Differential dominance of an allele of the *Drosophila tßh* gene challenges standard genetic techniques

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Abstract

The biogenic amine octopamine (OA) and its precursor tyramine (TA) are involved in controlling a plethora of different physiological and behavioral processes. The tyramine-ß-hydroxylase (tßh) gene encodes the enzyme catalyzing the last synthesis step from TA to OA. Here, we report differential dominance (from recessive to overdominant) of the putative null *tßh*^{nM18} allele in two

behavioral measures in Buridan's paradigm (walking speed and stripe deviation) and a proboscis extension assay in the fruit fly *Drosophila melanogaster*. The behavioral analysis of transgenic *tßh* expression experiments in mutant and wild type flies as well as of OA- and TA-receptor mutants revealed a complex interaction of both aminergic systems. Our analysis suggests that the different neuronal networks responsible for the three phenotypes show different sensitivity to *tßh* gene dosage. This conclusion entails important implications for standard transgenic techniques, commonly used in functional genetics.

Introduction

Pleiotropic genes play an important evolutionary role not only because they create functional and developmental relationships among traits, but also because they can become relevant for the maintenance of genetic variability in a population (Turelli and Barton, 2004; Lawson and Wolfe, 2011). Pleiotropy is also a prerequisite for differential dominance. Differential dominance occurs when dominance patterns for a single locus vary among traits, e.g., the same allele may behave recessively in one trait and dominantly in another (Ehrich et al., 2003; Kenney-Hunt and Cheverud, 2009). In wild populations, differential dominance is often accompanied by overdominance effects, thought to underlie the high level of heterozygosity found in these populations (Allison, 1954; Klingenberg et al., 2001; Ehrich et al., 2003; Turelli and Barton, 2004; Kenney-Hunt and Cheverud, 2009; Lawson and Wolfe, 2011). While heterozygosity tends to decrease in laboratory populations (Karlin, 1968; Motro and Thomson, 1982; Frankham, 1995; Saccheri et al., 1998; Madsen et al., 1999; Dennis, 2002; Nowak et al., 2007), the

differential dominance effects may persist in pleiotropic genes, including overdominance.

Differential dominance, found to be ubiquitous in quantitative and population genetics studies (Ehrich et al., 2003; Cheverud et al., 2004; Kenney-Hunt and Cheverud, 2009), may potentially wreak havoc in functional genetics, where a common strategy is to introduce transgenic alleles into homozygously null mutant individuals (Brand and Perrimon, 1993; Saga, 1998; Zars et al., 2000; Yokokura et al., 2004; Chun et al., 2010; Park et al., 2011; Kirshenbaum et al., 2016). For instance, if a mutation acts dominantly, rather than recessively, then the transgenic alleles will not rescue the phenotype even if the gene in question is responsible for it. In the case of overdominance, the outcome of such experiments may depend on the mechanism by which overdominance is achieved and could potentially range from no rescue to overdominant rescue, making these results difficult or impossible to interpret. Intermediate inheritance may make rescue experiments difficult to pin down statistically as successful or unsuccessful.

Because of the promiscuous role of biogenic amines in many different behavioral and physiological processes, the genes coding for their synthesis enzymes are prime candidates for pleiotropy and, hence differential dominance. The biogenic amine octopamine (OA) is structurally and functionally related to vertebrate noradrenaline (Evans, 1980; David and Coulon, 1985; Roeder, 1999). OA is synthetized from another biogenic amine, tyramine (TA) by tyramine-ß-hydroxylase (tßh, (Wallace, 1976). OA plays an important role in the initiation and maintenance of motor programs in insects in general

(Sombati and Hoyle, 1984; Baudoux et al., 1998; Duch et al., 1999; Ridgel and Ritzmann, 2005; Gal and Libersat, 2010). In skeletal muscles, OA concomitantly affects not only on the muscle tension (Evans and O'Shea, 1977) and relaxation rate (Evans and Siegler, 1982), but also on its metabolism: As a neurohormone being released into the hemolymph, it mobilizes lipids and stimulates glycolysis (Blau et al., 1994; Candy et al., 1997). OA appears to be involved in almost every behavioral and physiological process (Libersat and Pflueger, 2004; Roeder, 2005). In *Drosophila*, the X-linked *tßh*^{nM18} mutant has been an important tool to understand the role of TA and OA in many behaviors such as egg-laying (Monastirioti et al., 1996; Lim et al., 2014; Li et al., 2015) , aggression (Zhou and Rao, 2008), flight (Brembs et al., 2007; Pflüger and Duch, 2011; Ryglewski et al., 2017; O'Sullivan et al., 2018), and sugar responsiveness (Damrau et al., 2017a).

Loss-of-function *tßh*^{nM18} male mutants, with a complete depletion of octopamine, display reduced aggression: their fight initiation latency is increased, while lunging and holding frequencies are decreased. Furthermore, an acute silencing of octopaminergic neurons through the use of temperature-sensitive UAS-Shi^{ts}, phenocopies the *tßh*^{nM18} mutants, indicating the reduced aggression does not result from developmental defects in the mutants (Baier et al., 2002; Dierick, 2008; Hoyer et al., 2008; Zhou and Rao, 2008; Andrews et al., 2014). Interestingly, it was possible to rescue the aggression deficiency seen in *tßh*^{nM18} mutant flies by expressing TßH in a small subset of octopaminergic neurons (Zhou and Rao, 2008). These results suggest that the standard genetic rescue approach can be successful at least in this phenotype, even with a pleiotropic gene. Some of us have shown previously that another phenotype, sugar

sensitivity after starvation, can be similarly rescued (Damrau et al., 2017a). In the present work we studied *tßh*-associated differential dominance and rescue experiments using behavioral phenotypes as disparate as sugar sensitivity (Minnich, 1921; Dethier, 1976) and walking behavior in Buridan's paradigm (Bülthoff et al., 1982; Colomb et al., 2012). In Buridan's paradigm, we evaluated walking speed, a temporal parameter of movement control, as well as object fixation, a spatial measure of movement control. Fixation of visual cues is increased at higher contrast conditions (Osorio et al., 1990; Riemensperger et al., 2011). Interestingly, the sensitivity of the motion-sensitive neurons in the fly optic lobes was shown to increase when the fly is walking (Chiappe et al., 2010; Maimon et al., 2010; Rosner et al., 2010; Jung et al., 2011) or flying (Li et al., 2017). The gain increase in flight was found to be OA dependent (Suver et al., 2012; Strother et al., 2018).

Materials and Methods

Fly strains

tßh^{nM18} (Monastirioti et al., 1996; FBal0061578), ***oamb*** ((Han et al., 1998); OctαR, *oamb^{286}* FBti0038368, *oamb^{584}* FBti0038361), *honoka* (Kutsukake et al., 2000; Oct-TyrR, FBal0104701), *hsp-tßh* (Schwaerzel et al., 2003; FBal0152162), *Octß2R$^{Δ3.22}$* and *Octß2R$^{Δ4.3}$* (Damrau et al., 2014; CG6989, FBgn0038063), and w+;;UAS-*tßh* (Monastirioti, 2003; FBti0038601) were obtained from Henrike Scholz, Cologne; Hiromu Tanimoto, Martinsried; Andreas Thum, Konstanz; Martin Schwärzel, Berlin; and Amita Seghal, Chevy Chase. *TyrR^{f05682}* (*CG7431^{f05682}*, FBal0184987), *TyrRII^{Δ29}* (CG16766, FBgn0038541) and *TyrRII-TyrR^{Δ124}* were kindly provided prior to publication by Edward Blumenthal, Milwaukee. Receptor mutants and

their respective control lines were outcrossed for at least six generations into CS background.

Designation	Identifier	Associated target	Source or reference	Additional information
tßh^{nM18}	FBal0061578	*tßh*	(Monastirioti et al., 1996)	Gift from Henrike Scholz
oamb^{286}	FBti0038368	oamb	(Han et al., 1998)	Gift from Hiromu Tanimoto
oamb^{584}	FBti0038361	oamb	(Han et al., 1998)	Gift from Hiromu Tanimoto
honoka	FBal0104701	TyrR	(Kutsukake et al., 2000)	Gift from Andreas Thum
hsp-tßh	FBal0152162		Schwaerzel et al., 2003	Gift from Martin Schwärzel
Octß2R$^{\Delta 3.22}$	CG6989	*Octß2R*	(Scholz-Kornehl, 2015)	Gift from Martin Schwärzel before publication
Octß2R$^{\Delta 4.3}$	FBgn0038063	*Octß2R*	(Scholz-Kornehl, 2015)	N.A
w+;;UAS-*tßh*	FBti0038601		Monastirioti, 2003	Gift from Amita Seghal
TyrR^{f05682}	*CG7431*^{f05682}, FBal0184987	TyrR	(Zhang and Blumenthal, 2017)	Gift from Edward Blumenthal
TyrRII$^{\Delta 29}$	CG16766, FBgn0038541	TyrR	(Zhang and Blumenthal, 2017)	Gift from Edward Blumenthal
TyrRII-TyrR$^{\Delta 124}$	FBab0048326	TyrR	(Zhang and Blumenthal, 2017)	Gift from Edward Blumenthal

Table 1: Fly strains used in this work.

The *tßh*^{nM18} mutation is thought to be a null allele and abolishes OA synthesis. Consequently, the precursor of OA, TA, accumulates to approx. eight-fold over control levels (Monastirioti et al., 1996). Mutants for the *Octß2R* were created using recombination of FRT-containing P-elements (Parks et al., 2004), as described elsewhere (Scholz-Kornehl, 2015).

Fly care

Flies were kept on standard cornmeal/molasses-food in a 12/12 h light/dark cycle at 60% relative humidity and 25°C except for *hsp-tßh* and elaV-GAL4;tub-GAL80 crosses which were kept at 18°C without humidity control.

After hatching, experimental flies were collected into new food vials for two days. The day before testing, flies were CO_2-anesthetized, sorted by sex (females except for UAS-*tßh* experiments), and their wings were clipped at two thirds of their length. If not stated otherwise, animals recovered in the food vials overnight. Individuals were captured using a fly aspirator and transferred into the experimental setup on the following day.

Heat-shock

hsp-tßh flies were heat shocked for 30-45 min at 37°C with 3-4 h recovery time at 25°C before testing. elaV-GAL4;tub-GAL80;UAS-Tdc2 flies were heated at 33°C overnight with 30 min recovery time at room temperature before testing.

Buridan's paradigm

We used the Buridan's setup to test fly locomotion, details are described in (Colomb et al., 2012) (RRID:SCR_006331). Briefly, two black stripes (30 mm in width and 320 mm in height) were positioned opposite of each other 146.5 mm from the center of a platform (117 mm in diameter) surrounded by water and illuminated with bright white light from behind. The centroid position of the fly was recorded by custom tracking software (BuriTrack, http://buridan.sourceforge.net). If a fly jumped out of the platform, it was returned by a brush, and the tracker was restarted. All data are obtained from 5 min of uninterrupted

walk or the first 5 min of a 15 min walk. See 10.17504/protocols.io.c7vzn5 for fly preparation.

Data were analyzed using CeTrAn v.4 (https://github.com/jcolomb/CeTrAn/releases/tag/v.4) as previously described in (Colomb et al., 2012). Briefly, walking speed was measured in traveled distance over time. A median was calculated for the progression of one experiment; the mean of all medians is reported in the graphs. Speeds exceeding 50 mm/s are considered to be jumps and are not included in the median speed calculation (Colomb et al., 2012). Stripe deviation acted as a metric for fixation behavior. It corresponds to the angle between the velocity vector and a vector pointing from the fly position towards the center of the frontal stripe (for details see (Colomb et al., 2012). Therefore, the larger the stripe deviation, the less accurate the fly fixated the stripe and *vice versa*. The platform inside the arena was cleaned with 70% ethanol after each experiment to minimize odor cues.

Sugar sensitivity test

Sugar response was measured as described elsewhere (Damrau et al., 2017b). Briefly, flies were starved for 20 h with Evian® water. Immobilized by cold-anesthesia using a cold station (Fryka-Kälteteschnik, Esslingen am Neckar, Germany), a triangle-shaped copper hook was glued to head and thorax. 3 h later, the hook was attached to a rack so that free movement of flies' tarsi and proboscis was enabled. A filter paper soaked with sucrose solution was presented to all the tarsi. The proboscis extension response to a serial dilution of sucrose (0, 0.1%, 0.3%, 0.6%, 1%, 3% and 30%) was recorded. The total number of the fly's responses to all sucrose stimulations of increasing concentration was calculated (Scheiner et al.,

2004). Finally, the proboscis was stimulated by 30% sucrose solution. Flies not responding to proboscis stimulation or responding to the first stimulation (water only) were discarded from the analysis.

Relative performance by tßh gene dosage

For the summary of phenotypic performance according to the tßh gene dosage (Fig. 6), the mean or median of speed, stripe deviation, and total PER was normalized to the performance of wild type and mutant controls for each of the different tests. The order on the x-axis broadly reflects the number of gene copies in each experiment, whereby the position of the rescue and heterozygote experiments was set arbitrarily.

Statistics

The mean walking speed was calculated out of medians (see Buridan's paradigm, above) and plotted with the standard error of the mean. Sucrose response and stripe deviation are exhibited as boxplots representing the median (bar), the 25%-75% quantiles, data within (whiskers) and outside (outliers as black dots) the 1.5 times interquartile range. Statistical analyses were performed in R (RRID:SCR_001905). Walking speed data followed normal distribution whereas stripe deviation did not (Shapiro-Wilk test of normality, $p < 0.05$) so that we used the parametric Two-way ANOVA followed by TukeyHSD post hoc test and Welch Two-Sample t-Test, respectively, or non-parametric paired Wilcoxon rank sum test with Bonferroni-correction and Wilcoxon rank sum test, respectively. The p-value was additionally corrected for two repeated measurements of data achieved from Buridan's paradigm. The sample size of each group is indicated

within the graphs. Default alpha value was set to 0.005 (Benjamin et al., 2018).

Raw data and evaluation code available at DOI: 10.5281/zenodo.2625643

Results

Differential dominance of *tßh* mutation for different behavioral parameters

We examined the effects of the *tßh*^{nM18} mutation in two different experiments, assessing three different behavioral variables. We analyzed walking behavior in Buridan's paradigm (Bülthoff et al., 1982; Colomb et al., 2012), reporting both the median speed (a temporal measure of behavior) and stripe deviation (as a spatial measure assessing object fixation). The second experiment quantified sugar responsiveness after 20 h of starvation using proboscis extensions (Damrau et al., 2017b).

Homozygous mutants behaved significantly differently from genetic background-matched control flies in all three measures: homozygous female mutants showed reduced walking speed (Fig. 1A), fixated the stripes more closely (Fig. 1B), and were less likely to extend their proboscis to a sugar solution after starvation (Fig. 1C) compared to control flies with two intact *tßh* loci. Flies heterozygous for the *tßh*^{nM18} mutation did not behave similarly homogeneously across the three observed variables. In walking speed, heterozygous flies exceeded wildtype animals by about 20% (Fig. 1A), indicating overdominant inheritance. In stripe deviation, heterozygous flies behaved more similarly to the mutant flies than to the wild type control flies, evincing a dominant inheritance (Fig. 1B). In the proboscis extension experiment, starved heterozygous mutant flies extended their proboscis two times (the

median), compared to the one time for homozygous mutants and the three times for the wild type females (Fig. 1C). Despite thus coming to lie exactly halfway between the two homozygous groups, indicating intermediate inheritance, the heterozygote data are not statistically different from the wild type controls, indicating a recessive phenotype.

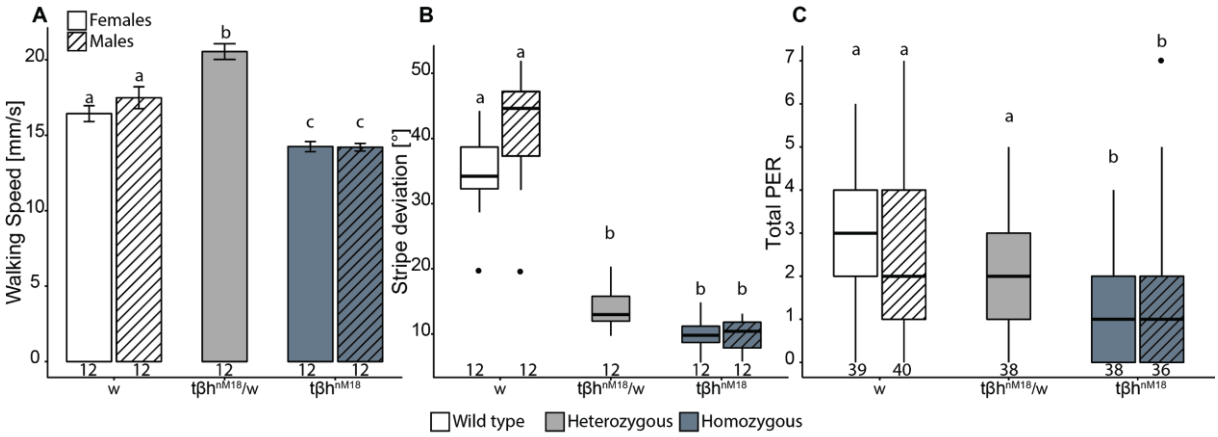

Fig. 1: Differential dominance of *tßh* mutation for different phenotypes.

Homo-, hetero- and hemizygous *tßh* mutants and their control were tested in Buridan's paradigm and the sugar sensitivity test. **A.** Median walking speed in Buridan's paradigm. Homozygous $tßh^{nM18}$ mutants walk slower than the wild type control, whereby heterozygous mutants walk faster than wild type (two-way ANOVA followed by TukeyHSD post hoc test, $p < 0.005$). Hemizygous mutant males walk slower than wild type males (Welch Two-Sample t-Test, $p < 0.005$). **B.** Stripe deviation, a measure of stripe fixation during walking, is reduced in homozygous $tßh^{nM18}$ mutants compared to heterozygous mutants and wild type (paired Wilcoxon rank sum test with Bonferroni correction for repeated measurement, $p < 0.005$) as well as in hemizygous males compared to their wild type control (Wilcoxon rank sum test, $p < 0.005$). **C.** The total number of proboscis extension response to a serial dilution of sucrose after 20 h of starvation was calculated. Homozygous and hemizygous $tßh^{nM18}$ mutants respond less to sucrose compared to wild type controls; heterozygous mutants are not statistically different from wild type but different from homozygous mutants. Significant differences are tested by paired Wilcoxon rank sum test with Bonferroni correction ($p < 0.005$). In **A**, bars and error bars indicate mean and standard error of the mean. In **B** and **C**, the Tukey boxplots represent the median (bar), 25%-75% quartiles (boxes), and total data range (whiskers) excluding outliers outside of the 1.5x the interquartile range (points). Numbers below graphs indicate sample size. Bars and boxes labeled with different letters are statistically significantly different.

Interestingly, the phenotypes of the heterozygous mutants cannot be explained by the presence of only one copy of the *tßh* gene, as the hemizygous wild type male flies also

only contain one copy (via their single X-chromosome), but behave indistinguishably from wild type females (Fig. 1).

As some aspects of Buridan's paradigm have been shown to be highly sensitive to genetic background (Colomb and Brembs, 2014) and the stripe deviation for the w+ control strain appeared unusually large, we repeated the locomotion experiment using flies with a different genetic background. We examined *tßh* hemizygous mutants and control males resulting from a cross to another wild type background. We found the same walking speed and stripe deviation phenotypes for *tßh* mutants in the W+/CantonS-background ($p < 0.05$, $n = 34$, Welch Two-Sample t-Test for speed, Wilcoxon rank sum test for stripe deviation; data shown at DOI: 10.5281/zenodo.2625643).

Taken together, we find that flies heterozygous for the $tßh^{nM18}$ mutation provide evidence for three different modes of inheritance, depending on the phenotype analyzed, conforming to the definition of differential dominance.

Driving tßh expression via GAL4-UAS

What consequences can differential dominance have on standard genetic techniques, commonly leveraged to understand gene function? To tackle this question, we started with a tried-and-tested method of transgenically expressing a wild type version of the mutated gene in various tissues using the GAL4-UAS system (Brand and Perrimon, 1993; Saga, 1998; Zars et al., 2000; Yokokura et al., 2004; Zhou and Rao, 2008; Chun et al., 2010; Park et al., 2011; Kirshenbaum et al., 2016). Here, we drove UAS-*tßh* in different cells in *tßh* mutant males: in all cells (actin-GAL4), in all neurons (nSyb-GAL4), in tyraminergic non-neuronal cells (TDC1-GAL4), in tyraminergic neurons (TDC2-GAL4) and in octopaminergic neurons (NP7088-

GAL4). All of those lines drive expression throughout development and in adulthood. Because we have already successfully used these techniques on sugar responsiveness (Damrau et al., 2017a), we focus on the walking measures from now on.

Despite the GAL4-UAS technique yielding rescue effects for a *tßh* phenotype before by others (Zhou and Rao, 2008), as well as in our laboratory for sugar sensitivity (Damrau et al., 2017a), neither the temporal nor the spatial walking measure showed any rescue for any of the targeted tissues (Fig. 2). In fact, for stripe deviation, some drivers yield even stronger stripe fixation than the mutant control strains (Fig. 2B). In both measures, some of the lines carrying the rescue construct alone already fail to show the mutant phenotype.

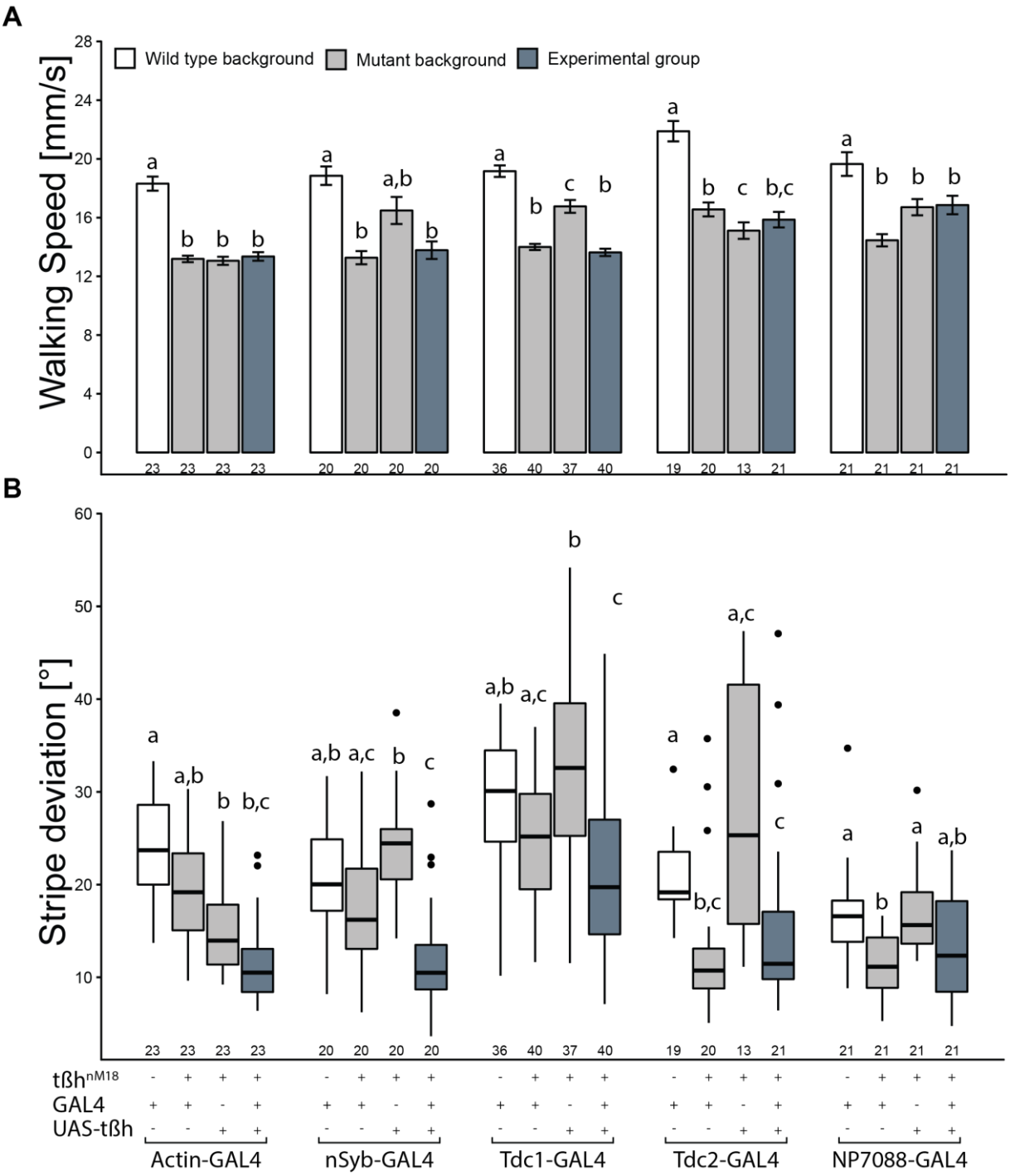

Fig. 2: UAS-*tßh* expression driven by different GAL4 lines cannot rescue the mutant locomotion phenotypes.

A. Median walking speed cannot be rescued by heterozygous GAL4-UAS-dependent *tßh* expression in mutants. All groups are different from the wild type control, except for the UAS-*tßh* control in the nSyb experiment (two-way ANOVA with TukeyHSD post hoc test and correction for multiple measurements, $p < 0.05$). **B.** Stripe deviation performance is already increased by the presence of the GAL4- or the UAS-construct. Ubiquitous Actin-GAL4 or pan-neuronal nSyb-GAL4 expression worsens the phenotype compared to the control lines (paired Wilcoxon rank sum test with bonferroni correction, $p < 0.05$). Explanation for bars, Tukey boxplots, numbers and letters is identical to Fig. 1.

Either the mutation in the *tßh* gene is not responsible for the phenotypes, or the differential dominance of the mutation can prevent GAL4-UAS-based expression effects from rescuing some mutant phenotypes (this work), but not necessarily all (Zhou and Rao, 2008; see, e.g., Damrau et al., 2017a).

Acute *tßh* expression differentially affects walking speed and stripe fixation.

Another commonly used technique to establish the specificity of the mutation for the phenotype is to ubiquitously express a wild type variant of the gene in the mutant background after development in the adult fly, i.e., right before the experiment. In our case, we expressed the *tßh* gene in homozygous $tßh^{nM18}$ mutant females under the control of the heat shock promoter *hsp-tßh* (Schwaerzel et al., 2003). A heat shock was induced for 45 min at 37°C and flies were allowed to recover for 3 h. After this treatment, rescue flies walked faster than controls (Fig. 3A), phenocopying the results of the heterozygote flies (Fig. 1A). These results did not quite reach the 0.005 alpha threshold, but passed the 0.05 threshold for suggestive effects. Given the behavior of the heterozygous flies, it is straightforward to assume an analogous overdominant effect in this case. This result also establishes the *tßh* gene as responsible for the mutant phenotype in this case. In contrast, expressing the *tßh* gene in this way left stripe fixation unaffected (Fig. 3B), similar to how heterozygous flies' stripe deviation was indistinguishable that of homozygous mutant flies (Fig. 1B). As published previously (Damrau et al., 2017a), sugar response after heat shock rescue was significantly improved (Fig. 3C, from Damrau et al., 2017a) without reaching wild type performance, similar to how heterozygous flies show an intermediate

number of proboscis extensions when compared with the two homozygous groups (Fig. 1C).

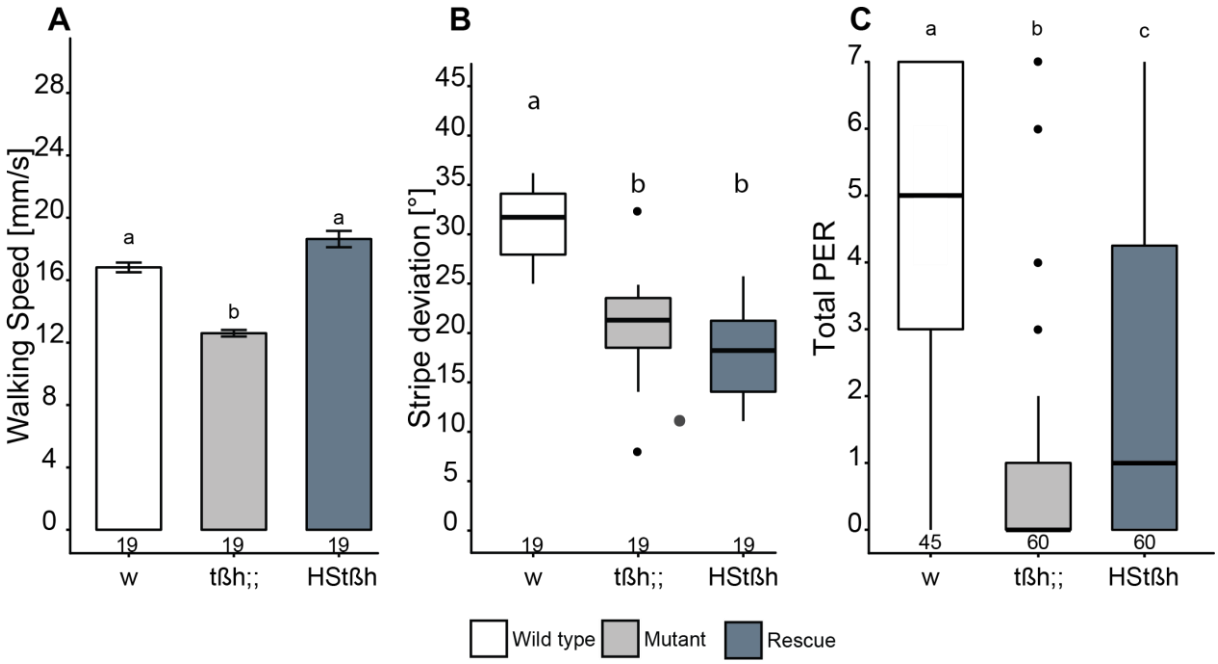

Fig. 3: Acute and ubiquitous *tßh* expression rescues walking speed and sugar response but not stripe deviation.

tßh expression was induced with a heat shock 3 h before the test. **A.** Median walking speed is increased beyond wild type levels after *tßh* induction (two-way ANOVA followed by TukeyHSD post hoc test, $p < 0.005$, $F = 69.8$). **B.** Stripe deviation is not affected by *tßh* induction. **C.** Sugar response is increased by *tßh* expression, but does not reach wild type levels (paired Wilcoxon rank sum test with bonferroni correction, $p < 0.005$, data already published in Damrau et al. (2017)). Explanation for bars, Tukey boxplots, numbers and letters is identical to Fig. 1.

Taken together, the results from heat shock-induced expression of *tßh* in the mutant background (Fig. 3) phenocopied those of the heterozygous flies (Fig. 1) throughout. This constitutes evidence that indeed the *tßh* gene is involved at least in the walking speed and sugar sensitivity phenotypes studied here and that differential dominance may be responsible for the difficulties rescuing the phenotypes in Fig. 2.

Overexpressing *Tdc2* and *tßh* differentially affects walking speed and stripe fixation.

The results so far seem to suggest that momentary levels of *tßh expression are* correlated with *tßh*-associated phenotypes and *not tßh* expression throughout development. To test this hypothesis, we increased the acute expression of the *tßh* and Tdc (tyrosine decarboxylase; synthesizes TA from tyrosine) enzymes in wild type animals (Fig. 4). While *tßh* overexpression is assumed to lead to OA production from TA and hence a decrease in TA titers, the Tdc overexpression should lead to increased TA production and hence a subsequent increase in OA concentration as well.

Overexpressing *tßh,* presumably decreasing TA levels and increasing OA levels*,* had no effect on walking speed (Fig. 4A), but decreased stripe deviation (Fig. 4B).*Tdc2*-overexpression, presumably elevating both TA and OA above wild type levels, reduced walking speed (Fig. 4B), but yielded a stripe deviation phenotype in the middle of the (large) range of variation found in driver and effector lines.

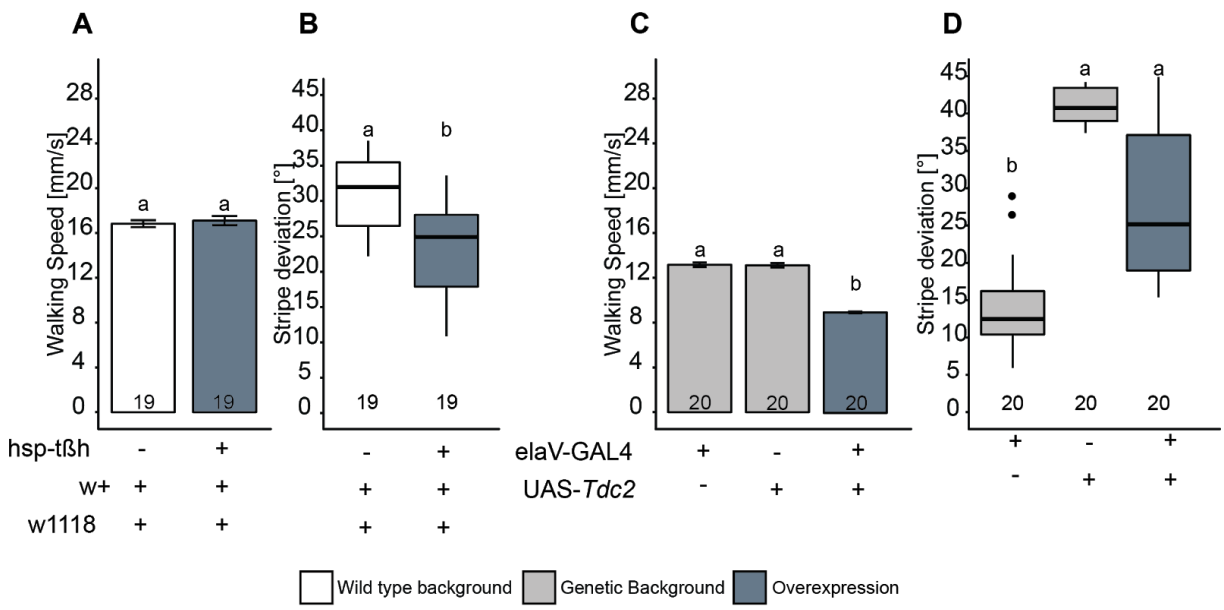

Fig. 4: Acute overexpression of TA and OA synthesis enzymes in wild type background affects flies' behavior in the Buridan's paradigm.

A. Median walking speed is not affected when *tßh* is overexpressed in wild type 3 h before testing via *hsp-tßh* (Welch Two-Sample t-Test, $p < 0.05$). **B.** In contrast, stripe deviation is reduced when *tßh* is overexpressed (Wilcoxon rank sum test with correction for multiple measurements, $p < 0.05$). **C.** Median walking speed is reduced after overexpression of Tdc2 (two-way ANOVA followed by TukeyHSD post hoc test, $p < 0.05$). **D.** The two parental controls show very different stripe deviation behavior, which prevents the interpretation of the performance of the overexpression group (paired Wilcoxon rank sum test with bonferroni correction, $p < 0.05$).
Explanation for bars, Tukey boxplots, numbers and letters is identical to Fig. 1.

These results hence suggest that overexpressing *tßh* selectively affected the spatial measure stripe deviation, while overexpressing *Tdc* seemed to mainly affect the temporal measure walking speed. Taking these data in with the results so far, there is now evidence that seems to suggest an involvement of the TA/OA system in all the phenotypes studied here, despite the varying results of the standard rescue experiments.

Differential involvement of OA and TA receptors on walking speed and stripe fixation.

To specifically affect the signaling of only one of the amines independently of the *tßh* locus, we manipulated the OA/TA system on the receptor level and examined several OA and TA receptor mutants, all outcrossed to the same genetic background.

We tested two alleles for each of two OA receptors *Oamb* and *Octß2R*, as well as one allele each for the three TA receptors *honoka*, *TyrR* and *TyrRII*, and a double receptor mutant for *TyrR* and *TyrRII*. While walking speed was affected in seven mutants (Fig. 5A, only $TyrRII^{\Delta 29}$ mutation had no effect), the stripe deviation was affected only in the two TA receptor mutants $TyrR^{f05682}$ and *honoka* in opposite directions (Fig. 5B). Interestingly, the double mutant $TyrRII\text{-}TyrR^{\Delta 124}$ showed no mutant phenotype.

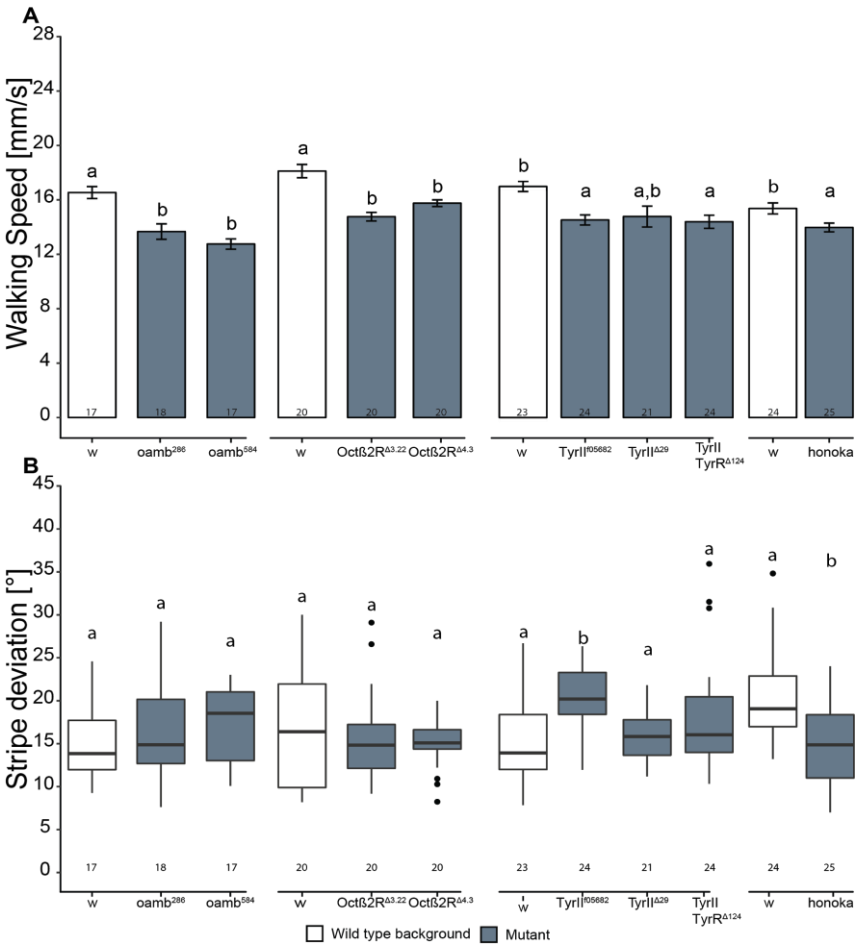

Fig. 5: Performance of flies mutant for TA and OA receptors in the Buridan's paradigm.

A. $oamb^{286}$, $oamb^{584}$, $Octß2R^{\Delta 3.22}$, $Octß2R^{\Delta 4.3}$, *honoka*, $TyrR^{f05682}$, and the double mutant $TyrRII\text{-}TyrR^{\Delta 124}$ walk slower than their respective controls, while $TyrRII^{\Delta 29}$ walking speed appeared at wild type level (Welch Two-Sample t-Test with correction for multiple measurements, $p < 0.05$). **B.** Stripe deviation is not affected in OA receptor mutants. In $TyrR^{f05682}$ and *honoka*, stripe deviation is significantly enhanced. Significant differences between control and the respective receptor mutant arte calculated by Wilcoxon rank sum test with correction for multiple measurements ($p < 0.05$). Explanation for bars, Tukey boxplots, number and letters is identical to Fig. 1.

These results support the hypothesis that TA and OA are involved in all the phenotypes studied here, despite the conflicting rescue data. However, in principle, one would need to rescue each of the receptor mutants as well to exclude effects from genetic variations outside of the targeted locus. With regard to the results of the rescue experiments shown here, we have abstained from such experiments and rely, instead, on the extensive outcrossing of the lines to homogenize the genetic background between mutants and controls, to decrease the chances of off-target differences.

Discussion

Differential dominance of the *tßh*^{nM18} mutation

A likely null mutation (Monastirioti et al., 1996) for the X-linked synthesis enzyme of the biogenic amine octopamine (OA), *tßh*^{nM18}, showed differential dominance in three different behavioral traits (Fig.1). Pleiotropic alleles often show differential dominance (Ehrich et al., 2003; Kenney-Hunt and Cheverud, 2009) accompanied by overdominance in some of the traits (Klingenberg et al., 2001; Cheverud et al., 2004). Such differential results exclude a single mechanism for the observed phenotypes, despite the shared involvement of a single gene. For instance, due to their homozygous sterility (Monastirioti et al., 1996) *tßh* mutant flies are usually kept in the heterozygous state. It is hence conceivable that any potential selective pressures may have increased fitness in the heterozygous state, providing a potential explanation for the increased walking speed in heterozygous mutants (overdominance; Fig. 1A). Additionally, since walking speed depends on multiple variables, a combination of recessive and dominant effects of the *tßh*^{nM18} allele on these variables may result in the overdominant phenotype,

as was proposed for morphological phenotypes (Klingenberg et al., 2001; Ehrich et al., 2003; Kenney-Hunt and Cheverud, 2009). However, these speculations do not explain the more recessive-intermediate (Fig. 1C) and, in the case of stripe deviation (Fig. 1B), dominant phenotypes observed in the other behavioral variables.

A staple in the genetic toolbox are rescue experiments, which serve to establish the spatiotemporal expression requirements of the gene in question for the phenotypes under scrutiny (e.g., Brand and Perrimon, 1993; Zars et al., 2000; Zhou and Rao, 2008). Such experiments are commonly carried out in order to arrive at necessity and sufficiency statements from which further mechanical understanding of gene function can follow (Gomez-Marin, 2017; but see also Yoshihara and Yoshihara, 2018). However, the implicit and all too often untested assumptions for these experiments is that the null mutations to be rescued follow recessive inheritance and that wild type level gene function can be restored with a single wild type allele.

Differential dominance affects the outcomes of standard genetic techniques

In this work, we not only introduced the wild type allele of the *tßh* gene in its genomic locus in heterozygous animals (and hence with certainly wild type spatiotemporal expression levels; Fig. 1), but we also deployed commonly-used used spatial (Fig. 2) and temporal (Fig. 3) transgenic rescue techniques, as well as transgenic overexpression in a wild type background (Fig. 4). While failed rescue experiments typically indicate that the mutated gene is not involved in the observed phenotype, the aggregate of all our experiments suggests that indeed the *tßh* gene seems

involved in the phenotypes we studied, despite several failed rescue experiments in the walking phenotypes.

Consequently, our data support the hypothesis that differential dominance significantly affected the outcomes of some of these standard experiments to an extent that make their interpretation difficult. For instance, it was impossible to pinpoint *tßh* gene function to a particular cell type, despite the evidence that *tßh* is indeed involved in walking behavior in Buridan's paradigm.

In order to provide an overview of the experiments carried out here, we ordered the gene dosage according to the number of *tßh* copies present in each animal, where a genomic version would count as a higher gene dosage than a transgenic version, regardless of actual expression levels, which are unknown. Plotting the relative performance in the three behavioral parameters against this classification of gene dosage, the wildly fluctuating consequences of manipulating gene dosage across parameters becomes apparent (Fig. 6).

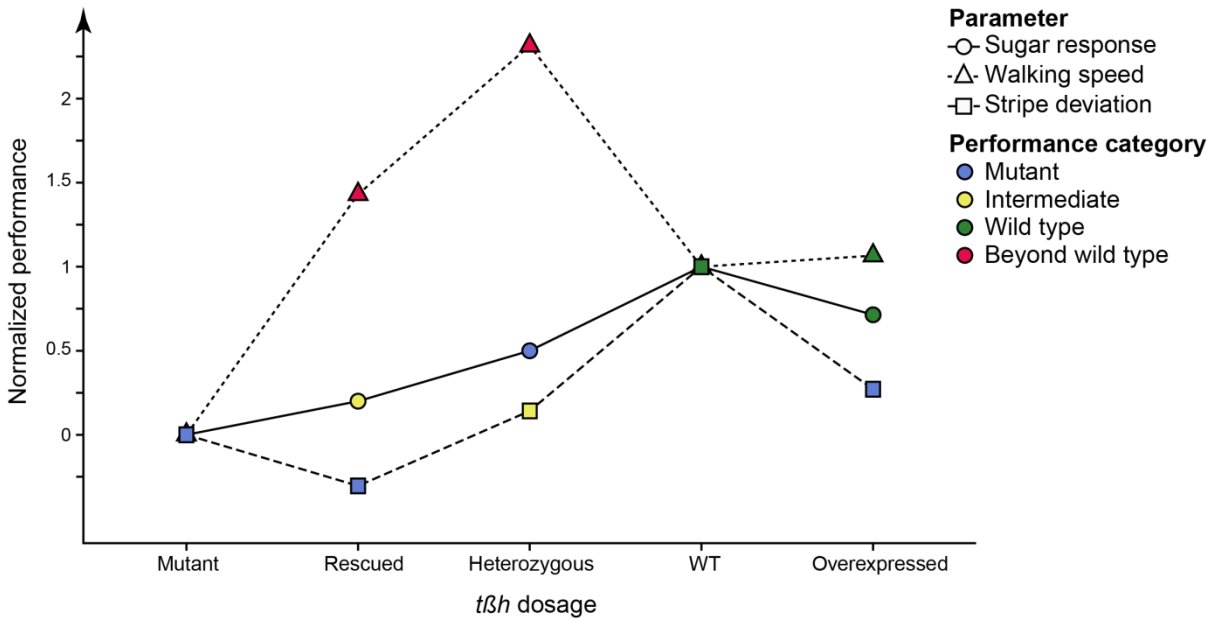

Fig. 6: Summary of phenotypic performance according to the *tßh* gene dosage.

Mean or median results were normalized to the performance of wild type and mutant controls for each experiment and depicted on the y-axis. The order on the x-axis broadly reflects the number of gene copies in each experiment, whereby the position of the rescue and heterozygote experiment was set arbitrarily. The color code indicates that the performance of the group is not statistically different from mutant (blue) or wild type (green), or that it is statistically different from both (yellow), or that it is significantly stronger than wild type (magenta).

While, for instance, any manipulation of *tßh* gene dosage leads to a significant reduction in stripe deviation (i.e., the flies fixate the stripe better), compared to the wild type controls, these same manipulations lead to walking speeds that are lower, higher, intermediate and indistinguishable from wild type. Only in one case is there even a resemblance of a monotonic curve, namely for the nominally recessive phenotype sugar responsiveness. The curve shapes suggest that the actual overall *tßh* gene dosage is a crucial, but non-monotonous determinant in control of the tested behavioral traits and that each trait is dependent on different gene dosages, according to its mode of inheritance.

It is straightforward to speculate that the acute sensitivity to *tßh* gene dosage may be representative for the sensitivity to actual OA/TA titers in the neuronal networks involved. However, for now, the exact translation of our gene dosages into relevant local amine concentrations remains experimentally undetermined.

Spatiotemporal control of walking

In addition to the effects of differential dominance on standard neurogenetic techniques, our experiments also yielded further insight into the spatiotemporal control of walking behavior (sucrose responsiveness is treated in detail in (Damrau et al., 2017a)). We discovered that *tßh* levels affect both how fast and in which direction flies are walking in Buridan's paradigm. In the absence of the *tßh* gene, flies walk more slowly and fixate the stripes better than control flies independently of their sex (Fig.1) or genetic background (Damrau et al., 2014). This latter test is important, since fixation scores were shown to be highly dependent on the flies' genetic background ((Colomb and Brembs, 2014); Fig. 4D). Acute and ubiquitous transgenic expression of *tßh* affected both walking speed and stripe fixation (Figs. 3, 4), confirming that those effects are *tßh*-specific.

Stripe fixation

In particular, any manipulation of the *tßh* gene increased fixation behavior of the flies, beyond wild type levels. Perhaps most strikingly, median stripe deviation was the lowest value for every single rescue construct we tested (Figs. 2B, 3B). This is unexpected, as part of such fixation behavior can be interpreted as an outcome of a fly's light/dark preference (Gorostiza et al., 2016). Gorostiza et al. (2016) discovered a correlation between dark-

preference in a T-Maze and tighter fixation behavior in Buridan's paradigm. With inhibited OA-ergic neurons, transgenic flies showed a lower dark-preference, while activated OA-ergic neurons increased dark preference. The results by Gorostiza et al. show a clear bi-directional involvement of OA in dark-preference and one would expect analogous manipulations of OA via its synthesizing enzyme to have analogous consequences. It is straightforward to hypothesize that the observed discrepancy may be explained by the involvement of TA in addition to OA in stripe fixation (and, by extension, light/dark preference).

Indeed, in our array of receptor mutants tested, OA receptor mutant flies do not fixate the stripes any different from control flies, while flies mutant for the TA receptors *honoka* and *TyrR*^{f05682} fixate the stripes less strongly than wild type controls (Fig. 5B). In other words, decreased TA signaling can lead to decreased stripe fixation (Fig. 5B), while the increased TA levels in *tßh* mutants (Monastirioti et al., 1996) can explain some of the increased fixation behavior in these flies (Fig. 1B). Although we have not tested all known OA receptors (Farooqui, 2012), considering the TA receptor and other data on stripe deviation, this suggests that TA may act independently of OA on this behavioral trait with an increased TA activity leading to a stronger stripe fixation. We thus conclude that our manipulations of the OA synthesis enzyme *tßh* affected stripe fixation, at least in part, via an involvement of the OA precursor TA.

Walking speed

The contrast to stripe fixation (a spatial measure of walking behavior) could not be more stark in walking speed (a

temporal measure of walking behavior). While any manipulation of the *tßh* locus increased stripe fixation, irrespective of whether we removed or added *tßh* copies, adding or removing *tßh* genes both increased and decreased walking speed. For instance, removing one copy (i.e., in the heterozygous state) increased walking speed, while removing both (homozygous mutants) decreased walking speed (Fig. 1A). Thus, while one may assume that walking more slowly would enable flies to fixate the stripe more accurately, in these experiments walking speed and stripe fixation are decoupled. While transgenic expression of *tßh* rescue constructs throughout development had no effect on walking speed (Fig. 2A), acute expression before the experiment yielded a phenocopy of the heterozygous flies: increased walking speed beyond wild type controls (Fig. 3A). These results seem to indicate that momentary levels of TA/OA are critical for walking speed, but transgenic expression of *tßh* during development can abolish this sensitivity. This hypothesis is contradicted by our overexpression results, showing the opposite pattern: acute *tßh* expression after wild type development does not affect walking speed (Fig. 4B), while transgenic *Tdc2* expression throughout development reduces walking speed (Fig. 4A). These overexpression results in walking speed are mirror-symmetric compared to those in stripe fixation, supporting the potential opponent role OA/TA may be playing in both parameters.

Due to these opposite results between spatial and temporal control of walking behavior and the highly varying nature of the walking speed results, one may speculate whether walking speed is either controlled by OA alone, or by OA in conjunction with TA. As we find that both OA and TA

receptor mutants are affected in walking speed (Fig. 5A), we conclude that both OA and TA signaling are involved in the control of walking speed.

TA and OA as opponent systems

Manipulating *tßh* expression modifies the balance of OA and TA in opposite directions (Monastirioti et al., 1996). Therefore, the *tßh* dosage dependence may be explained by the alteration of a fine balance between relative TA and OA concentrations, as particularly obvious in the walking speed parameter. In *Drosophila* larvae, it was suggested that the relative increase in OA levels but not the absolute endogenous amount is important for regulation of starvation-induced locomotion (Koon and Budnik, 2012). However, the interaction between the two neuromodulators seems to be more complex than a simple balance (Hoyle, 1985; Scheiner et al., 2002; Schwaerzel et al., 2003; Saraswati et al., 2004; Alkema et al., 2005; Fox et al., 2006; Fussnecker et al., 2006; Brembs et al., 2007; Pflüger and Duch, 2011).

Conclusion

We found differential dominance of the *tßh*^{nM18} mutation in sugar response, stripe fixation and walking speed. Interestingly, the commonly used genetic approaches to narrow down the functionality of *tßh* on the cellular level did not work in the locomotion-related behaviors, probably because the *tßh* gene dosage is crucial for these phenotypes. Therefore, our data indicate that common GAL4-UAS rescue experiments can only yield valid results for cases where the heterozygous mutants show a wild type phenotype. Moreover, we conclude that TA/OA signalling is involved in the spatiotemporal control of

walking, with different mechanisms for spatial and temporal parameters, respectively.

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