The Role of Monoaminergic Neurotransmission for Metabolic Control in the Fruit Fly Drosophila Melanogaster

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Hormones control various metabolic traits comprising fat deposition or starvation resistance. Here we show that two invertebrate neurohormones, octopamine (OA) and tyramine (TA) as well as their associated receptors, had a major impact on these metabolic traits. Animals devoid of the monoamine OA develop a severe obesity phenotype. Using flies defective in the expression of receptors for OA and TA, we aimed to decipher the contributions of single receptors for these metabolic phenotypes. Whereas those animals impaired in octß1r, octß2r and tar1 share the obesity phenotype of OA-deficient (tßh-deficient) animals, the octß1r, octß2r deficient flies showed reduced insulin release, which is opposed to the situation found in tßh-deficient animals. On the other hand, OAMB deficient flies were leaner than controls, implying that the regulation of this phenotype is more complex than anticipated. Other phenotypes seen in tßh-deficient animals, such as the reduced ability to perform complex movements tasks can mainly be attributed to the octß2r. Tissue-specific RNAi experiments revealed a very complex interorgan communication leading to the different metabolic phenotypes observed in OA or OA and TA-deficient flies.

Keywords: octopamine receptor, tyramine receptor, insulin, body fat distribution, insulin release

INTRODUCTION

Hormones are known to have a major impact on various metabolic traits. Among these hormones biogenic amines take a special position as they modulate these metabolic traits at different levels. Two of these amines, octopamine (OA) and tyramine (TA) are specifically relevant in invertebrates (Roeder, 1999, 2005). They act as functional equivalents of the vertebrate hormones/transmitters epinephrine and norepinephrine; similar to their roles in vertebrates, in which epinephrine- or norepinephrine-mediated signaling leads to a variety of metabolic changes (Debuyser et al., 1991; Bachman et al., 2002), OA and TA appear to be similarly potent in order to control metabolic traits in invertebrates (Lange, 2009; Li et al., 2016). Although TA and OA have been shown to act as independent neuroactive compounds, they share a large number of similarities (Roeder et al., 2003; Saraswati et al., 2004; Lange, 2009). Most importantly, OA producing cells always contain TA, as the latter one serves as a biological precursor for OA (Roeder, 2002, 2005; Cole et al., 2005). On the other hand, only very few neurons in the insect brain produce TA but no OA, making it hard
to examine how the different actions of both compounds are disentangled under physiological conditions (Monastirioti et al., 1995; Busch et al., 2009; Selcho et al., 2014). Beside the countless modulatory actions in invertebrates that can be attributed to these monoamines (Roeder, 1999, 2002), they have also been shown to regulate various metabolic traits. OA in particular appears to take a central position in regulating metabolism associated traits. It was shown that OA signaling is highly relevant in controlling behaviors with a direct impact on energy expenditure comprising the regulation of physical activity or the timing of sleep. Moreover, it was shown recently that OA directly affects the metabolic resting rate, therewith directly influencing fat storage (Li et al., 2016).

Although this role of both monoamines has mainly been studied in fruit flies, it appears also to apply to other insect and even to other invertebrates such as nematodes (Suo et al., 2006). As already mentioned, the vertebrate counterparts epinephrine and norepinephrine act in very similar ways as OA and TA do. In both systems, the corresponding hormones are released in times of stress and act as major transducers that orchestrate the organism’s stress reaction (Atgié et al., 1998; Adamo and Baker, 2011; Even et al., 2012). Release of these compounds should thus increase physical activity and resting metabolic rates reducing body fat stores. Diminished release of these compounds has exactly the opposite effects; it reduces activity and the metabolic rate, which leads, long-term, to more body fat.

Release of OA and TA modulates various behaviors and metabolic traits in a well-coordinated manner in order to shift the animal’s physiology to a high performance, high energy-expenditure state (Li et al., 2016). Thus, they appear to take a central position in the regulatory network responsible for inter-organ communication (Rajan and Perrimon, 2011). This comprises both types of behaviors, those that are associated with energy intake as well as those associated with energy expenditure. Food intake as the only energy source is also under the control of OA-mediated signaling (Zhang et al., 2013). Directly associated with this effects is the enhanced physical activity that is seen during periods of starvation, which appears to be devoted to enable efficient searches for novel food sources (Yang et al., 2015). OA and TA control movement activity and movement performance at different levels. In larval muscles, both compounds act antagonistically to each other. Whereas OA enhances the contraction properties of skeletal muscles, TA has the opposite effect (Saraswati et al., 2004; Selcho et al., 2012; Ormerod et al., 2013). Insect flight, which is the most energy-demanding physical activity, is also tightly controlled by OA signaling (Blau et al., 1991; Brembs et al., 2007), further showing the central role of monoaminergic neurotransmission for energy-demanding behaviors in general. Another behavior with a major impact on the balance between energy intake and expenditure is sleep, which thus takes a central position for energy homeostasis. In Drosophila, it was shown that the amount of sleep is directly correlated with starvation resistance (Slocumb et al., 2015). OA acts as a wake-promoting agent and impairments in the biosynthesis of OA are associated with enhanced daily sleep (Crocker and Sehgal, 2008; Crocker et al., 2010). At least in part, these effects of OA and/or TA are mediated through their modulatory action on insulin release from insulin producing neurons, which is thought to be mediated through the OAMB receptor located on these cells (Erion et al., 2012; Luo et al., 2014; Li et al., 2016). Recently, we could show that OA has a direct impact on energy expenditure-related metabolic traits, namely, it enhances the resting metabolic rate, thus reducing body fat. Consequently, reduced OA signaling leads to lowered metabolic rates and increased body fat with all its downstream consequences such as reduced life span and increased starvation resistance (Li et al., 2016). Reproduction, which critically depends on matching metabolic parameters, is also tightly controlled by OA signaling (Lee et al., 2003, 2009; Li et al., 2015), further demonstrating the role of OA to orchestrate numerous physiological actions within the organism.

Despite this body of information, we know little about the molecular mechanisms that are responsible for transducing the effects of either of these two monoamines into a suitable physiological reaction. Most importantly, the specific roles of the four OA and three TA receptors in this process remains to be elucidated (El-Kholy et al., 2015). Thus, we analyzed a set of transgenic animals impaired in expression of one of these different receptors each and employed RNAi experiments with the most relevant receptor genes targeted to major metabolic organs (brain, fat body and oenocytes).

**MATERIALS AND METHODS**

**Fly Stocks and Maintenance**

The fly stocks used in this study were as follows: TDC2 βR54 flies were generously provided by Jay Hirsh (University of Virginia, Charlottesville, VA, USA; Cole et al., 2005), TylfΔ18 flies were generously provided by Henrike Scholz (University of Cologne, Köln, Germany) and OAMB-defective flies by Kyung-An Han (University of Texas, El Paso, TX, USA; Lee et al., 2003). The Prome(E(800)-Gal4 (oenocyte-Gal4) line was obtained from Joel Levine (University of Toronto, Toronto, ON, Canada; Billeter et al., 2009). The octβ1Rβ2819, octβ2Rβ05679, octβ3RMB04794, TAR1P1281, TAR2MB03023 and TAR3MB09692 mutant lines used in this study were generated by the Gene Disruption Project (Bloomington Stock Center, Indiana, Bloomington, USA). The UAS-dsRNAi lines of octβ1R (#47895), octβ2R (#104050), octβ3R (#6099), TAR1 (#26876), TAR2 (#2857) were obtained from the Vienna Drosophila Resource Center. Other transgenic strains including nshy-GAL4 (#51635), ppl-GAL4 (#58768) were obtained from the Bloomington Drosophila Stock Center. All flies, unless otherwise stated, were raised on standard yeast/cornmeal/agar medium at 25°C with about 50%–60% relative humidity under a 12 h/12 h light/dark cycle as described previously (Rahn et al., 2013; Li et al., 2015). RNAi-mediated knockdown of OAR/TARs genes in different tissues was achieved by crossing UAS-receptor RNAi line to the tissue-specific promoter GAL4 line and the F1 generation flies were kept at 29°C to enhance the RNA interference, the parental lines crossed to w1118 were used as controls.

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RT-PCR Analysis
Total RNA was extracted from the brains of 15 females kept on normal food. RT-PCR was essentially performed following recently described methods (Li et al., 2015). The following primers were used: Rpl32 forward (5′-CCG CTT CAA GGG ACA GTA TC-3′), Rpl32 reverse (5′-GAC AAT CTC CTT GGC CTT CT-3′), Dilp2 forward (5′-CTG ATG ATG TGG GAG GA-3′), Dilp2 reverse (5′-GAC AAC TGG AGG GTA TG-3′), OAMB-F (5′-CGG TTA ACG CCA GCA AGT G-3′), OAMB-R (5′-AGG ATG CAC GAA ATA ACG GCT GC-3′), Octβ1R-F (5′-GGC AAC GAG TAA CCG TTT GG-3′), Octβ1R-R (5′-TCA TGG TAA TGG TCA CCG GC-3′), Octβ2R-F (5′-TCC TGG GGT ACA CAC TCT CCA-3′), Octβ2R-R (5′-CCA CCA ATT GCA GAA CAG GC-3′), Octβ3R-F (5′-TGG GGT CAA CAA GGC CTA CG-3′), Octβ3R-R (5′-GTG TTA TGC GGC GCT GTT AAG GA-3′).

Starvation Resistance Assays
The starvation resistance assays were performed on constant conditions mentioned above. Four to five-day-old adult flies were placed in vials containing 1% agar, and dead flies were recorded every 2–3 h until all flies died. For each genotype, at least 100 flies were used in this assay.

BODIPY Staining and Body Fat Determination
The whole fly bodies were collected and fixed in 4% paraformaldehyde for 30 min at room temperature. After washing with phosphate-buffered saline, the flies were repeatedly frozen in liquid nitrogen and thawed on ice three times, followed by staining with a solution containing 1 µg/ml BODIPY dye (Invitrogen, Darmstadt, Germany) for 1 h in the darkness before observation by epifluorescence microscopy (Olympus, Hamburg, Germany).

Glucose and Trehalose Measurement
The hemolymph glucose and trehalose measurement were performed using Glucose (HK) Assay Kit (Sigma, Steinheim, Germany) with minor modifications as described previously (Li et al., 2016). The hemolymph sample was pooled from 15 flies per genotype and added to 50 µl of glucose assay reagent. After incubation for 15 min at room temperature, the glucose levels were calculated according to the standard curve established by measuring absorbance at 340 nm. For trehalose measurement, 0.25 µl of porcine kidney trehalase (Sigma, Steinheim, Germany) was added to convert trehalose to glucose. After incubation at 37°C overnight, the absorbance was measured again, and the amount of trehalose was calculated.

Immunohistochemistry for dILP2 Measurements
Immunohistochemistry was performed as previously described (Li et al., 2016). The brains were dissected in Drosophila Ringer’s solution and immediately fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Subsequently, the samples were washed with PBST (0.3% Triton X-100 in PBS) and blocked in blocking-buffer (10% goat serum in PBST) for 30 min at room temperature, followed by incubation with the primary antibody (1:200 rabbit anti-dILP2, a gift from Eric Rulifson, UCSF, USA) overnight at 4°C with subsequent application of the secondary antibody (1:500 donkey anti-rabbit IgG, Jackson Immunolabs, Suffolk, UK) for 3 h at room temperature. After three washings, the brains were mounted on slides and images were obtained using a fluorescent microscope equipped with an apotome (Carl Zeiss Image AxioVision, Göttingen, Germany). To facilitate the quantification of dILP2 fluorescence intensities in the region of pars intercerebralis, series of sections were gathered under identical thickness, exposure time and all other relevant settings. Fluorescence intensity was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical Analyses
All statistical analyses were accomplished using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Starvation survivorship was analyzed by log-rank (Mantel-Cox) assays. Other parameters were evaluated using the unpaired two-tailed Student’s t test and one-way ANOVA. All data were presented as mean values ± SD.

RESULTS
Although TA is the biological precursor of OA, both monoamines act as independent neuroactive compounds in a wide variety of behavioral paradigms. Recently, we could show that differences between animals defective in tβh (TA, but no OA) and tdc2 (no OA, no TA) could be observed regarding their body fat storage (Li et al., 2016). We analyzed these phenotypic peculiarities in more detail and could show in the current work that other metabolically relevant traits also differ between both types of animals. Whereas hemolymph carbohydrate levels are lower in both sexes of the tβh-defective animals, we could observe sex-specific differences in tdc2-defective animals where
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FIGURE 1 | Regulation of metabolic traits in flies without octopamine (OA) and tyramine (TA; tdc2-deficient). Measurement of hemolymph carbohydrate levels (glucose and trehalose) in adult male and female flies of the control (w1118) and the TDC2 Ro54 genotypes (A). Relative fluorescence of dILP2 immunoreactive cells in the pars intercerebralis of control animals and TDC2 Ro54 animals of both sexes (B). Brains of female flies of control and TDC2 Ro54 animals were analyzed as in (B), but treated either with buffer, or with TA or OA prior to immunohistochemistry (C) (Mean values ± SD; N ≥ 5, *p < 0.05, ***p < 0.001).

males show the same phenotype as tβh-defective animals did, whereas females show the opposite phenotype (Figure 1A). This sex-specific discrepancy was also observed for food intake by tdc2-deficient females, which show reduced food intake while males did not show these alterations. Most impressive were the differences in metabolic rates, where only OA-deficient animals show a substantial reduction, while tdc2-deficient ones have an unaltered metabolic resting rate (Li et al., 2016).

Regarding insulin secretion, tdc2-deficient animals showed a slight reduction in the dILP2 content of the IPCs, which is equivalent to the situation under OA deficiency (Figure 1B). Feeding OA and TA to these animals led to a slight reduction in dILP2 release (Figure 1C).

In order to learn more about their relevance for various metabolic traits, we choose a series of Drosophila lines carrying insertions in the respective genes coding for OA and TA receptors that should effectively impair expression of functional proteins. OA and TA transmit their effects via a total of seven G-protein coupled receptors, with four being specifically tuned to react to OA and three to TA. They can be further subgrouped regarding their primary structures into a more alpha-adrenergic subtype (OAMB), those sharing similarities with ß-adrenergic receptors (Octß1R-Octß3R) and two classes of TA receptors (Maqueira et al., 2005; El-Kholy et al., 2015). The TAR1 (also known as TyrR, Oct/TyrR) and the other two TARs (TAR2, also known as TyrR1 and TAR3) do not cluster together (El-Kholy et al., 2015). A recent analysis utilizing promoter reporter

FIGURE 2 | Body fat content of flies deficient in different receptors receptive for OA or TA. BODIPY staining of the octß2R-deficient animals (A, bottom) are shown in comparison with labeling of matching controls (A, top). Quantitative total body triacylglycerol (TAG) assay was performed with adult females of the different genotypes (B) (Mean values ± SD; N ≥ 5, *p < 0.05, ***p < 0.01).
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lines revealed the spatial distribution of the different receptors in the different tissues of the fly. Flies without OA (∆βh-deficient animals) show an impressive metabolic phenotype, they are obese with fat deposits increased by more than 30% compared with matching controls (Li et al., 2016). We measured the triglyceride levels in different fly lines defective in expression of the corresponding receptors and observed that flies impaired in expression of the Octß1R, the Octß2R and the TAR1 showed a significantly enhanced fat deposition as observed by BODIPY staining of the corresponding animals, while the OAMB-deficient ones had reduced fat levels. As an example, we show the staining of the octß2r-deficient flies (Figure 2A). In order to quantify this effect, we measured triglyceride levels of these animals and obtained a similar result (Figure 2B). Results from this quantitative approach were almost congruent with that obtained using the fat staining approach. As the OAMB has already been described in greater detail (Erion et al., 2012), we excluded the OAMB from all downstream studies.

Differential fat deposition is assumed to directly influence important metabolic traits such as starvation resistance (Ballard et al., 2008). Thus, we analyzed starvation resistance in these animals and identified significant differences to matching control populations (Figure 3). Whereas the Octß1R, Octß2R and TAR1 (Figures 3A,B) show statistically significantly increased starvation resistances, the TAR2 had a lower resistance, whereas the resistances of the Octß3R and TAR3 were not different from the controls (Figures 3A,B).

One component that has a major effect on the fat content of flies is the level of insulin release from cells in the pars intercerebralis. We first analyzed which of the corresponding receptors are expressed in this peculiar brain region that contains different neurosecretory cells including those that produce and release the most important insulins in the fly (dilp2, dilp3, dilp5), but also those that produce, e.g., DH44, DH31 or SiFatamide. Thus, we isolated the pars intercerebralis region manually and used the resulting material as a template for RT-PCR. From the receptors tested, the OAMB, the Octß1R, the Octß2R and the TAR1 showed specific signals, which implied that they are indeed expressed in neurosecretory cells of the brain (Figure 4A). We reanalyzed some of the doubtful candidates genes using promotor-Gal4 lines and showed expression in lateral parts of the pars intercerebralis for some of them, which implies that most of the OA and TA receptors are present in the pars intercerebralis, presumably to modulate hormone release from the corresponding cells in this highly specialized brain region (Figures 4B–E).

Based on this information, we analyzed the dILP2 content in insulin-producing cells of the pars intercerebralis of female
flies of the corresponding lines (Figure 5A). Only flies with insertions in the \textit{octß1R} or \textit{octß2R} genes had statistically significantly different dILP2 levels in their insulin-producing cells. Directly associated with the insulin release is usually the hemolymph sugar level. We measured the combined sugar levels (glucose + trehalose) in the hemolymph of the corresponding flies and identified higher glucose levels for \textit{octß1R}-insertion-carrying flies (Figure 5B). The control of traits that are...
directly associated with energy expenditure is obviously highly relevant in this context. The ability to perform complex movement tasks, such as climbing vertical planes, was addressed (Pfeiffenberger et al., 2010; Li et al., 2016). For this, we used a simple negative geotaxis assay. OA (tβh-defective) deficient animals showed a massively reduced ability to perform this task. While flies impaired in the tar2 receptor showed an increased climbing activity, the tar1-defective lines showed reduced abilities. Most impressive were the impairments seen in lines with impaired octß2r expression, as they showed movement impairments that almost matched those seen in animals devoid of OA (Figure 6).

In order to elucidate the mechanisms underlying differential fat contents of the different receptor-deficient lines, we performed RNAi experiments with selected receptor lines. Silencing expression in the CNS using the nsyb driver line revealed slightly increase body fat especially for those animals with reduced octß1r and tar1 expression in the nervous system (Figure 7A). Targeting this intervention to oenocytes led to slightly reduced body fat in octß2r-deficient flies (Figure 7B). Silencing expression in the fat body (ppl-Gal4, Figure 7C) increased body fat only in case of tar1 silencing (Figure 7C). Moreover, we analyzed the effects of RNAi-mediated silencing in the oenocytes (Figure 8A) and the fat body (Figure 8B) on the release of dILP2 from insulin-producing cells in the pars intercerebralis. If we analyzed the dILP2 concentration in insulin producing cells in response to RNAi-mediated gene-silencing of the corresponding receptor genes, we observed no changes in response to manipulation in the oenocytes (Figure 8A), but a profound reduction in response to manipulation in the fat body for octß1r, octß2r and tar1 (Figure 8B).

**DISCUSSION**

To allow for a suitable organismal reaction in response to different internal or external situations, behavioral and metabolic traits have to be well-orchestrated. The monoamines OA and TA are central mediators of this complex interorgan communication system. Thus, they occupy the same position that epinephrine and norepinephrine take in vertebrates. Consequently, impairing their signaling properties incurs a great variety of metabolic alterations. Among these modifications, those associated with body fat stores are most conspicuous. Despite the structural similarities between OA and TA, they obviously act differentially in the regulation of major metabolic traits. Whereas animals devoid of OA (tβh-deficient) are obese, those without OA and TA (tdc2-deficient) are not, implying that the effects of both monoamines on this major metabolic trait are opposed. In order to learn more about the underlying mechanisms, we focused on the corresponding receptors, as results obtained with those animals defective in synthesis of either OA (TβH) or OA + TA (TDC2) are not easy to interpret. Tβh-deficient animals produce no OA, but contain higher amounts of TA, tdc2-defective animals, on the other hand, have neither OA nor TA, which complicates direct assignments of specific phenotypes to either OA or TA.

It was our assumption that the major metabolic phenotype of Tβh-deficient animals, the high fat content, is mediated via interaction with only one specific receptor, which turned out not to be the case. A total of two out of four different line defective in OA receptors (Octß1R, Octß2R) and one line defective in TA receptors (TAR1) showed an increased body fat content, thus phenocopying OA-deficient animals (see Table 1). Moreover, animals defective in expression of the oamb receptor gene are leaner than matching controls are. As expected, the increased body fat observed in some of the flies came with enhanced starvation resistances. This observation implies that mechanisms required to modulate body fat content are more complex than anticipated, thus mirroring the situation found in vertebrates, where regulation of body fat is mediated by sets of α- and β-adrenergic receptors rather than by only single representatives of this family.

It is not completely understood how signaling through these receptors controls body fat content. Different mechanisms have to be taken into account, comprising behaviors that are directly
linked with energy intake or energy expenditure (Crocker and Sehgal, 2008; Li et al., 2016). Moreover, controlling release rates of neurohormones or conveying direct effects on peripheral organs such as the fat body or skeletal muscles may also be relevant in this context (Crocker et al., 2010; Nässel et al., 2015). Regarding the expression profiles of the different OA and TA receptors, all options listed above have to be taken into account. Among these possible actions of OA and TA, the control of insulin release is most interesting, as it would add another mechanism to the list of almost identical functions shared by OA/TA and epinephrine/norepinephrine. It has been proposed that the OAMB receptor mediates the effects of OA on insulin release via direct control of release rates (Luo et al., 2014). The TAR1, which also is involved in body fat control (El-Kholy et al., 2015), which making a direct interaction possible.

Taken together, we aimed to understand the various facets of OAergic and TAergic control of metabolic traits in more depth using animals with defective expression of peculiar OA or TA receptors. It became apparent that a complex network comprising different receptors in different tissues is responsible for the control of metabolic traits such as body fat content. Whereas some actions of OA and TA can be attributed to specific receptor subtypes, this is not possible for others. The reduced ability to perform complex movement tasks appears to depend on OA signaling mediated via the Octß2R in skeletal muscles. The regulation of other metabolic traits appear to be much more complex and involve complex remote control effects, which might have been expectable especially as OA as well as TA are thought to take a central role in interorgan communication.

### AUTHOR CONTRIBUTIONS

YL, CF and TR conceived the study and wrote the manuscript, YL, LT, JF, SN, SE-K, FS performed experiments, YL, CG and IB evaluated the data.

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