Octopamine Signaling Via *Oamb* is Essential for A Well-Orchestrated Climbing Performance of Adult Drosophila Melanogaster

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Abstract

The biogenic amine octopamine (OA) orchestrates many behavioural processes in insects. OA mediates its function by binding to OA receptors belonging to the G protein-coupled receptors superfamily. Despite the potential relevance of OA for controlling locomotion, our knowledge about the role of each octopaminergic receptor still limited. In this study, RNA interference (RNAi) was used to knockdown each OA receptor type in almost all *Drosophila* melanogaster tissues using a tubP-GAL4 driver to investigate the loss of which receptor affects the climbing ability of adult flies. The results demonstrated that oamb-deficient flies had impaired climbing ability more than those deficient in other receptors receptive for OA. Targeted RNAi-mediated kockdown of oamb in the nervous system or muscular system decreased the climbing ability, indicating that within Drosophila legs, OA through oamb orchestrated the nervous system control and muscular tissue responses. Oamb-deficient adult males showed morphometric changes in the length and width of leg parts. Transmission electron microscopy revealed that the leg muscles *oamb*-deficient flies have severe ultrastructural changes compared to those of control flies. The severe impairment in the climbing performance of *oamb*-deficient flies correlates well with the completely distorted leg muscle ultrastructure in these flies. Taken together, we could conclude that OA via *oamb* plays an important role in the locomotor activity of *Drosophila*.

Introduction

Locomotor activity is an integrative characteristic of the functional state of the nervous system as it is implicated directly or indirectly in most types of insect behaviours such as foraging and mating. Distinct brain structures like the mushroom bodies or the central complex have been shown to be required for the control of locomotor activity (Martin et al. 1998; Strauss and Heisenberg 1993). In adult *Drosophila*, locomotor activity can be measured by different tools. Negative geotaxis performance is an innate escape behaviour in which adult flies ascend food vials as a response to tapping the vials. It could be used as a tool to evaluate the locomotor activity of *Drosophila*. This behaviour requires nervous system signaling commands via different biogenic amines.

Octopamine (OA) is one of these biogenic amines that acts as a neurohormone, neurotransmitter and neuromodulator in invertebrates. This catecholamine was first discovered in the salivary glands of *Octopus vulgaris* (Erspamer and Boretti 1951). OA is synthesised from the amino acid tyrosine. Tyrosine is converted by decarboxylation to tyramine by the enzyme tyrosine decarboxylase, and then tyramine is converted by hydroxylation to OA with the help of tyramine beta-hydroxylase. OA orchestrates many diverse physiological and behavioural processes including aggression, fight-flight response, circadian rhythm and locomotion (Roeder 1999). OA is essential for modulating the function of skeletal and visceral muscles (Orchard and Lange 1987). OA in invertebrates has the same function of noradrenaline in vertebrates. OA exerts these functions by binding to and activating OA receptors belonging to the G protein-coupled receptor superfamily. Drosophila possesses four OA receptors: Oa2 (also referred as Octß1R : CG6919), oamb (CG3856), Octß2R (CG33976, CG6989) and Octß3R (CG42244, CG7078) (Maqueira et al. 2005). OA stimulates glycogenolysis, modifies muscle contraction, supports long-term

flight and regulates “arousal” in the central nervous system (Brembs et al. 2007). Despite the potential relevance of OA for controlling locomotion, our knowledge about the role of each octopaminergic receptor still limited.

The GAL4/UAS system enables scientists to study either gain-of-function or loss-of-function of the gene (s) of interest (Brand and Perrimon 1993), facilitating studying gene expression in *Drosophila* (Fischer *et al.* 1988). The GAL4/UAS system has been used in RNA interference (RNAi) technology. This technology enables the knocking down or knocking out a specific gene of interest.

The main goal of this work was to reveal which one of the *Drosophila* OA receptors is important for adult locomotion. We achieved this by knocking down each OA receptor everywhere within the insect body using a universal driver and performing the negative geotaxis assay with the corresponding adults. Next, we explored whether the effect of OA originates from its effect on the nervous system or muscular system, and specific GAL4 drivers were crossed to OA receptor (s) followed by evaluation of the climbing ability performance of F1 male adults. Finally, we examined the morphometric and skeletal muscle ultrastructure changes in the legs of adult flies that displayed the knocking down of OA receptors. The findings of the current study provide evidence that OA via oamb orchestrates nervous system commands with muscular system response, consequently controlling balanced locomotion. Moreover, the current study is the first that link the leg muscles development with neurohormonal control. Information regarding biogenic amine receptors is very important for comparative, evolutionary physiology and biochemistry as well as serving as possible specific targets for insecticides.

Results

Crossings of three *GAL4* lines with each OA receptor were used to reliably dissect the most important receptor in negative geotaxis performance. First, RNAi targeted against the corresponding receptor genes was crossed with the tubulin promoter *GAL4*-driver line *tubP-GAL4* that drives expression in most organs of the fly. Data obtained revealed that despite all receptors receptive for OA have significant adverse effect on climbing performance, *oamb* -deficient flies showed the most severe impairment, which was highly significantly different ($P = 0.005$) from that of control flies (Fig. 1). The control male flies showed the native characteristic strait-line walking behaviour to climb the cylinder. By contrast, *oamb* -deficient male flies showed impairment in moving up against gravity. Some flies attempted several times to climb up the cylinder for a short distance, but they failed to maintain their equilibrium and fell into the bottom of the cylinder. Furthermore, few flies showed complete defective locomotion behaviour. They remained on the cylinder bottom walking very slow throughout the experiment. Therefore, we focused on whether the underlying mechanism of octopamine signalling via oamb in climbing performance originated from the nervous system or muscular system. To achieve this, the *oamb* RNAi line was crossed to *elav-GAL4* and *Mhc-GAL4* lines, respectively. The results showed that targeting the RNAi effect of *oamb* either to the nervous system or muscular system significantly (P 0.001) decreased the climbing ability of adult males compared with control flies (Fig. 2A,B). Because *oamb*-deficient flies had the weakest performance in climbing ability, we focused on the morphometric changes in *oamb*-deficient adult leg parts.

The length and width of each leg segment were measured. The results indicated that the lengths of all parts of forelegs (P = 0.001, 0.006 for coxa and trochanter ; P = 0.000 for femur, tibia and tarsi)

and hind legs (P = 0.000 for coxa, trochanter, femur; P = 0.046, 0.047 for tibia and tarsi respectively). *oamb* -deficient flies were significantly different from those of control flies. In addition, the width of the trochanter and femur of the forelegs (P = 0.000, 0.002 for trochanter and femur respectively) and the coxa, trochanter and femur of the hind legs were significantly (P = 0.000, 0.002, 0.032, respectively) different from those control flies (Fig. 3).

In this study, ultrastructure examination of the metathoracic leg of control flies revealed that the tubular oblique muscles consist of spindle-shaped myofibrils, arranged in parallel and had the known striated pattern of successive sarcomeres (Fig. 4A & B). All muscle fibres were ensheathed in a sarcolemma. The sarcolemma invaginates into fibres at regular distances. Each sarcomere extends between adjacent Z-discs of the myofibril. The Z line generally looks like a series of dense bodies and disconnected through the sarcomere. Their roundish mitochondria are present between the myofilaments and contain an electron-dense matrix. In *oamb*-deficient flies, the muscles displayed disorganisation of all components. The sarcomeres and the entire myofibrils showed loss of striations and structure (Fig. 4C & D). The sarcoplasmic reticulum around each myofibril was noticeably disintegrated such that division of the muscle fibres into myofibrils was indistinct. The myofibrils broke down such that there were spaces in the muscle fibre. The Z line split into smaller fragments that were dispersed among the sarcomeres. Although mitochondria were distributed along damaged myofibrils, some were enlarged with an irregular contour, some were small with a rounded contour and others were absent from certain areas of the muscle fibre. Their cristae were electron lucent.

Discussion

OA is a catecholamine that acts as a neuromodulator in invertebrates (David and Coulon 1985; Roeder 1999, 2002, 2005). The role of neuromodulators was explained by “orchestration hypothesis,” which assumed that neuromodulator release into specific neuropils configures neural commands to produce a coordinated network activity (Sombati and Hoyle 1984). OA mediates its function by binding to G protein-coupled receptors, which included cyclic AMP production or Ca^{2+} release. OA regulates many physiological processes including aggression, fight-flight response, circadian retime and locomotion (Riemensperger et al, 2005; Schwaerzel et al, 2003; Unoki et al, 2005; Zhou et al, 2008).

Each leg segment of the multi-jointed legs of adult insects contains a stereotyped arrangement of muscles. Contractions of these muscles through motor neurons control the coordination of locomotion. OA plays an important role in this process. In locusts, OA is delivered in time for enhancement of leg muscle contraction (Duch et al. 1999). In this study, we focused on the role played by octopaminergic receptors in balanced locomotion using RNAi of each receptor with three GAL4 drivers. Targeted RNAi-mediated knockdown using the tubulin-promoter GAL4 driver showed that *oamb*-deficient flies had the weakest performance in the negative geotaxis assay. The present study revealed significant impairment

in negative geotaxis performance when *oamb* deficiency was directed to the muscular or nervous system. The walking speed of *oamb*-deficient flies was lower than that of control *flies. Moreover, most of leg parts of oamb*-deficient flies was smaller than those of control flies. These findings are interesting as they provide evidence that *oamb* acts in leg muscles as a neuromodulator/neurotransmitter that is important for normal leg muscle architecture and size and for the coordination of muscle contractions required for balanced locomotion. A possible explanation for these structural changes is based on the known physiological role of *oamb* mediated signalling that induces Ca^{2+} oscillations due to Ca^{2+} release from intracellular stores (Balfanz et al,2005). This reaction is controlled by kinase and phosphatase activities (Hoff et al, 2011). Furthermore, blocking phosphatase activity in *oamb* expressing cells completely abolished Ca^{2+} oscillations (LIT). In mammals, it has been shown that Ca^{2+}-dependent pathways control muscle development (Damm and Egli, 2014). Lowering intracellular calcium levels inhibits the differentiation of skeletal myoblasts into mature myotubes (Porter et al, 2002). Moreover, it has been shown in mammals that adrenergic receptors signalling regulated myoblast differentiation (Saini et al, 2010; Church et al, 2014). This might explain, why the lack of *oamb*-mediated signalling events during development leads to the observed structural changes. The neuromodulatory role of OA in skeletal and visceral muscle contraction was reported before (Evans 1981). *Oamb* expression was reported in adult *Drosophila* leg muscles (El-Kholy et al. 2015). *Oamb*, the invertebrate counterpart of mammalian α-adrenergic receptor, is also expressed in the oviduct muscles of female insects and regulates their contraction by elevating the cytosolic Ca^{+2} level (Lee et al. 2003) and in the tracheal system (El-Kholy et al. 2015). OA through *oamb* regulates other physiological processes such the induction of insulin release from insulinproducing cells causing changes in the amount of daily sleep and changes in fat storage, leading to lean adult flies (Crocker and Sehgal 2008; Crocker et al. 2010; Erion et al. 2012; Luo et al. 2014; Li et al. 2016).

To verify the role of *oamb* in the formation of normal leg muscle architecture, leg muscles of male *Drosophila* were analysed using transmission electron microscopy. The results revealed that in *oamb* -deficient flies, the leg muscles displayed abnormal morphologies of sarcomeres, disorganised myofibrils and mitochondrial abnormalities. For these reasons, male *Drosophila* flies hypothesize to show severe defective locomotion behaviour. The sarcomeres and the entire myofibrils showed loss of striations and structure. The sarcoplasmic reticulum around each myofibril was noticeably disintegrated; consequently, division of the muscle fibres into myofibrils was indistinct. Moreover, the myofibrils broke down forming spaces in the muscle fibre. The thickness of myofibrils that could be observed in the leg muscles of *oamb* -deficient flies was significantly less than that the leg muscles of control flies.

Z-lines as anchoring structures for myofilaments dictate the final length of sarcomeres. Z-lines would be expected to participate in the organisation of myofilaments during the initial stages of myofibril assembly. The results of this study showed that Z-lines within the leg muscles of *oamb* -deficient flies were split into smaller fragments that were dispersed among the sarcomeres, causing loss of the normal muscle architecture and consequently impairment of the negative geotaxis performance of

corresponding flies. Recently, Sujkowski et al. (2020) reported that *oamb* expression in *Drosophila* is required for adaptations in skeletal muscles in legs.

Mitochondria play an important role in the muscular system. In *Drosophila*, myofibers have a greater dependency on mitochondria and lipid oxidation and, thus, the numbers of healthy mitochondria can influence the capacity to maintain muscle mass and function. In skeletal muscles, mitochondria regulate energy haemostasis by producing ATPs required for muscle contraction through oxidative phosphorylation. In addition, mitochondria contribute to Ca^{+2} homeostasis (De Stefani et al. 2011; Eisner et al. 2014), redox signalling (Finkel 2011; Sena and Chandel 2012), release of pro-apoptotic factors (Frezza et al. 2006), synthesis of haeme molecule and regulation of nuclear gene expression (Picard et al. 2014; Chae et al. 2013). In this study, ultrastructural changes were detected in the shape of mitochondria within the leg muscles of *oamb* -deficient flies. The mitochondria were electron lucent, enlarged with an irregular contour or small with a rounded contour and others were absent from certain areas of the muscle fibre. These changes could be the reason behind the observed disorder of muscles.

Ultrastructure changes in mitochondria occur in muscle disorders (Kelley et al. 2002; Chen et al. 2010; Crane et al. 2010; Sao *et al.* 2010; Austin and St-Pierre 2012), particularly in muscular dystrophy, while in degenerating fibres, the number of mitochondria is reduced and they disappear from severely atrophied muscle fibres.

Taken together, the data presented in this study reveal that octopamine signalling via *oamb* plays an essential role in adult *Drosophila* movement ability as well as in normal leg muscle architecture formation and interorgan communication between the nervous system commands to motor neurons and muscular tissue responses. It became apparent that information regarding biogenic amine receptors is very important form many points of view including comparative, evolutionary physiology and biochemistry as well as serving as possible specific targets for insecticides.

Materials And Methods

Drosophila strains and rearing

All *Drosophila* lines were obtained from Bloomington Drosophila Stock Center (Bloomington Stock Centre, Indiana University, Bloomington, USA). These lines included RNAi effector lines generated by the Transgenic RNAi Project for the knockdown of *Drosophila* OA receptors and three GAL4 driver lines (*tubP*-GAL4, *elav*-GAL4 and*Mhc*-GAL4). UAS effector lines and GAL4 driver were mated to a *w1118* control line and each RNAi experiment was done with these 2 controls in parallel. At least two available constructs for the same receptor-coding gene have been tested to be sure the observed phenotype is not due to off-target effects and the results presented in the manuscript were to the strongest construct which show high significant difference. All lines were reared on standard *Drosophila* medium (14–15 g agar, 18.5 g yeast, 61 g glucose, 30.5 g sucrose, 101 g corn meal/L) at 25 °C except for F1 of the crossings was kept

at 27-29 °C to enhance the RNA interference effect. All lines were kept at 50%-60% relative humidity with an 18/6-h light/dark cycle.

Climbing assay (negative geotaxis)

A negative geotaxis assay was performed using only male adult flies to avoid the effect of weight changes in adult female flies due to the physiological process including ovulation and oogenesis. Briefly, 10 males were placed into a 100 mL empty glass cylinder. The flies were tapped to the bottom of the cylinder and left to climb the cylinder. This procedure was repeated five times. The upward movement of the flies was recorded until the last climbing fly reached the top of the vial. The upward movement of flies was recorded with a digital video camera. The videos were cut into snapshots at the rate of 5 frames/second. These frames were analysed using ImageJ 1.46r software (National Institutes of Health, Bethesda, MD, USA) to trace the upward paths of the flies. The walking speed was calculated by dividing the length of the path (cm) by the time required in each path (s) (Rothenfluh and Heberlein 2000; Khurana*et al.* 2010).

Effect of OA receptor knockdown on climbing ability

OA receptors were silenced using the GAL4/UAS system. Three GAL4 drivers were used. These were the universal driver tubulin promoter GAL4 (*tubP*-GAL4), which induces gene repression in almost all insect's body cells, nervous system-specific driver *elav-GAL4* and muscular system-specific driver myosin heavy chain (*Mhc*-GAL4). Each one of these driver lines was independently crossed to the *Oa2*,*oamb*, *Octß2R* or *Octß3R* lines.The climbing ability of 10 F1 adult males was studied as described above. Adult F1 males of w^{1118} crossed to – GAL4 and – UAS lines were used as controls.

Morphometric analysis of adult legs

Fore and hind legs were removed by forceps from adult flies and then transferred to glass slides using a fine brush for microscopic examination using an Olympus BX61 light microscope at magnification 2.5×10. Photographs were taken and the length and width of each leg segment (coxa, trochanter, femur, tibia and tarsi) were measured using ImageJ software.

Transmission electron microscopy

Ten hind legs were removed by forceps. Then, the legs were fixed in 5% glutaraldehyde in sodium cacodylate buffer (pH 7.4) for 12–24 h. The specimens were washed and fixed overnight in 15% buffered osmium tetroxide. Samples were incubated overnight in 0.5% aqueous uranyl acetate solution, then dehydrated, filtered and embedded in spur resin (Davidson 1981). Semi-thin (1 µm) sections were stained with methylene blue Atur II and examined using an Olympus BX61 light microscope. Ultra-thin sections (60 nm) were cut using an ultramicrotome (Ultracut S, Leica), and stained with 2.5% uranyl acetate and lead citrate and were examined using JEOL, JEM 100-SX electron microscope. Sarcomere length (Z-Z

distance), myofibril and sarcoplasmic reticulum thickness and mitochondrial area were measured using Image J software.

Statistical analysis

The normality of the obtained data was tested to check if data were parametric or non-parametric using Kolmogorov-Smirnov test. Statistical differences in means of different biological attributes were calculated by one-way ANOVA followed by student *t*-test. The level of significance was set at $P < 0.05$ (Minitap 18.1 software).

Declarations

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CONTRIBUTION TO THE FIELD

Biogenic amines act as neurohormones, neuromodulators and neurotransmitters controlling many physiological processes in both vertebrates and invertebrates. Some commercially available pesticides act on biogenic amines such as Rotenone, neonicotinoids and formamidine . Biogenic amines-based pesticides have adverse effects on human, especially nervous system causing neurological diseases. For this reason, the biogenic amine octopamine and its related receptors represent good insecticide targets as being exclusive to invertebrates. Consequently, detailed information about the contribution of single receptor in vital physiological and behavioral processes may help to choose the most effective target for specific insecticides design tools. In this study, we focus on the contribution of octopaminergic receptors in locomotion, as a crucial behavior for survival. Results of tissue specific RNAi-based experiments revealed that *oamb*-deficient flies showed impaired negative geotaxis and leg muscles atrophy. In addition, this study revealed for the first time, the role of *oamb* in the development of normal leg muscles architecture. Previous studies stated that agonists and antagonists for octopamine receptors act as potential targets for insecticides (Evans and Maqueira, 2005) , here our results specify OA receptor oamb for molecular docking for synthesis of specific insecticides due to the essential role of this receptor in locomotion and skeletal muscle development. In addition to this, the data provide new insights into what may be a potential future prospect for human muscle atrophy therapeutics by targeting the α-adrenergic receptors, the counterparts of invertebrate *oamb*.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

SE-K suggested the study idea and designed the experiments, BA and SE-K performed experiments and evaluate the data, all authors wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

Figure 1

Climbing speed of adult *Drosophila* males deficient in four different receptors receptive for octopamine as compared to controls. The experiment was performed in triplicate. Mean ± SD; N=10; *P 0.05, **P 0.01, ***P 0.001.

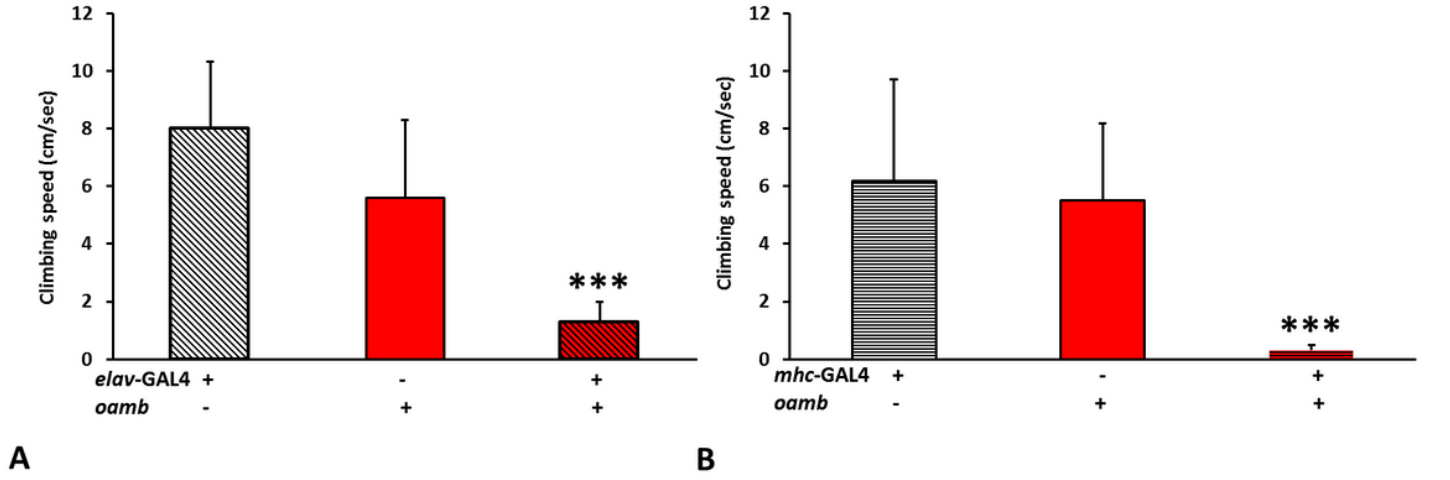

Figure 2

The climbing speed of *oamb*-deficient adult *Drosophila* males as compared to controls. The experiment was performed in triplicate**.**RNAi-mediated gene kockdown was directed to the nervous system using *elav-GAL4* driver (A) and to the muscular system using *mhc-GAL4* driver line (B). Three asterisks indicate high significance ($P \leq 0.001$).

Figure 3

Length and Width of fore and hind legs' parts of *oamb*-deficient as well as matching control adult *Drosophila* males.Mean ± SD; N=10; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Figure 4

Transmission electron micrograph of the leg muscular tissue in *oamb*-deficient adult *Drosophila* males . A and B TEM micrograph of male flies of matching controls of RNAi experiment. The muscles display features typical of insect skeletal muscle. C and D, TEM micrograph of *oamb*deficient flies leg muscles. M, mitochondria.Myo, myofibrils.Z, z-line.