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RESEARCH ARTICLE

Influence of an L-type SALMFamide neuropeptide on locomotory performance and muscle physiology in the sea cucumber *Apostichopus japonicus*

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ABSTRACT

Neuropeptides in the SALMFamide family serve as muscle relaxants in echinoderms and may affect locomotion, as the motor behavior in sea cucumbers involves alternating contraction and extension of the body wall, which is under the control of longitudinal muscle. We evaluated the effect of an L-type SALMFamide neuropeptide (LSA) on locomotory performance of *Apostichopus japonicus*. We also investigated the metabolites of longitudinal muscle tissue using ultra performance liquid chromatography and quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) to assess the potential physiological mechanisms underlying the effect of LSA. The hourly distance, cumulative duration and number of steps moved significantly increased in sea cucumbers in the fourth hour after injection with LSA. Also, the treatment enhanced the mean and maximum velocity by 9.8% and 17.8%, respectively, and increased the average stride by 12.4%. Levels of 27 metabolites in longitudinal muscle changed after LSA administration, and the increased concentration of pantothenic acid, arachidonic acid and lysophosphatidylethanolamine, and the altered phosphatidylethanolamine/phosphatidylcholine ratio are potential physiological mechanisms that could explain the observed effect of LSA on locomotor behavior in *A. japonicus*.

KEY WORDS: Echinoderm, Neuropeptide, Muscle relaxant, Locomotion, Longitudinal muscle, Metabolic physiology

INTRODUCTION

Neuropeptides are the oldest and most ubiquitous signaling factors between cells (Elphick et al., 1991b). They are secreted by peptide neurons and exert their effects on target cells by binding to their corresponding receptors, which typically are G protein-coupled receptors (Strand, 1999; Fricker, 2012; Gomes et al., 2013). They also constitute one of the largest groups of neurotransmitters that are involved in regulating various physiological and behavioral processes, such as energy metabolism, homeostasis, feeding and locomotor behaviors in animals (Fricker, 2012; Ding et al., 2020). Mature neuropeptides are stored in secretory vesicles, and neuronal depolarization triggers the fusion of secretory vesicles with the cell membrane to release the peptides into the extracellular environment (Fricker, 2012). Neuropeptides are degraded to amino acids by extracellular peptidases via the activation of their specific receptors (Fricker, 2012).

The SALMFamides are a family of structurally related neuropeptides that were discovered in echinoderms (Elphick et al., 1995, 2015). SALMFamide-1 (S1) and SALMFamide-2 (S2) were first identified from the radial nerve cords of the sea stars *Asterias rubens* and *Asterias forbesi* (Elphick et al., 1991a). These two SALMFamides are C-terminally amidated, and S1 is an octapeptide (GFNSALMFamide), whereas S2 is a dodecapeptide (SGPYSFNSGLTFamide). Both have the C-terminal motif FNSxLxFamide (where x is variable), indicating that S1 and S2 may have evolved as a result of gene duplication or intragenic DNA replication (Elphick et al., 2015). *In vitro* pharmacological studies revealed that both S1 and S2 can cause the relaxation of tube feet, apical muscles and the cardiac stomach in sea stars (Elphick et al., 1995; Melarange et al., 1999; Elphick and Melarange, 2001; Melarange and Elphick, 2003). In *A. rubens*, S1 and S2 are derived from different precursor proteins. The precursor of S1 contains seven SALMFamides that have a C-terminal LxFamide motif (where x is variable); thus, these neuropeptides are called L-type SALMFamides (LSAs). Although S2 has a C-terminal LxFamide motif, its precursor consists of seven neuropeptides with a C-terminal FxFamide motif; thus, the peptides in this precursor are known as F-type SALMFamides (Saleuddin, 2020).

In the sea cucumber *Apostichopus japonicus*, two precursors (AjL-SALMFaP and AjF-SALMFaP) that contain SALMFamides have already been identified (Elphick, 2012; Elphick et al., 2013). In contrast to the structures of SALMFamides in sea stars, there are three types of C-terminal motifs of neuropeptides in AjL-SALMFaP (LPFamide, IAFamide and ILLamide), and AjF-SALMFaP has the C-terminal motifs FxFamide LxFamide and FxLamide (Saleuddin et al., 2020). Among these neuropeptides, an LSA neuropeptide (RMGFTGNTGILL-NH_2) was identified by mass spectrometric analysis of circumoral nerve ring extracts. It is derived from AjL-SALMFaP, but it has rarely been studied till now (Chen et al., 2019).

Apostichopus japonicus is distributed widely along the shallow waters of northeastern Asia (Liao, 1980) and is an important fishery resource in China, Japan, Korea and Russia (Sloan, 1985). As an indispensable benthic organism in the marine environment, the activities of *A. japonicus*, such as feeding, moving and reproducing, may play important roles in the energy cycle of the marine ecological system (Pan et al., 2015). In addition, the relatively simple nervous system, slow locomotor velocity

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(<3 mm s^{-1}) and coelomic fluid that might be the vehicle for hormonal or neurosecretory activity make *A. japonicus* a perfect model species for studying the behavioral endocrinology of marine invertebrates (O'Shea and Schaffer, 1985; Byrne, 2001; Pan et al., 2015).

Previous expression and pharmacological studies on SALMFamides in sea cucumbers indicated their involvement in intestine and longitudinal muscle relaxation (Díaz-Miranda and García-Arrarás, 1995; Díaz-Miranda et al., 1995; Ohtani et al., 1999). However, little is known about the behavioral actions of SALMFamides and their underlying physiological mechanisms in sea cucumbers, especially the neuropeptides derived from the LSA precursor. The aim of this study was to investigate the effect of the LSA RMGFTGNTGILL-NH_2 on locomotory performance in *A. japonicus* and to evaluate changes in metabolite levels and metabolic pathways in longitudinal muscles that might underlie the physiological mechanisms that are involved in the effect of LSA on locomotion. We used infrared photography and professional behavioral analysis software to evaluate the locomotor behavior and applied ultra-performance liquid chromatography and quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) to investigate the metabolomic characteristics of longitudinal muscles in *A. japonicus*.

MATERIALS AND METHODS

Synthesis of LSA

The neuropeptide LSA is derived from the LSA precursor AjL-SALMFaP in *A. japonicus*, and its sequence and molecular formula are RMGFTGNTGIL-NH_2 and $C_{56}H_{95}N_{17}O_{15}S_1$, respectively (Rowe et al., 2014). Amidation of the C-terminal leucine in LSA was identified by mass spectrometric data (Chen et al., 2019). The solid-phase peptide synthesis method was used to produce LSA, and the structure of the synthesized LSA was established by mass spectroscopy. These procedures were performed by Shanghai Jill Biochemical Co., Ltd (Shanghai, China). The molecular mass of the synthesized LSA was 1278.55 g mol^{-1}, and its purity was 99.32%. LSA was stored at −20°C until use.

Animal rearing and maintenance

Sea cucumbers, *Apostichopus japonicus* (Selenka 1867), were obtained from an outdoor pond at the port of Zhuwang, Laizhou, China (37°15.656′N, 119°53.985′E). They were transferred to our laboratory in Qingdao and placed in 1500 l cylindrical aquariums filled with aerated sand-filtered seawater (water temperature 15±0.5°C, salinity 30‰, dissolved oxygen content 8.5 mg l^{-1}, pH 8.0). Sea cucumbers were fed a mixed diet (70% sea mud, 30% *Sargassum* powder) once a day, and the diet was put into each aquarium evenly at 08:00 h. Before feeding, feces and residual food were siphoned carefully, and half of the water in each aquarium was exchanged. The animals were maintained in these aquariums for 2 weeks.

LSA treatment, behavioral video acquisition and data analysis

Twenty-four healthy sea cucumbers were removed from the aquariums, weighed and randomly allocated to the control and LSA-treated (LSA) groups (99.6±13.8 g, n=12 individuals/group). Each sea cucumber was placed in one of 24 identical glass tanks (50×50×50 cm, water depth 17 cm) in order to record video of the locomotor behavior of each individual. A white acrylic plate was placed on the bottom of each experimental tank to highlight the sea cucumber and facilitate behavioral data analysis. An infrared camera (Hikvision DS-2CD3310D-I, 4 mm, Hangzhou, China) was fixed above each tank by a bracket, and the field of view was adjusted manually. The study was carried out under full dark conditions. Before the start of the experiment, sea cucumbers were placed in their tanks for 24 h to allow them to adapt to the experimental conditions (Ding et al., 2019). The locomotor behavior of each sea cucumber was recorded for 3 h before injection. LSA was dissolve in 1× phosphate-buffered saline (PBS) solution and diluted to 10^{-3} mol l^{-1} with sterilized sea water; each sea cucumber in the LSA group was injected with this LSA solution on the middle of the back of the body (injected volume/weight=0.1%) (Kato et al., 2009), and the final concentration of LSA in the sea cucumber was 10^{-6} mol l^{-1}. The sea cucumbers in the control group were injected with a mixed solution of 1× PBS and sterilized sea water (injected volume/weight=0.1%), and the concentration of 1× PBS was identical to that of the LSA solution. Under dim red light, a 1 ml syringe (JIANSH®, Luohe, China) with a 0.3 mm microneedle was used for injection (Pinillos et al., 2001). The locomotory activities of each sea cucumber were recorded for 9 h after injection (Ding et al., 2020).

EthoVision XT (10.1) software (Noldus Inc., Wageningen, The Netherlands) was used to quantify the locomotory performance of each sea cucumber. The behavioral indicators of locomotion in this study included total distance moved, cumulative duration of movement per hour, and mean and maximum velocity after injection. In addition, the number of steps (one contraction and relaxation of the sea cucumber body was defined as one step) that the sea cucumber moved per hour was counted, and the average stride length, frequency and velocity were calculated. One-way analysis of variance followed by Tukey's *post hoc* multiple comparison tests conducted with SPSS 20.0 software were used to analyze the behavioral data. A probability level of P<0.05 was considered to be statistically significant.

Longitudinal muscle tissue collection and UPLC-Q-TOF-MS metabolomics analysis

In this experiment, 48 healthy sea cucumbers were divided randomly into two groups (control and LSA groups, each with n=24) and used to take samples of longitudinal muscle tissues for UPLC-Q-TOF-MS analysis. The sea cucumbers in the control and LSA groups were treated in the same way as described above for the behavioral study. The locomotion experiment revealed that significant differences between the control and LSA groups occurred 4 h after injection. Therefore, the longitudinal muscle tissues of each sea cucumber were collected at this time point. Before dissection, sea cucumbers were anesthetized using magnesium sulfate (0.4 mol l^{-1}), which is considered to be an effective method with minimal negative effects on this species (Zhou et al., 2014). About 2 g of muscle tissue were removed from each sea cucumber, washed with ultrapure water, dried with absorbent paper, placed in a sterile tube and stored in a −80°C freezer. Muscle tissues from three specimens in each group were combined as one sample for the UPLC-Q-TOF-MS metabolomics test, which was used to detect the change of muscle metabolites after LSA administration. The procedures were the same as those described by Ding et al. (2019).

After inputting the raw UPLC-Q-TOF-MS data of longitudinal muscles into Progenesis QI software, a data matrix, including mass-to-charge ratio (m/z) values, peak intensity and retention time (RT), was produced. The main parameters were at default settings. Low-weight ions were eliminated (relative standard deviation >30%), and then we used the method of quality control-based robust LOESS signal correction to correct the data. The significantly changed

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Table 1. Longitudinal muscle metabolites with concentrations that differed significantly between the control and L-type SALMFamide neuropeptide (LSA) groups

Metabolite	Ion mode	Mass (Da)	RT (min)	VIP	$FC_{(LSA/control)}$	*P*
6-Carboxy-5,6,7,8-tetrahydropterin	neg	212.070	0.631	5.197	1.294	0.023
(E)-C-HDMAPP	neg	259.023	0.669	3.503	1.868	0.018
L-Hypoglycin A	pos	124.075	1.497	1.487	1.687	0.003
Pantothenic acid	pos	242.100	1.497	4.558	1.868	0.004
Pro Met Ser Glu	pos	463.186	1.497	2.876	2.034	0.007
PS(O-20:0/20:3(8Z,11Z,14Z))	neg	808.585	10.555	2.812	1.214	0.031
PE(19:0/22:2(13Z,16Z))	neg	858.621	10.987	3.876	0.692	0.019
Ganodosterone	pos	834.642	11.646	5.851	1.222	0.015
Cer(d18:0/22:0)	pos	646.612	12.643	1.301	1.273	0.015
Margaroylglycine	pos	672.589	13.461	2.552	1.383	0.044
N-Glycoloylganglioside GM2	pos	766.618	13.783	1.028	1.770	0.044
PC(20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	pos	844.624	13.999	2.519	1.169	0.041
LysoPE(0:0/14:1(9Z))	neg	424.237	4.745	1.013	1.872	0.006
Arachidonic acid	neg	303.233	5.975	2.486	1.387	0.003
15-HETE-T	neg	408.221	6.072	1.511	0.332	0.030
N-Arachidonoyl taurine	neg	410.237	6.757	2.076	0.507	0.039
5,6-Dehydro arachidonic acid	neg	301.217	8.090	1.437	0.534	0.021
PA(14:1(9Z)/18:1(9Z))	neg	625.423	8.090	1.202	0.234	0.019
Decanoylcholine	neg	259.244	8.820	1.308	0.519	0.011
Sodium tetradecyl sulfate	neg	361.169	8.820	1.187	0.616	0.022
NNAL-*N*-glucuronide	neg	385.147	8.820	1.638	0.482	0.021
Tiracizine	neg	403.158	8.820	3.476	0.442	0.015
Muricoreacin	neg	629.455	8.820	2.687	0.508	0.015
PS(20:3(8Z,11Z,14Z)/0:0)	neg	528.273	9.053	1.338	1.348	0.044
PC(19:0/0:0)	neg	536.372	9.195	1.150	0.334	0.018
Cer(d16:1/22:0)	pos	616.566	9.541	1.096	2.047	0.002
MG(0:0/20:3(11Z,14Z,17Z)/0:0)	pos	778.616	9.600	1.596	1.574	0.018

Data include the ion mode (positive, pos; or negative, neg), mass (compound molecular weight), retention time (RT), variable importance in the projection (VIP), fold-change (FC) and *P*-value of the metabolites. PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; MG, monoacylglyceride.

metabolites between the control and LSA groups were identified by partial least squares discriminant analysis (PLS-DA), orthogonal partial least squares discriminant analysis (OPLS-DA), fold-change (FC) analysis and the *t*-test on the UPLC-MS data. PLS-DA is a supervised analysis method, and it affords a comprehensive reflection of the distinction between the control and treatment groups (Boulesteix and Strimmer, 2007). Based on PLS-DA, OPLS-DA corrects the orthogonal transformation, which can filter out the noise irrelevant to classification information and improve the analytical ability and effectiveness of the model. In addition, the variable importance of projection (VIP) values was also calculated in the model. The metabolic biomarkers of the present study meet three requirements: VIP>1, FC>1.2/<0.8333, q<0.05. Finally, significant metabolites were searched in the Kyoto Encyclopedia of Genes and Genomes (KEGG) biochemical databases to annotate related metabolic pathways.

Ethical note

All procedures of the present study were performed under the Regulations of the Administration of Affairs Concerning Experimental Animals of China, as well as the Regulations of the Administration of Affairs Concerning Experimental Animals of Shandong Province. This work was approved by the Animal Welfare Committee of the Institute of Oceanology, Chinese Academy of Sciences (permit no. IOCAS 2019.06-2022.06).

RESULTS

Influence of LSA on locomotory performance of *A. japonicus*

Fig. 1A shows the hourly distance moved after injection. The total distance moved per hour during the 9 h following injection ranged from 140.11 to 230.98 cm in the LSA group and 112.97 to 164.40 cm in the control group, and increased significantly in the LSA group relative to the control at the third (Fig. 1A; P=0.034) and fourth (Fig. 1A; P=0.002) hour. The average distance moved per hour was 177.73±28.43 cm in the LSA group and 137.62 ±18.5 cm in the control group after injection. Fig. 1B shows the cumulative duration of movement per hour after injection. The hourly cumulative duration of movement within the 9 h after injection was higher in the LSA group than in the control group in each hour. Again, a significant difference between the two groups occurred at the fourth hour (Fig. 1B; P=0.011). The average cumulative duration of movement per hour during the 9 h after injection was 21.27±1.36 and 25.53±3.32 min in the control and LSA groups, respectively. In addition, the mean and maximum velocities of the LSA group were, respectively, 9.82% and 17.78% higher than those of the control group, but the differences were not statistically significant (Fig. S2, P=0.139; Fig. S3, P=0.117).

Fig. 1C shows the hourly number of steps taken after injection. The hourly mean number of steps taken by sea cucumbers within 5 h of injection was higher in the LSA group than in the control group in each hour, and the number of steps traveled increased significantly in the LSA group versus the control group during the fourth hour (Fig. 1C; P=0.034). The average numbers of steps taken after injection was 29.16±2.04 and 33.73±4.93 in the control and LSA groups, respectively. The treatment enhanced the mean locomotor stride by 12.44%, but the difference was not statistically significant (Fig. S1; P=0.051). In addition, the average duration of each step and step velocity in each hour also did not differ significantly between the two groups (Figs S4 and S5; P>0.05). The dataset of locomotory performance in this study has been uploaded to figshare (https://doi.org/10.6084/m9.figshare.14174099.v1).

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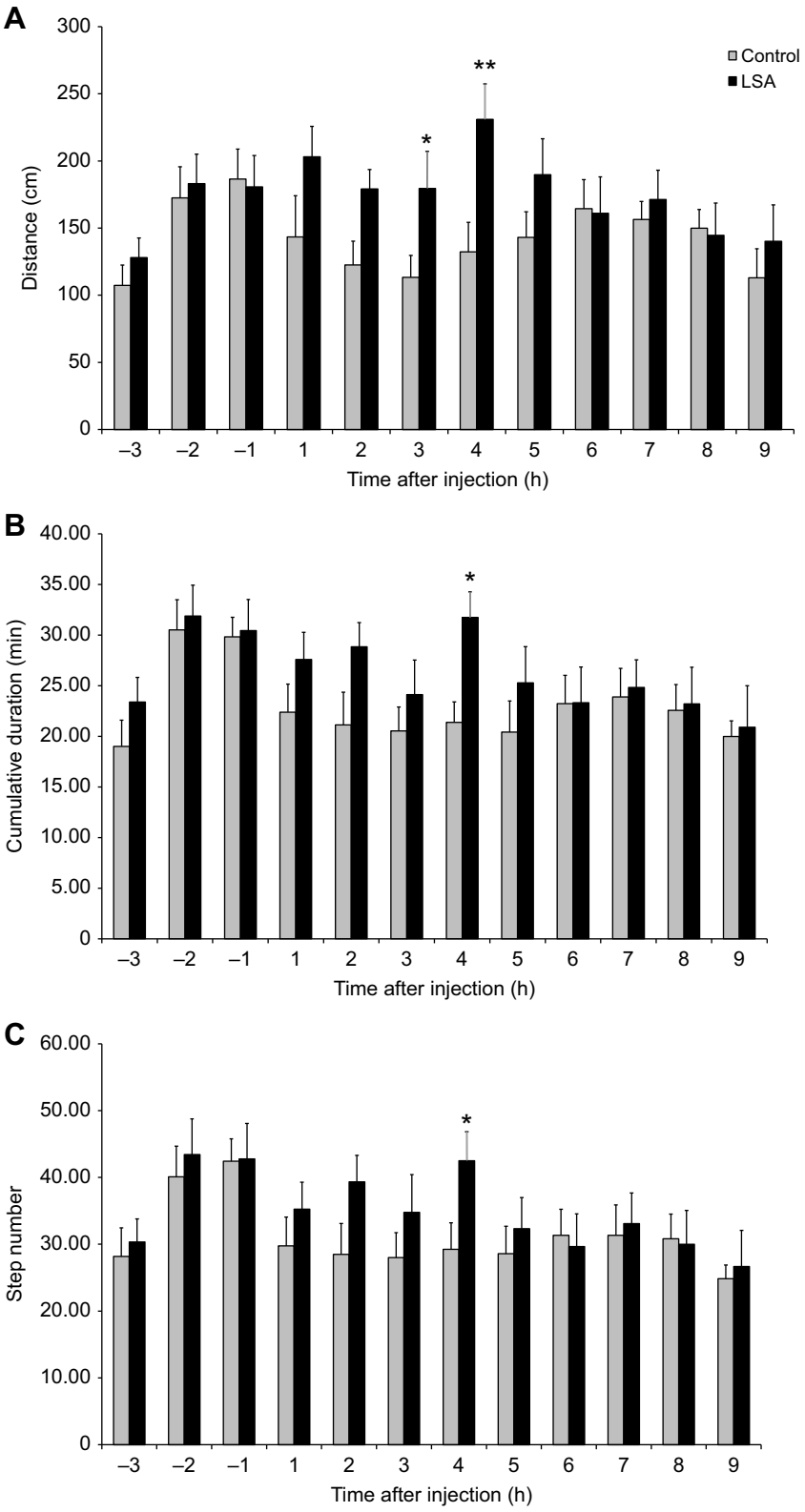

Fig. 1. Effect of L-type SALMFamide neuropeptide (LSA) on locomotory performance of *Apostichopus japonicus*. (A) Hourly total distance traveled, (B) cumulative duration of movement and (C) total number of steps taken by *A. japonicus* in the control and LSA groups. Each bar and vertical line represent the mean ±s.e.m. (n=12 for each group). One-way analysis of variance followed by Tukey's *post hoc* multiple comparison tests were used in analyses. Asterisks indicate a significant difference from the control in each hour (*P<0.05, **P<0.01).

Influence of LSA on longitudinal muscle physiology of *A. japonicus*

Both PLS-DA and OPLS-DA are supervised discriminant statistical methods that were used to identify the metabolic differences in longitudinal muscle tissues between the control and LSA-treated groups. The abscissa in the PLS-DA diagram represents the first principal component PC1 (t_1), and the ordinate represents the second principal component PC2 (t_2) (Fig. 2A). $R2X_1$ and $R2X_2$ are the cumulative interpretation rates of the model, and they were 0.237 and 0.134 for the PLS-DA of our results. OPLS-DA filters out the noise associated with the classification information of PLS-DA, which improves the analytical power and effectiveness of the model.

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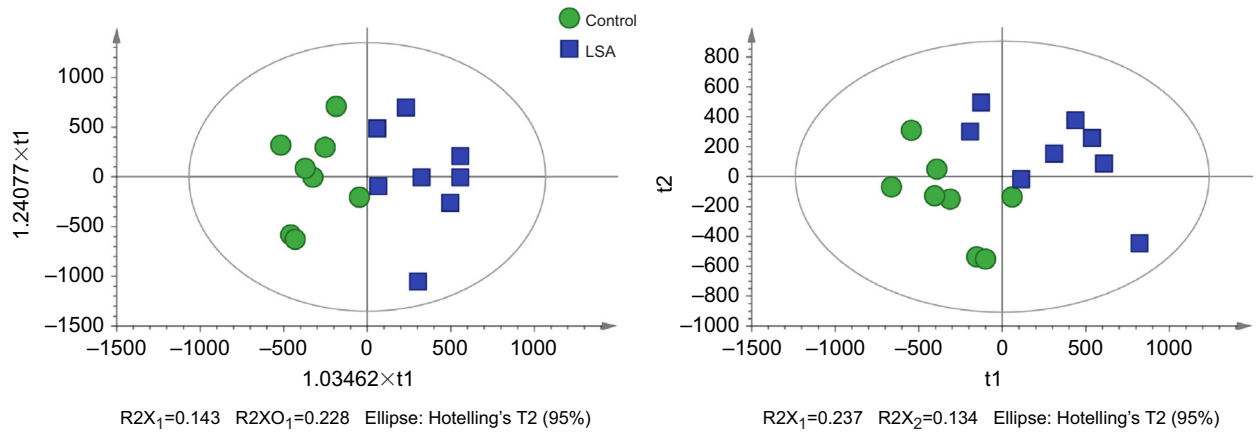

Fig. 2. Metabolite differences between control and LSA groups. (A) Partial least squares discriminant analysis (PLS-DA) and (B) orthogonal partial least squares discriminant analysis (OPLS-DA) scores. The *x*-axis and *y*-axis represent the first principal component (PC1) and the second principal component (PC2), respectively.

It maximizes the difference between groups. Each point in the figure represents a sample, and the control (green dot) and LSA (blue square) groups clearly are differentiated from each other (Fig. 2B). Fig. 3 shows a heat map of overall altered metabolites. The rows represent the changed metabolites and the columns represent the longitudinal muscle tissue samples. Different colors indicate different abundance intensities (mean value acquired from all detected samples from the same group). Both Figs 2 and 3 show significant metabolite differentiation between the control and LSA groups.

Analysis using *t*-tests ($P<0.05$) and the OPLS-DA model (VIP>1.0) identified 27 key metabolites from the positive (10) and negative (17) ion patterns that showed significant changes; levels of 16 of them increased and levels of 11 decreased after LSA administration (Table 1). The increased metabolites included pantothenic acid, lysophosphatidylethanolamine (LysoPE), arachidonic acid (ARA), phosphatidylcholine [PC(20:0/22:6)], *N*-glycoloylganglioside GM2 and Cer(d16:1/22:0). Levels of metabolites such as phosphatidylethanolamine (PE), PC(19:0/0:0), *N*-arachidonoyl taurine, tiracizine and muricoreacin were

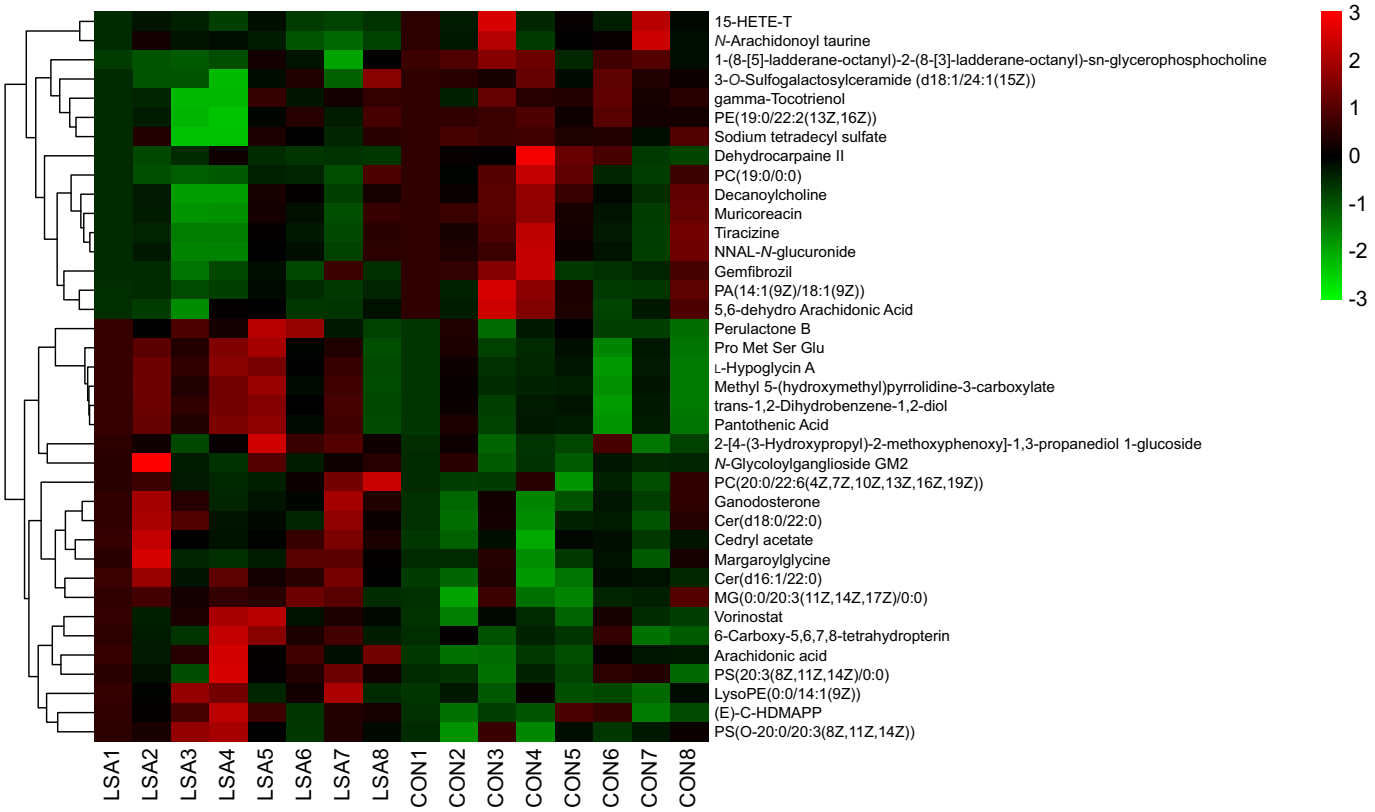

Fig. 3. Heat maps of overall differential metabolites from the control and LSA groups. Each row represents a differential metabolite and each column represents a muscle sample. Different colors represent different abundance intensities (mean value acquired from all detected samples from the same group).

decreased in the LSA group. In addition, metabolic pathway enrichment analysis revealed that these metabolites participate in the following five metabolic pathways: linoleic acid metabolism, pantothenate and CoA biosynthesis, β-alanine metabolism, biosynthesis of unsaturated fatty acids, and arachidonic acid metabolism. The first four pathways were disturbed significantly after LSA treatment (Fig. 4, $P<0.05$). The dataset of metabolomics in this study has been uploaded to figshare (https://doi.org/10.6084/m9.figshare.14174099.v1).

DISCUSSION

Effect of LSA on locomotor behavior of *A. japonicus*

Our behavioral data indicated that LSA participated in the regulation of locomotor activity of *A. japonicus*, and this was particularly true for exercise endurance. The significantly greater distance, number of steps and cumulative duration of movement traveled per hour at the fourth hour after injection suggested that LSA can stimulate locomotor endurance within a certain period of time in this species. Similarly, the pedal peptide-type neuropeptide of *A. japonicus* can accelerate the cumulative duration of movement in the seventh hour after injection (Ding et al., 2020), and the time period expected for LSA to regulate the locomotion of *A. japonicus* is reasonable in this study. The effects of neuropeptides on locomotory performance have been widely studied in invertebrates. For example, neuropeptide F1a and neuropeptide F2 can inhibit stage-related locomotor behavior of locusts, and the downstream nitric oxide synthase regulates these two neuropeptides to adjust locomotory plasticity by controlling the synthesis of nitric oxide in the brain of locusts (Hou et al., 2017). The effect of neuropeptide DH31 on the circadian rhythm of locomotion in *Drosophila* was discovered by the gene knockout method (Goda et al., 2019). Yang et al. (2016) reported that the GdFFD neuropeptide can suppress the locomotor ability of *Aplysia*, mainly by enhancing the activity of the parapedal commissural nerve in the central nervous system. The increased locomotor behavior of nematode npr-1 mutants is caused by progressive amplification of multiple types of sensory neurons, including stretch sensing, touch sensitive and nociceptive neurons, which promote movement by secreting neuropeptides and glutamate (Choi et al., 2015).

Although the effects of diverse neuropeptides on invertebrate locomotion have been widely reported, studies of the effects of SALMFamide neuropeptides on locomotor behavior are scarce. In echinoderms, SALMFamide neuropeptides can give rise to the relaxation and eversion of the cardiac stomach in *A. rubens* (Melarange et al., 1999). They can also result in intestinal contraction in the sea cucumber *Stichopus japonicus* (Ohtani et al., 1999). In addition, nerve fibers immunoreactive to S1 and S2 are present in the tube feet and apical muscles of *A. rubens*, suggesting that they are involved in the locomotor systems of sea stars (Newman et al., 1995). Two SALMFamide neuropeptides identified in the sea cucumber *Holothuria glaberrima* can induce relaxation of longitudinal muscle bands and intestinal muscle tension (Díaz-Miranda and García-Arrarás, 1995; Diaz-Miranda et al., 1995).

The results of our study revealed that the stride of *A. japonicus* increased slightly after LSA injection, indicating that LSA might be involved in the modulation of muscle relaxation in the process of locomotor behavior. This result is consistent with the conclusion of previous studies that SALMFamide neuropeptides act as muscle relaxants in echinoderms. In addition, the distance and number of steps traveled and cumulative duration of movement per hour were significantly higher at the fourth hour post-treatment in the LSA group compared with the control, suggesting that LSA can promote the locomotor endurance of *A. japonicus* within a certain period of time. The increased mean and maximum velocity, and average step velocity in the LSA group revealed that this neuropeptide can improve the locomotor efficiency of sea cucumbers to some extent. In summary, the results of this study indicated that LSA is involved in the regulation of locomotor behavior in sea cucumbers; more precisely, it promotes locomotor endurance.

Possible physiological mechanisms underlying the stimulation of locomotor activity by LSA in *A. japonicus*

In the present study, the locomotory performance of *A. japonicus* was significantly increased relative to the control at the fourth hour after LSA injection; thus, we selected this time point to evaluate LSA-induced changes in metabolite levels in longitudinal muscle tissues. The control and LSA groups exhibited different muscle metabolite profiles, and it was obvious that a combination of metabolic pathways, rather than a single one, was involved in the stimulation of locomotor activity of sea cucumbers. The levels of pantothenic acid, LysoPE and ARA were significantly higher in the muscle tissues of sea cucumbers in the LSA group, whereas the level of PE decreased significantly. Additionally, the ratio of PE to PC was possibly altered after LSA administration. These metabolites are categorized into four main pathways: linoleic acid metabolism, pantothenate and coenzyme A (CoA) biosynthesis, β-alanine metabolism, and biosynthesis of unsaturated fatty acids. All of these metabolites and metabolic pathways may be involved in the physiological mechanisms responsible for the observed LSA-induced promotion of locomotory endurance and efficiency in *A. japonicus*.

Pantothenic acid, also known as vitamin B5, consists of pantoate (a, γ-dihydroxy-β-β dimethylbutyrate) and β-alanine (β-Ala) and is a category B water-soluble vitamin. It occurs in almost all types of animal and plant tissues and is especially common in liver, eggs, yeast and grains. A small portion of pantothenic acid exists in free form, but most of it binds to CoA or acyl carrier protein (ACP, a component of a fatty acid synthase complex) to become a biologically active vitamin B5 complex (Tahiliani and Beinlich, 1991). CoA and ACP can be used as carriers for acyl groups in enzymatic reactions and participate in the synthesis of fatty acids and cholesterol or sterols, oxidation of fatty acids and pyruvic acid or α-ketoglutarate, and bioacetylation (Gonthier et al., 1998).

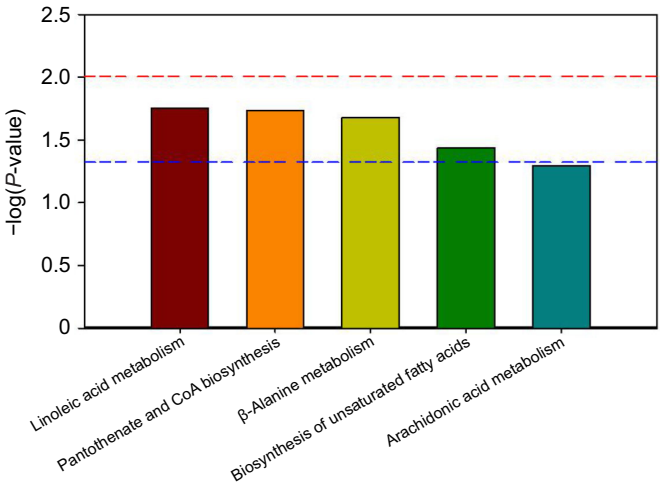

Fig. 4. KEGG pathway enrichment of overall differential metabolites from the control and LSA groups. The *x*-axis shows the enriched pathway, and the *y*-axis shows the significance level of pathway enrichment. Values above the red and blue dashed lines represent $P<0.01$ and $P<0.05$.

Thus, pantothenic acid participates in the production of energy in organisms and controls the metabolism of fatty acids, which is essential for almost all forms of life. In most tissues in organisms, pantothenic acid is transported into cells to synthesize CoA. The main conversion process is: pantothenic acid→pantothenylcysteine→pantatheine→4′-P-pantetheine-dephospho CoA→CoA. CoA plays a key role in the supply of energy to living organisms. It is a cofactor for more than 70 enzyme reaction pathways in organisms, including carbohydrate decomposition, fatty acid oxidation, amino acid decomposition, pyruvate degradation and the tricarboxylic acid cycle, and these processes provide nearly 90% of the energy during the life of the organism. We found that the content of pantothenic acid in the muscle tissues of the LSA group was increased significantly relative to that of the control group. Considering the key role of pantothenic acid in fatty acid decomposition and body function, this result suggested that the increased level of pantothenic acid led to an enhanced energy supply to the muscle, which may explain how LSA promoted locomotor endurance and efficiency in *A. japonicus*.

ARA is a polyunsaturated omega-6 fatty acid that is widely found in various organisms. Previous studies have shown that ARA is present in the sea urchin *Diadema savignyi* and in *A. japonicus*, and it accounts for the highest proportion (>50%) of polyunsaturated fatty acids in the egg and body wall in *D. savignyi* (Kim et al., 2018; Ding et al., 2020). ARA also is a precursor for various bioactive lipid mediators and has potential roles in muscle anabolism and ion channel activation (Ding et al., 2020). Additional intake of ARA is likely to enhance muscle adaptability during the recovery of muscle after acute training in humans (Mitchell et al., 2018). In addition, cytochrome P450 epoxidase can oxidize ARA into four regioisomeric epoxy eicosatrienoic acids that can relax the blood vessels, and the K^+ ion channel of vascular smooth muscle can be activated by cytochrome P450 metabolites of ARA (Hu and Kim, 1993). ARA and other fatty acids can directly activate K^+ ion channels in smooth muscle cells, and K^+ ion channels are closely related to intercellular signaling (Ordway et al., 1989). ARA also can increase the sensitivity of smooth muscle to Ca^{2+} by inhibiting myosin phosphatase activity (Gong et al., 1992). We found that the concentration of ARA in the longitudinal muscle tissues of *A. japonicus* increased after LSA injection, which agrees with the effect of pedal peptide-type neuropeptide on muscle tissues of *A. japonicus* reported by Ding et al. (2020). LSA may activate K^+ ion channels in the muscle cells of sea cucumbers, thereby affecting the signal transduction function of muscle cells and enhancing muscle adaptability. This process is a potential physiological mechanism by which LSA promoted the locomotor endurance and efficiency of *A. japonicus*.

PE and PC are the major phospholipids in almost all cell membranes, and the composition of cell membrane phospholipids is closely related to muscle function, which in turn is related to mitochondria, cell growth, muscle contraction, locomotory performance and insulin sensitivity. (Heden et al., 2016). A study of hepatopathy revealed that the ratio of PC to PE is a key factor regulating cell membrane integrity and that a reduction of this ratio will cause loss of integrity of the cell membrane and result in hepatopathy (Li et al., 2006). After specifically knocking out the enzymes related to PC and PE, researchers found that the synthesis of PE decreased, the value of PC/PE increased, and reduced endoplasmic reticulum/sarcoplasmic reticulum Ca^{2+}-ATPase activity and skeletal muscle ultimately resulted in reduced motor behavior (Funai et al., 2013, 2016; Selathurai et al., 2015). Therefore, glucose metabolism, muscle contraction and locomotor behavior may be associated with the ratio of PC to PE. After acute and long-term physical exercise, the PC/PE ratio in skeletal muscle is reduced (Lee et al., 2018), and the ratio also plays an important role in skeletal muscle metabolism (Wilson et al., 1981; Stuart et al., 1988; Goodyear and Kahn, 1998). In addition, PC and PE convert to lysophosphatidylcholine (LysoPC) and LysoPE, catalyzed by phospholipase A2. PC contains two fatty acids, whereas LysoPC and LysoPE contain only one fatty acid (Alberts et al., 2002). Our metabolomics data revealed that PE and PC(19:0/0:0) levels decreased and PC(20:0/22:6) and LysoPE levels increased significantly in the longitudinal muscle of *A. japonicus* after LSA administration. Although it is difficult to determine the trend of the PC/PE value, a change in this ratio would result in alteration of the fluidity and permeability of the muscle cell membrane in *A. japonicus*, which would influence the physiological function of the muscle cells. Wu et al. (2012) previously demonstrated that the PC/PE ratio is involved in regulating behavioral plasticity of animals. Because the phospholipid composition is crucial for muscle contraction and locomotor behavior in animals and SALMFamides serve as muscle relaxants in echinoderms, changes in membrane fluidity and the physiology of muscle cells caused by changes in the PC/PE value may be a physiological mechanism underlying the improved locomotor endurance and efficiency observed in LSA-treated *A. japonicus*.

Conclusion

We found that LSA participates in the regulation of locomotor behavior of *A. japonicus*. The significantly greater distance and number of steps moved and cumulative duration of movement per hour after injection indicated that this neuropeptide can improve the locomotor endurance of *A. japonicus*. The increased mean and maximum velocities and stride also suggested that LSA enhanced the locomotor efficiency of sea cucumbers. The metabolomics analysis of longitudinal muscle tissues suggested that the increased level of pantothenic acid led to an enhanced energy supply to the muscle. A higher concentration of ARA may activate K^+ ion channels, thereby affecting the signal transduction function of muscle cells and enhancing muscle adaptability. In addition, the increased LysoPE and altered value of PE/PC would result in a change of membrane fluidity and permeability, and consequently influence the physiological function of the muscle cells. Therefore, the alteration of these metabolites would be the physiological mechanisms responsible for the behavioral effects of LSA in *A. japonicus*.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.Z.; Methodology: K.D., P.Z., Q.F., X.G.; Software: K.D., X.F.; Validation: X.G.; Formal analysis: K.D., X.F., X.L.; Investigation: K.D., P.Z., Q.F., S.Z., X.G.; Resources: L.Z.; Data curation: L.Z., X.F., S.Z., X.G., X.L.; Writing - original draft: K.D.; Writing - review & editing: K.D., L.Z.; Visualization: L.Z., P.Z., Q.F., S.Z., X.G., X.L.; Supervision: L.Z., P.Z., X.L.; Project administration: L.Z.; Funding acquisition: L.Z.

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Data availability

The locomotory performance dataset is available from the figshare repository: https://doi.org/10.6084/m9.figshare.14174099.v1

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