type animals with mutant $DopR^{f02676}$ animals suggested that the receptor may be required for proper expression of the grooming behavior (Fig. 1a–f). Visual comparison of undusted flies (Fig. 1b, c) to flies that have been coated with brilliant yellow dye and not allowed to groom (Fig. 1d, e) display the baseline conditions for groomed vs. ungroomed flies. This comparison along with control experiments at time 0′ (Fig. 2) suggest that $DopR^{f02676}$ flies do not show any physical differences in size or retention of dye that would bias a behavioral comparison. Given the 30 min of grooming time after dusting, the majority of $DopR^{f02676}$ flies (Fig. 1g) frequently showed a visible difference in comparison to wild-type animals (Fig. 1f). Excess dye remaining on *DopR* animals after grooming was typically most notable on the legs, wings and thorax.

Grooming behavior was quantified as a comparison between the remaining amount of dye on an animal after 30 min of grooming, relative to the value of an ungroomed condition for each genotype (Fig. 2a, c, e). Grooming was measured by recording absorbance values at the peak absorbance for the dye (397 nm) across all individuals of a given genotype or condition. We also tested absorbance in undusted flies to assess any differences arising from the flies alone and observed no difference between wild-type and mutant animals (data not shown). Wild-type animals (+/+ 30′: mean value = 1.664) display a significant amount of grooming ($P < 0.0001$) when compared with ungroomed wild-type animals (+/+ 0′: mean value = 2.847) (Fig. 2a). $DopR^{fo2676}$ flies (–/–30′: mean value = 2.174) also showed a significant amount of grooming ($P < 0.0001$) relative to ungroomed mutant animals (–/–0′: mean value = 3.005). No difference was observed in comparing wild-type animals to mutants at time 0. However, the relative amounts of grooming as compared between +/+ 30′ and $DopR^{f02676}/DopR^{f02676}$ 30′ showed a significant difference ($P = 0.0007$), suggesting that $DopR^{f02676}$ mutant animals engage in less grooming behavior than wild-type animals or are less efficient in grooming. Expressing grooming behavior as percent difference (described in *Materials and methods*) compares relative amount of grooming behavior for each genotype (Fig. 2b), and suggests that $DopR^{f02676}$ animals show approximately 20% less grooming when expressed as a percentage of wild-type behavior. $DopR^{attp}$ null mutants were also tested in comparison to genomic rescue lines that were generated to assess *DopR* in courtship conditioning behaviors (Keleman *et al*. 2012). The $DopR^{attp}$ null animals also display a grooming deficit in comparison to genomic rescue at 30 minutes ($P < 0.0001$). The null phenotype appears to be greater than the strong $DopR^{f02676}$ hypomorph, in comparing the percent difference to relative controls (Fig. 2b, d). This difference may reflect higher grooming levels of the rescue background (blue bar in Fig. 2d) more than the absolute difference in grooming between $DopR^{attp}$ and $DopR^{f02676}$ (red bars in Fig. 2b, d). The mean values, measures of variance and statistical significance for all experiments are described, compiled and available in Appendix S1.

Given the significant deficit in grooming for *DopR* mutants, one may also predict that downstream intermediates of GPCR signaling may also be involved in regulating this behavior. We tested a hypomorphic mutant allele of the calcium-dependent adenylate cyclase, *rutabaga*, in the

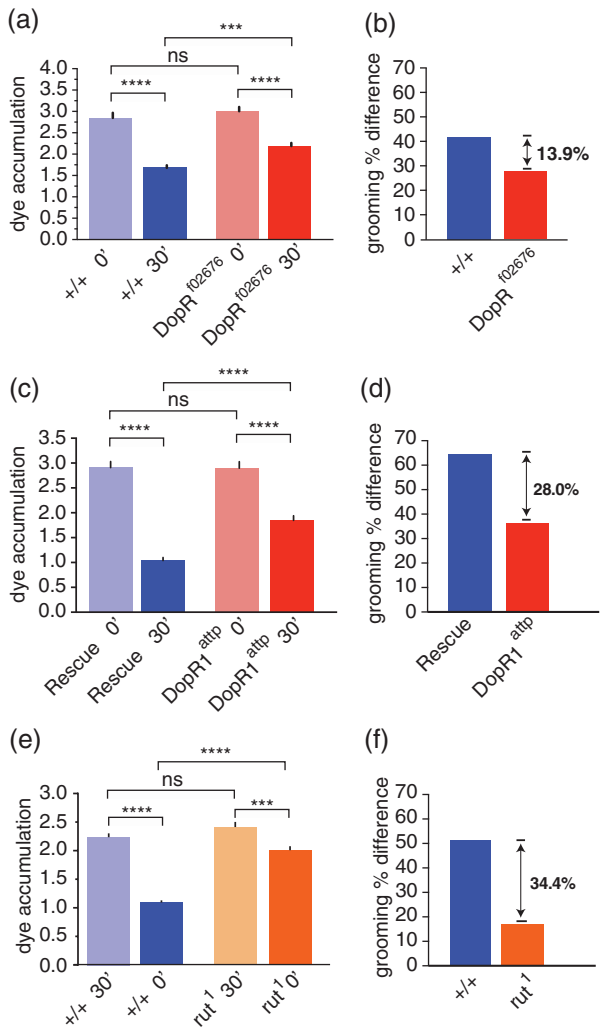

Figure 2: Dopamine receptor (*DopR/dDA1/dumb*) required for modulation of grooming behavior. (a, c, e) Grooming of wild-type (blue) and mutant flies (red/orange) measured at 0 or 30 min after dusting. SEM is measured for each genotype and condition. (a, b) $n=43$ flies per genotype and condition. (a) Wild-type and $DopR^{f02676}$ flies both exhibit grooming behavior (+/+ 30′ compared with +/+ 0′, $P<0.0001$; $DopR^{f02676}$ 30′ compared with $DopR^{f02676}$ 0′, $P<0.0001$). *DopR* flies fail to groom as well as wild-type animals ($DopR^{f02676}$ 30′ compared with +/+ 30′, $P<0.001$). Acute dusting of each genotype at 0′ results in equivalent accumulation of dust (ns = not significant). (b, d, f) Grooming percent difference is calculated for each genotype (dye accumulation at 0′ − dye accumulation at 30′/dye accumulation 0′ × 100) providing a relative value for comparing grooming behaviors. (c) $DopR^{attp}/DopR^{attp}$ null flies display a grooming deficit ($DopR^{attp}$ 30′ compared with +/+ 30′, $P<0.0001$). (d) Grooming percent difference for $DopR^{attp}$ null. (c, d) $n=45$ flies for each genotype or condition. (e) rut^1 homozygous flies display a grooming deficit (rut^1 30′ compared with +/+ 30′, $P<0.0001$). (f) Grooming index for rut^1 flies. (e, f) $n=30$ flies for each genotype or condition. Statistical analyses by one-way ANOVA and Bonferroni correction. ns = non-significant.

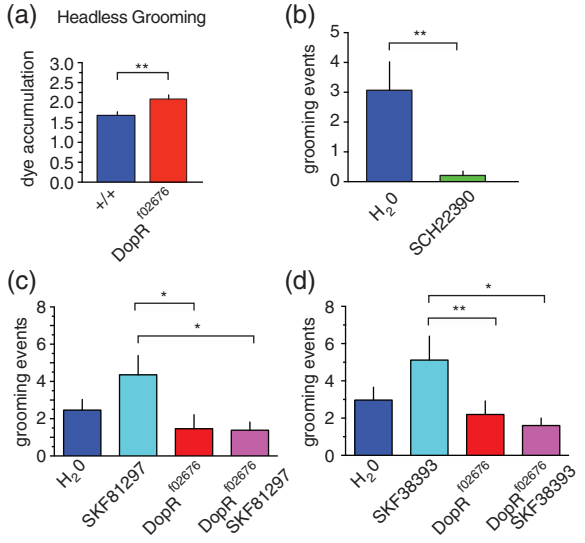

Figure 3: Pharmacological stimulation of headless flies modulates grooming. (a) Headless flies display grooming behavior and headless $DopR^{f02676}$ flies (blue 30′ grooming) as well as wild-type (red 30′ grooming) fail to groom. SEM measured for each genotype. Two-tailed unpaired *t*-test, $P=0.0038$. $n=30$ per genotype. (b–d) Total grooming events measured for each condition over 2-min observation period after pharmacological application. (b) Application of D1 dopamine receptor antagonist SCH22390 on wild-type flies prevents grooming behaviors. SEM measured for each condition. Two-tailed unpaired *t*-test, $P=0.0051$. $n=33$ per genotype. (c) Application of D1 dopamine receptor agonist SKF81297 displays asymmetric effects on wild-type and $DopR^{f02676}$ flies. SEM measured for each condition. Statistical analysis by one-way ANOVA and Bonferroni correction (*$P<0.05$). $n=39$ per genotype and condition. (d) Application of D1 dopamine receptor agonist SKF38393 displays asymmetric effects on wild-type and $DopR^{f02676}$ flies. SEM measured for each condition. $n=34$ per genotype and condition. Statistical analysis by one-way ANOVA and Bonferroni correction (**$P<0.01$).

grooming assay and also observed a significant difference ($P<0.0001$) in comparing the +/+ 30′ grooming condition to the rut^1 homozygous mutants (Fig. 2e). The grooming index for the rut^1 condition displayed a 34% deficit in grooming relative to controls (Fig. 2f).

Given the recent work identifying grooming circuitries (Seeds *et al.* 2014), along with potent pharmacological interventions on headless flies that predict a role for dopamine in grooming behaviors (Yellman *et al.* 1997), we began to investigate these behaviors in headless adult animals. Using the same protocol as was used for our standard grooming assay (Fig. 2), we tested whether headless $DopR^{f02676}$ homozygous mutant flies also display a grooming deficit, relative to wild-type animals. Indeed, we observed that acutely decapitated $DopR^{f02676}$ mutants show significant differences ($P=0.0038$) in grooming relative to headless wild-type animals (Fig. 3a).

We further assessed a requirement for dopaminergic function in grooming behaviors by applying D1 family dopamine

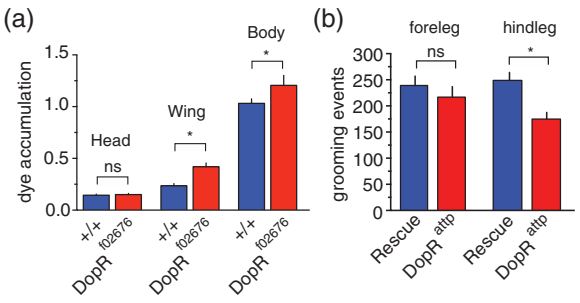

Figure 4: Dopamine receptor function potentiates hind leg grooming. (a) Grooming of individual regions of wild-type (blue) and $DopR^{f02676}/DopR^{f02676}$ (red) measured at 30 min after dusting and subsequent dissection. *P* value for wing grooming = 0.0204. *P* value for body = 0.0302. *n* = 33–35 for all conditions (details in Appendix S3). SEM is measured for each genotype and condition. Statistical analysis by one-way ANOVA and Bonferroni correction. (b) Wild-type (blue) and $DopR^{attp}/DopR^{attp}$ (red) total foreleg and hind leg grooming events measured during 20-min observation period after dusting. *n* = 20 for each genotype. Statistical analysis by one-way ANOVA and Bonferroni correction. *P* value = 0.015 for hind leg comparison. ns = non-significant.

receptor agonists and an antagonist directly to the neck connective of decapitated adult flies. For all pharmacological experiments (Fig. 3b–d), the total grooming events including leg, thorax, wing and abdomen grooming were scored and compared between individual conditions. Similar to observations from Yellman *et al.* (1997), application of the selective D1 family dopamine receptor antagonist to the neck connective of headless wild-type animals shows a consistent loss of grooming behavior ($P = 0.0021$) that accompanies loss of postural control and akinesia for the majority of animals during the 2-min observation period (Fig. 3b). Application of selective D1 family dopamine receptor agonists (SKF81297 and SKF38393) showed increased grooming behavior in wild-type animals and an absence of any potentiating effect in the *DopR* mutant animals (Fig. 3c, d). There were no significant differences in mutant animals when comparing blank to agonist conditions, whereas a significant difference was observed when comparing wild-type plus agonist condition to mutant plus agonist condition (*P* value: SKF81297 = 0.0293 and SKF38393 = 0.0317).

As grooming behavior represents an integrative whole of multiple, individual motor programs, we assessed whether the DopR dopamine receptor modulates a specific step or multiple steps in grooming. We scored the cleanliness of three discrete body regions by performing simple dissections immediately following the standard grooming assays, and measured absorbance for each individual region (Fig. 4a). Significant differences in grooming were observed when comparing wild-type vs. *DopR* mutant wings ($P = 0.0204$), and also for the 'body', which for this experiment includes legs, abdomen and thorax ($P = 0.0312$). No differences were observed for grooming values of wild-type vs. *DopR* mutant heads.

These results were supported by direct observation of grooming behavior in wild-type and $DopR^{attp}$ mutants (Fig. 4b). Foreleg grooming events include cleaning of eyes, antennae and head as well as rubbing the forelegs themselves, and hind leg events include grooming of the abdomen, wing, thorax and hind legs. Scoring the behavioral videos suggested no difference in foreleg grooming frequency between wild-type and *DopR* mutant animals. We also assessed basic locomotor behavior by tracking the animals using ETHOVISION XT tracking software, and no differences were observed between mutant and wild-type animals (data not shown). We observed a significant deficit in hind leg grooming frequency for $DopR^{attp}$ mutants relative to wild-type ($P = 0.0006$). This represents approximately a 30% drop in hind leg grooming activity as normalized to wild-type behavior. Similar results were also observed for the $DopR^{f02676}$ allele (Appendix S3).

DopR protein has been observed in discrete circuits of the adult *Drosophila* brain, with areas of highest expression including the mushroom bodies and central complex (Kim *et al.* 2003; Kong *et al.* 2010; Lebestky *et al.* 2009). DopR expression in the thoracic ganglion appears to coincide with the broad innervation of the neuropil by dopaminergic neurons, as marked by tyrosine hydroxylase antibody (Fig. 5). DopR protein was expressed broadly in all thoracic ganglion neuropils (prothoracic, accessory mesothoracic, mesothoracic, metathoracic and abdominal) as well as in the cellular cortex surrounding the neuropil present within the neurolemma sheath. Weak DopR immunochemical signal observed in mutant animals (Fig. 5d) may reflect non-specific background staining of secondary antibody, a similar epitope within the thoracic ganglion, or the small residual amount of DopR protein present in the $DopR^{f02676}$ mutants (Kim *et al.* 2003; Kong *et al.* 2010; Lebestky *et al.* 2009). Quantitation of the DopR signal intensity for three identical bounded regions within both wild-type and mutant neuropil highlights differences in signal abundance (Supplemental Materials). Additional projections and z-stack movies of DopR and TH colocalization highlight the penetrance of dopaminergic innervation throughout the neuropil and the broad expression of DopR throughout these domains (Videos S1–S4).

Previous work has utilized the $DopR^{f02676}$ allele as an effective rescue condition for restoring dopamine receptor function in neurons (Kong *et al.* 2010; Lebestky *et al.* 2009). When crossed to *Gal4* lines, the transposon *UAS* element within the second intron of the *DopR* locus is capable of expressing a truncated, functional form of the DopR protein. $DopR^{f02676}/+$ displays a dominant phenotype in grooming behavior (Fig. 6a, b). This is similar to the dominant effects observed for sleep, arousal and ethanol-stimulated locomotor behavior (Kong *et al.* 2010; Lebestky *et al.* 2009). The pan-neuronal *Elav-Gal4* transgene proved to be an effective circuit driver in modulating dopaminergic function, as expression of the *DopR-IR* RNAi construct creates a similar grooming deficit as observed for the *DopR* alleles (Fig. 6c, d). We then utilized the *Elav-Gal4* line to investigate the restoration of DopR function in a $DopR^{f02676}$ mutant background. The *elav-Gal4/+;*$DopR^{f02676}/+$ genotype (green) displays significant restoration of the grooming deficit relative to both wild-type (blue) and *elav-Gal4/+* transgene (yellow) controls (Fig. 6e, f).

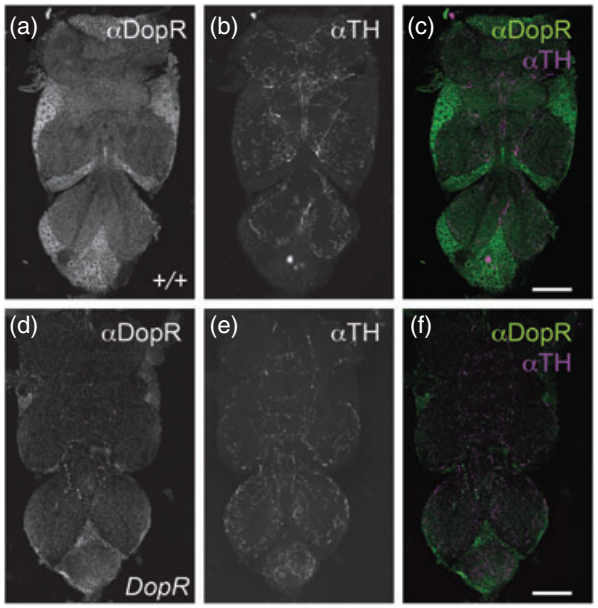

Figure 5: Expression of the dopamine receptor protein in the adult thoracic ganglion. (a–c) Wild-type thoracic ganglion. (a) DopR protein expression and (b) TH protein expression in dopaminergic neurons. c) Colocalization of αDopR and αTH in the adult thoracic ganglion of a wild-type fly. (d–f) *DopR*^{f02676} thoracic ganglion. (d) DopR protein expression and (e) TH protein expression in dopaminergic neurons. (f) Colocalization of αDopR and αTH in the adult thoracic ganglion of a wild-type fly. (a–f) Each image is a projected stack of three serial optical slices, corresponding to 3.45 μm volume through the thoracic ganglion. Scale bar = 33 μm. Complete projections of ganglia available in Appendix S3.

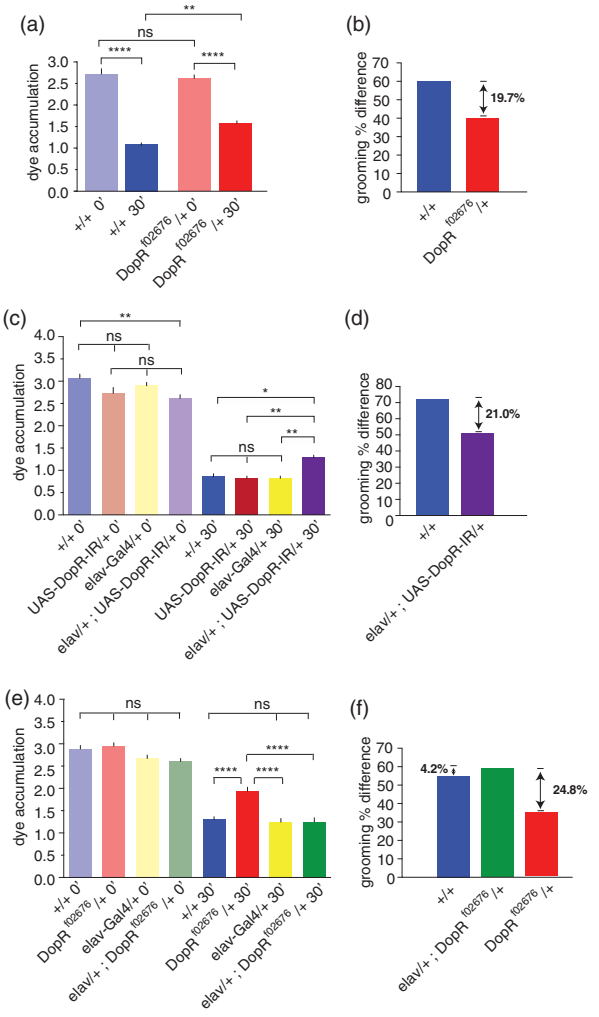

Figure 6: Broad neuronal DopR expression restores grooming in *DopR* mutant. (a) *DopR*^{f02676}/+ heterozygous flies display a grooming deficit (*DopR*^{f02676}/+ 30′ compared with +/+ 30′, $P = 0.0027$). (b) Grooming percent difference for *DopR*^{f02676}/+. (a, b) $n = 30$ flies for each genotype or condition. (c) *elav-Gal4* driving expression of *UAS-DopR* RNAi results in a grooming deficit (purple). Comparison of +/+ to *elav-Gal4/+;UAS-DopR-IR/+*, $P = 0.0115$. (d) Grooming percent difference for wild-type and *elav-Gal4/+;UAS-DopR-IR/+*. (c, d) $n = 25–28$ flies for each genotype or condition. (e) *elav-Gal4* driving expression of DopR from the *UAS* element within the transposon of the *DopR*^{f02676} hypomorphic allele results in restoration of grooming (green). Comparison of *DopR*^{f02676}/+ to all genotypes, $P < 0.0001$. (e, f) $n = 45$ flies for each condition. (a, c, e) SEM is measured for each genotype and condition. Statistical analysis by one-way ANOVA and Bonferroni correction. * = $p < 0.05$. ** = $p < 0.01$. **** = <0.0001. ns = non-significant.

Discussion

Our genetic, pharmacological and immunohistochemical data suggest a role for the *Drosophila* type I family dopamine receptor, DopR, in modulating grooming behavior. Given the complexity of executing fine motor control of independent grooming programs for different body parts, and the further complexity of programs that assess completion of grooming to potentiate progress along the hierarchy to the next step, it is likely that many molecular switches and neurotransmitter systems will be engaged in completing this highly orchestrated behavioral suite. An elegant suppression hierarchy has been proposed to explain the serial release of suppression, or disinhibition, along these circuits that allows a linear progression of the grooming behavior (Seeds *et al.* 2014).

The first step in the grooming hierarchy is the grooming of the eyes, antennae and head by the forelegs. Upon completion, the fly uses its hind legs to clean the abdomen, wings and thorax. We did not observe quantitative differences for dye accumulation in the cleaning of the head between wild-type and *DopR*^{f02676} mutants (Fig. 4a). Perhaps, the removal of *DopR* hinders release of the suppression of body grooming circuits by the head grooming regulatory circuits, forcing the fly to spend disproportionately more efforts cleaning the head and leaving the remaining tasks of cleaning the body and wings undone. This would result in a

higher absorbance value for body and wings, while the head values are potentially equivalent, or a lower absorbance value for *DopR* mutants suggests hyperactive head grooming. Our data documenting the frequency of foreleg and hind leg grooming in wild-type vs. $DopR^{attp}$ *and* $DopR^{f02676}$ mutants (Fig. 4b, Appendix S3) argue against such role for *DopR* in the disinhibition of later programs, as we did not observe a higher engagement of foreleg grooming frequency in the *DopR* mutants relative to wild type. It is likely that the initial step in the grooming program is unaffected. However, we observed significant differences in grooming with regard to the body (legs/thorax/abdomen) and the wings by the hind legs. Taken together, the data may point to a specific role for *DopR* in positively regulating one or more of the hind leg motor subprograms after head cleaning is completed.

Both spontaneous and dust-induced headless grooming behaviors are robustly observed in wild-type animals, and the observed deficit of grooming behavior in *DopR* mutants is also observed in headless *DopR* flies. Application of agonists for type I dopamine receptors also appears to potentiate a difference in grooming behaviors when comparing wild-type and *DopR* mutants (Fig. 3c, d). These results suggest that the primary *DopR* phenotype is because of the function within thoracic ganglion, as opposed to DopR circuits in the central brain. One may suppose that potentiating and inhibitory circuits for grooming are present in the central brain, but if *DopR* was required solely in the central brain for positive regulation of grooming, it would be unlikely to observe our pharmacological results that display sensitivity to agonists for the neck connective of headless animals. Furthermore, the broad expression of DopR in all segments of the thoracic ganglion supports a role for DopR function within the thoracic ganglion, but does not prospectively identify individual candidate circuitries that modulate grooming behavior. Future studies will investigate the requirement for *DopR* in individual circuits of the *Drosophila* central nervous system, with specific attention dedicated to targeting hind leg circuitries.

The deficit in grooming behavior observed for $DopR^{attp}$ and $DopR^{f02676}$ mutants is less than that observed for the downstream adenylate cyclase (*rutabaga*) mutants (Fig. 2). This may suggest that other monoaminergic neurotransmitter systems or alternate non-aminergic GPCR pathways also regulate grooming behaviors. The Hirsh Laboratory had identified octopamine as another potential stimulator of hind leg grooming in headless animals, although this treatment also showed a robust simultaneous stimulation of locomotion (Yellman *et al.* 1997). There are five known octopamine receptors in *Drosophila*, including *Octopamine β1, β2* and *β3 Receptor*, *Oamb* and *Oct-Tyr*, an octopamine-tyramine receptor. Many of these receptors have behavioral phenotypes when their expression is reduced, including effects on locomotion (Koon & Budnik 2012), starvation stress response, metamorphosis (Ohhara *et al.* 2015), sleep (Crocker *et al.* 2010; Kayser *et al.* 2015), courtship conditioning (Zhou *et al.* 2012), learning and memory (Burke *et al.* 2012) and olfaction (Kutsukake *et al.* 2000). Further investigation of the octopaminergic circuits and cognate receptors in grooming behaviors may be warranted, as well as a broader candidate survey to phenotypically characterize GPCRs that are expressed in the thoracic ganglion and their respective circuits (Fredriksson & Schioth 2005; Hauser *et al.* 2006; Jenett *et al.* 2012).

Dopamine has been identified as a regulator of grooming behaviors in mice, and both pharmacological (Hoffman & Beninger 1985; Starr & Starr 1986) and genetic evidence has implicated D1 family dopamine receptors in grooming behavior (Cromwell *et al.* 1998). Additionally, lesioning studies in rodents that target dopaminergic regions in the substantia nigra with 6-OHDA, a neurotoxic compound with partial selectivity for dopaminergic neurons, show disruption of the natural serial order of grooming chained events (Berridge 1989, Berridge & Cromwell 1990; Berridge & Fentress 1987). In comparing the stereotyped grooming behavioral patterns in both rodents and *Drosophila*, although the number of limbs involved and the individual acts are distinctly different, there are clear similarities in the execution of stereotyped, chained grooming patterns that reach a natural cyclic conclusion and either terminate or repeat. And given the identical molecular candidates in play for both model systems, it will be illuminating to continue to expand our research to broaden this comparative investigation. At a neuroanatomical level, it is tempting to examine the large structural differences between brains of flies and mammals and dismiss any potential homologous relationships of underlying circuitries, but more evidence and investigation into evolutionary relationships between molecules, neural networks and the systems of organization within the invertebrate and mammalian brain suggest many similarities, and future comparative investigations will allow a richer, reciprocal understanding of brain circuitry and its regulation (reviewed in Anderson & Adolphs 2014; Hartenstein & Stollewerk 2015; Kaiser 2015; Tessmar-Raible *et al.* 2007; Tomer *et al.* 2010).

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Statistics for figures.
Appendix S2: Grooming chamber design files.
Appendix S3: DopR Protein Expression in Thoracic Ganglion.

Video S1: Wildtype DopR Protein Expression (Anterior Thoracic Ganglion).
Video S2: Wildtype DopR Protein Expression (Posterior Thoracic Ganglion).
Video S3: DopRf02676 mutant. DopR Protein Expression (Anterior Thoracic Ganglion).
Video S4: DopRf02676 mutant. DopR Protein Expression (Posterior Thoracic Ganglion).