Biochemical and Biophysical Research Communications xxx (2018) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Soy undecapeptide induces *Drosophila* hind leg grooming via dopamine receptor

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ARTICLE INFO

*Article history:*
Received 19 March 2018
Accepted 21 March 2018
Available online xxx

*Keywords:*
Soy peptide
Drosophila
Behavior
Dopamine receptor

ABSTRACT

β-Conglycinin α subunit (323-333) [βCGα(323-333)] is an exogenous neuromodulating undecapeptide found from enzymatic digest of β-conglycinin, a soy major storage protein by mice behavior tests. We investigated effect of βCGα(323-333) on *Drosophila* behavior. Oral administration of βCGα(323-333) in *Drosophila* increased hind leg grooming, which may act through specific sets of neurons. It was reported that dopamine receptor (*DopR*) meditates hind leg grooming, and we tested involvement of *DopR* in βCGα(323-333)-induced hind leg grooming by using *DopR* knockout flies. In the wild type but not in the *DopR*-knockout flies, βCGα(323-333) increased hind leg grooming. These results suggest that βCGα(323-333) induces hind leg grooming via activating the *DopR*. This is the first report showing that exogenously administered peptide changes fly behaviors.



## 1. Introduction

There is a number of endogenous peptide hormones and neuropeptides, which exhibit a variety of physiological effects. They are produced from precursor proteins and become active forms. In contrast, it was revealed that exogenous bioactive peptides are also produced after enzymatic digestion from various food proteins which were not recognized as such precursor proteins. Recently, we found that food-derived bioactive peptides interact with the nervous system and exhibit emotional behaviors [1,2]. Bioactive peptides originated from food proteins sometimes show emotional behaviors at a low dose comparable to pharmaceutical drugs.

We previously found that digest of β-conglycinin (βCG), a major soy storage protein, by chymotrypsin, present in the gastrointestinal tract, exhibits anxiolytic-like effect after oral administration in mice. Based on the structure-activity relationship and the comprehensive peptide analysis of the digest, we also identified an undecapeptide, corresponding to βCG α subunit(323-333), [βCGα(323-333)], exhibiting anxiolytic-like effect in mice [3]. In this study, we tried to test effect of this βCGα(323-333) on behavior using a model organism other than mice. Rodents such as mice and/or rats were generally used for screening for anxiolytics and antidepressants. A number of established behavioral tests in rodents are known: the open-field test, the elevated plus-maze (EPM) test, the light/dark exploration test, the forced swim test and the tail suspension test. In contrast, only a few studies have been found to measure emotional changes in insects such as *Drosophila*, which have been used as an attractive genetic tractable model to study various genetic disorders.

Here, we investigated effect of βCGα(323-333) on fly behavior. Following administration of βCGα(323-333) leads to an increased hind leg grooming, which was suppressed by *DopR* knockdown, suggesting the reduction of anxiety may work through dopamine receptor. Therefore, we propose here a novel mechanism in which behavioral change by soy β-conglycinin-derived peptide βCGα(323-333) may be mediated via the dopamine receptor.

## 2. Materials and methods

### 2.1. Fly husbandry and lines

Following flies were used in this study: Canton-S as a wild-type, Act5C-Gal4/CyO (DGRC #5176), and UAS-DopR1-IR (kind gift from Keleman Laboratory, Janelia, USA) [4]. All flies were reared in an incubator with 12:12 day: night cycle (lights on at 07:00 h and off at 19:00 h), maintained the temperature at 25 °C and humidity

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https://doi.org/10.1016/j.bbrc.2018.03.162
0006-291X/

40–60%. We used commercially available *Drosophila* food (Instant Drosophila medium, Carolina Biological Supply Company, Burlington, North Carolina 27215). Three to five day-old flies were used.

### 2.2. Genetics

*DopR*1 knockdown flies were generated by crossing the driver line Act5C-Gal4/CyO with UAS-DopR1-IR. CyO positive flies were used as a control.

### 2.3. Peptide treatment of Drosophila

βCGα(323-333) was originally derived from chymotryptic digest of soy β-conglycinin. Purification of this peptide was previously described [3]. Different concentrations of peptide (10 mM, 1 mM) were used for the behavioral assay.

### 2.4. Preparation of 96 well agar plate

250 μl of 1% agar solution was poured into each well of a 96 well plate. The plate was prepared about 2 h s in advance of the behavioral test and left at the room temperature (RT).

### 2.5. Preparation of 5 ml tube and transparent plastic sheet

Using needle (Terumo needle, 18 G X 1/2) a hole was made at the middle position of a 5 ml tube's lid. Then the glass capillary filled with peptide solution was inserted half way through this hole for the flies to drink. To allow fresh air flow into the tube, two more small holes were made on top of the lid by using another needle (22G X 1/2). A transparent plastic sheet was cut 8.0 cm long and 7.0 cm wide to cover the 96 well plate. This will keep the transfer process smooth and prevents flies from flying way. The plastic sheet is reusable.

### 2.6. Behavioral assay

The outline of the experimental procecure was shown in Fig. 1. All behavioral experiments were performed between 7 and 9 pm at RT, keeping the humidity 40–60% and luminosity range 330–350 lx.

On the day of behavioral experiment about 30–35 Canton-S male flies were isolated under $CO_2$ anesthesia, and left them for starvation and thirst for 9–10 h in the incubator. Just prior to assay, the flies in the vial was given cold anesthesia (ice-chilled) for 1–2 min and carefully transfer 5 flies into each 5 ml tube using a homemade ice chamber (fill ice to a yellow tip box's lid and place a glass plate on top). A glass capillary (Narishige, G-100 thin-walled glass capillary) was filled with 90% of peptide solution and mark the capillary wall up to the level of the solution. The capillary tube was then inserted half way through a hole made at the middle position of the 5 ml tube lid. The flies were then allowed to drink both control and peptide solution for 45–60 min. In order to measure the peptide mediated behavioral changes, we first feed Canton-S wild-type flies the control solution (milliQ water mixed with 0.125 mg/ml brilliant blue dye) and quantify behavioral activities. In the control experiment we observed the fly uptake of $H_2O$, which suggests that our starvation method could allow us to measure the uptake amount of a single fly and thereby its behavioral impact. Approximately a single fly can drink upto 1 μl of solution.

Following feeding the flies, the capillary tube was removed after 1 h and further anesthetized (ice-chilled) the flies keeping them in 5 ml tube for few minutes. Carefully transferred all flies using the same homemade ice chamber, one fly into each well of 96 well plate. After transferring every 5 flies by using forceps, covered the wells of 96 well plate with transparent plastic sheet to prevent flies from flying away. After placing all the necessary flies into 96 well plate, waited 10 min for the flies to recover from cold anesthesia. Using 9.7 inch iPad (Apple Inc, USA), a 10 min video was recorded to analyze the fly behavioral activities.

The 96 well plate was then left into −30 °C for 2 h for the flies to be dead. By using a light microscope, the fed flies were noted down for behavioral analysis. Manually quantified the activity of each fly from the recorded 10 min video. Free Stopwatch software (http://free-stopwatch.com/) was employed to quantify the time dedicated to each behavioral activity.

### 2.7. Statistical analysis

All values were expressed as mean ± S.E.M. Student's t-test was used to assess differences between control and sample administered group.

## 3. Results and discussion

### 3.1. Designing method to fly behavioral assay

After placing them into 96 well plate, flies were devoted either groom and walk, or no movement. Grooming event was initiated while flies cleaned their head, eyes, antennae, and mouth part using the front legs and the rest of the body parts with hind legs [5]. Hence, we categorized the grooming phenotype into front leg and hind leg grooming (Fig. 2A). Periodically, the flies were stopped to groom their legs itself. Besides this, each fly spent a minimum time to walk or no movement (Fig. 2B). From our recorded 10 min video, we observed that the flies spent ~43% of time for grooming and walking, while ~57% of time for no movement (Fig. 2B). During grooming, they spent more time on front leg grooming (~59%) compared to hind leg grooming (~41%) (Fig. 2C). On the contrary if we feed flies βCGα(323-333), the hind leg grooming is significantly increased compared to control group (water only) (Fig. 2E). This behavioral study was designed to measure the sole effect of compound/peptide effect on fly regardless of other food components. In mouse, grooming was reported as anxiety marker [6]. This will allow us to dissect precisely the underlying peptide mediated neural behavioral mechanism in insect model.

### 3.2. Soy undecapeptide βCGα(323-333) induces hind legs grooming

Recent discovery with various natural food derived bioactive peptides exhibited the potency to act on nervous system, and thereby play an important role in changing neural behavior [1,7–11]. Given the recent work identifying neuromodulating activities of βCGα(323-333) [3], we began to test behavioral effect on *Drosophila*. βCGα(323-333) was dissolved in water and feed Canton-S flies in order to investigate its effect on fly behavior. Our initial observation showed an increased hind leg grooming compared to control (no peptide) at a dose of 10 mM (Fig. 3). However, lower concentration (1 mM) did not show any significant difference (data not shown). This result suggests the stimulation of specific sets of neuron by βCGα(323-333) to increase hind leg grooming. We then decided to explore the βCGα(323-333) mediated underlying mechanism of neural behavior.

### 3.3. βCGα(323-333)-induced grooming is mediated through dopamine $D_1$ receptor

Dopamine is known to regulate insect grooming [12–14]. In order to explore the underlying neural mechanism of βCGα(323-

Please cite this article in press as: M.R. Karim, et al., Soy undecapeptide induces *Drosophila* hind leg grooming via dopamine receptor, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.03.162