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Development of an *In Vitro* Bioassay System to Examine the Effects of Bioactive Substances on the Chromatophores' Movement Using the Epidermis of the Bigfin Reef Squid *Sepioteuthis lessoniana*

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Abstract: To examine the neurophysiological regulation of squid chromatophores, an *in vitro* bioassay system was developed using the epidermis of the bigfin reef squid *Sepioteuthis lessoniana*. The squid epidermis was sectioned under anesthesia with 0.1% ethanol and stretched over a kimwipe soaked with artificial seawater. In cephalopods, chromatophores have three types of pigments: orange, yellow, and dark brown; however, only chromatophores with dark brown pigment were analyzed in this study. After stabilizing the contraction-relaxation movement of the chromatophore, the following bioactive substances were added to the separated epidermis: acetylcholine, α-melanocyte-stimulating hormone, and noradrenaline (each 1 mM); and the chromatophores' movement was observed under a microscope while taking videos. The video-based analysis of squid chromatophores indicated that acetylcholine and α-melanocyte-stimulating hormone had no effect on the squid chromatophores movement under present conditions. Conversely, noradrenaline (1 mM) promoted contraction-relaxation squid chromatophores' movement. The accelerated movement of chromatophores was inhibited by the addition of yohimbine (1 mM) but not by propranolol (1 mM). To the best of our knowledge this is the first report determining that noradrenaline promotes contraction-relaxation movement of the chromatophore *via* α-2 receptors in the bigfin reef squid because yohimbine is known to be an α2-adrenergic receptor blocker. In current study, squid chromatophores responded well to bioactive substances including inhibitors. Collectively, our *in vitro* bioassay provides an effective model to investigate the mechanism of contraction-relaxation movement of squid chromatophores.

Keywords: Squid chromatophore, *In vitro* bioassay, Noradrenaline, Yohimbine, Acetylcholine, α2-Adrenergic receptor

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Introduction

The squid giant axon has been used for neurophysiological studies and contributed to neurotransmission through calcium and potassium channels (Katz and Miledi, 1967; Dipolo, 1974; Griggs *et al.*, 1996). Additionally, the neurotransmitters such as acetylcholine and noradrenaline are highly contained in the squid ganglia (Lam *et al.*, 1974; Juorio and Barlow, 1976) and regulate their behaviors (Jozet-Alves *et al.*, 2012; Thomas *et al.*, 2021). Therefore, these neurotransmitters play a significant role in the neurophysiology of squids.

Conversely, the squid chromatophore has a specific structure in which the pigment-containing sac (elastic sacculus) gets stretched with the contraction of muscle fibers (Loi *et al.*, 1996; Mattiello *et al.*, 2010; Williams *et al.*, 2019). This special structure allows the squid to immediately change its body color (Mattiello *et al.*, 2010; Williams *et al.*, 2019). The movement of squid chromatophores has reportedly been regulated by neurotransmitters, such as acetylcholine, glutamate, and peptide hormones (Loi *et al.*, 1996; Mattiello *et al.*, 2010). Previous studies reported that glutamate (Mattiello *et al.*, 2010) and α-melanocyte-stimulating hormone (Loi *et al.*, 1996) induced chromatophore expansion in the cuttlefish *Sepia officinalis*. However, the detailed mechanism of these bioactive substances mediated by receptors has not been investigated.

This study is focused on the bigfin reef squid *Sepioteuthis lessoniana* (Fig. 1), whose chromatophore contraction-relaxation continues for a relatively extended period (at least 4 h) after being taken out from seawater (supplementary video S1). Using this squid, an *in vitro* bioassay system was developed to investigate the effect of bioactive substances such as acetylcholine, noradrenaline, and α-melanocyte-stimulating hormones on squid chromatophores. The receptor-mediated effects of the α2 blocker (yohimbine) (Jabir *et al.*, 2022) and β2 blocker (propranolol) (Han *et al.*, 2016) on chromatophore movement were examined.

Materials and Methods

Animals:

The bigfin reef squid *S. lessoniana* (Fig. 1) were collected *via* fishing at Tsukumo Bay in Noto Peninsula, Ishikawa Prefecture, Japan. Since our facility faces Tsukumo Bay, the squids we caught were reared and acclimated for 3–6 h in our facility's tanks before being used for our experiments.

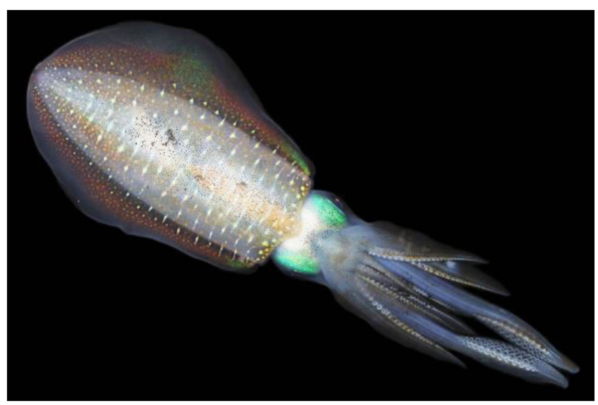

Fig. 1: Photograph of the bigfin reef squid *Sepioteuthis lessoniana*

This study was conducted in strict accordance with the recommendations in the ethical guidelines of Kanazawa University. All the experiments were performed under anesthesia to minimize pain and discomfort.

Reagents:

Acetylcholine chloride, L-noradrenaline, yohimbine hydrochloride, and propranolol hydrochloride were purchased from Wako Pure Chemical Corporation (Osaka, Japan). α-melanocyte-stimulating hormone (Sigma-Aldrich Inc., St. Louis, MO, USA) was used in our experiments.

Development of an in vitro bioassay system using the epidermis of the bigfin reef squid to observe the chromatophores' movement:

The squid epidermis was dissected out (approximately 1.5–2 cm^2) under anesthesia with 0.1% ethanol and stretched over a kimwipe soaked with artificial seawater (Allen seawater: NaCl 3%, $MgSO_4 \cdot 7H_2O$ 0.358%, $MgCl_2 \cdot 6H_2O$ 0.272%, $CaCl_2 \cdot 2H_2O$ 0.06%, KCl 0.039%, $NaHCO_3$ 0.01%) (Suzuki *et al.*, 2016). After letting the movement of the chromatophores stabilized, bioactive substances were added to the separated epidermis and the contraction-relaxation movement of the chromatophores was observed and video recorded under the microscope (SMZ745T-1K, NIKON Corporation, Tokyo, Japan) using a camera (FLOYD, WRAYMER Inc., Osaka, Japan). In cephalopods, chromatophores have three types of pigments: orange, yellow, and dark brown (Loi *et al.*, 1996); however, only dark brown chromatophores were analyzed in this study.

Effects of bioactive substances on chromatophore movement with an in vitro bioassay system using the epidermis of the bigfin reef squid:

After letting the contraction-relaxation movement of the chromatophores stabilized, their movement was counted before adding the reagent for 10s by video-based analysis. Thereafter, the chromatophores' movement was counted every 30s until 290s after adding each reagent. In each experiment, we focused on three chromatophores and calculated the number of times each chromatophore moves per 10s.

Based on the above procedure, the effects of bioactive substances on chromatophore contraction-relaxation movement were examined. Acetylcholine chloride, L-noradrenaline, yohimbine hydrochloride, propranolol hydrochloride, and α-melanocyte-stimulating hormone were each diluted with distilled water to 1 mM and were added to the epidermis containing the chromatophore. Firstly, the effects of acetylcholine chloride, L-noradrenaline, and α-melanocyte-stimulating hormone on chromatophores' movement were examined. Secondly, the inhibitory action of blockers (yohimbine hydrochloride and propranolol hydrochloride) was analyzed by simultaneously adding blockers to the above reagents.

Results

Effects of acetylcholine and α-melanocyte-stimulating hormone on chromatophore movement in the bigfin reef squid:

The contraction-relaxation frequency of chromatophores by acetylcholine and α-melanocyte-stimulating hormone was counted by video-based analysis. As a result, no changes in the chromatophores' movement were observed by adding either acetylcholine (1 mM) or α-melanocyte-stimulating hormone (1 mM) as shown in Figures 2 and 3, respectively.

Effects of noradrenaline on chromatophore movement in the bigfin reef squid:

The chromatophore movement by adding noradrenaline was examined by the video-based analysis and it was found that noradrenaline promoted the contraction-relaxation movement of chromatophores in the bigfin reef squid (Fig. 4). By adding noradrenaline (1 mM), chromatophores in the bigfin reef squid showed intense contraction-relaxation movements than before adding noradrenaline (supplementary video S2).

Analysis of noradrenaline action on chromatophore movement with blockers:

In order to investigate the action mechanism of noradrenaline, the influences of blockers (yohimbine and propranolol) were examined. It

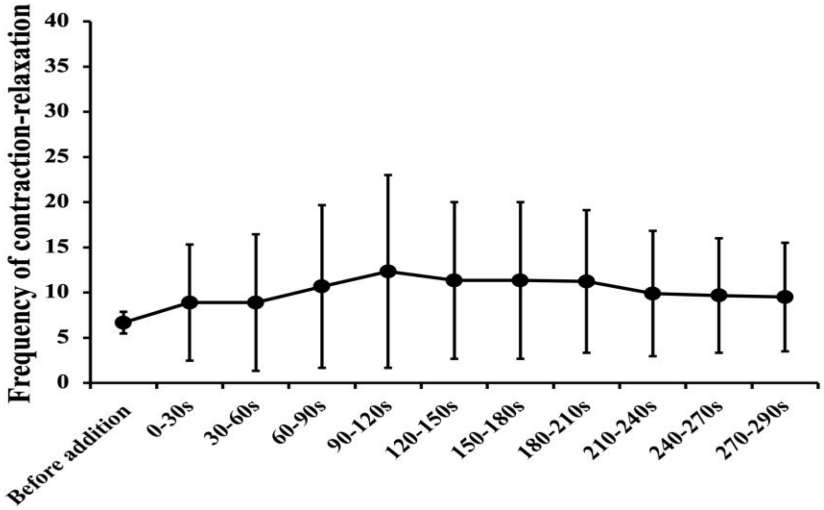

Fig. 2: Changes in frequency of contraction-relaxation by adding acetylcholine (1 mM). The frequency of contraction-relaxation of the chromatophores per 10 sec was counted. All results (n = 3) are expressed as means ± standard error.

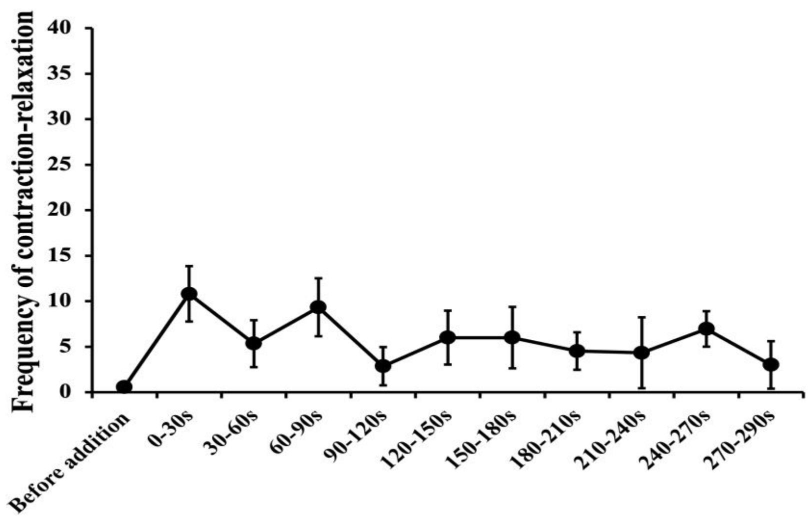

Fig. 3: Changes in frequency of contraction-relaxation by adding α-melanocyte-stimulating hormone (1 mM). The frequency of contraction-relaxation of the chromatophores per 10 sec was counted. All results (n = 3) are expressed as means ± standard error.

was found that yohimbine inhibited the contraction-relaxation movement of chromatophores (Fig. 5, supplementary video S3), whereas propranolol did not affect the chromatophore movement (Fig. 6, supplementary video S4).

Supporting Video information:

Supplementary video associated with this article can be found at following link: http://ijzi.net/Chromatophores.html

Discussion

In this study, an *in vitro* bioassay system was developed using the epidermis of the bigfin reef squid *S. lessoniana* to examine the neurophysiological regulation of squid chromatophores. This bioassay was evaluated by taking videos of the contraction-relaxation movement of the chromatophore and counting the frequency of these movements.

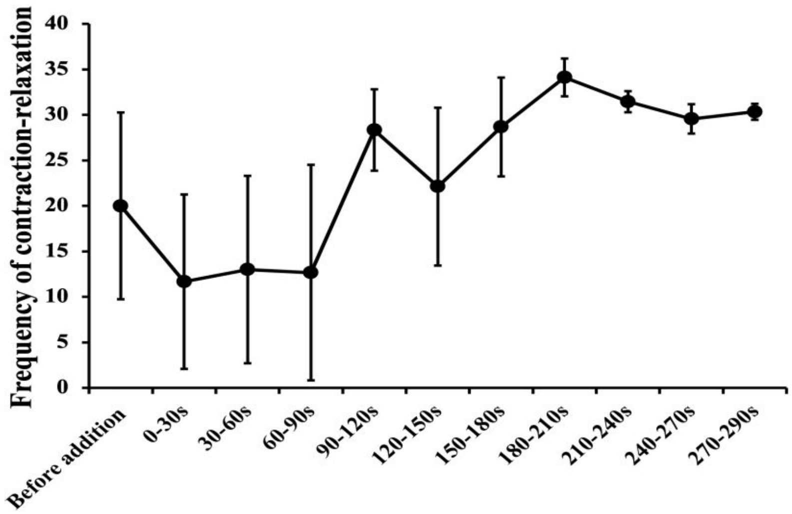

Fig. 4: Changes in frequency of contraction-relaxation by adding noradrenaline (1 mM). The frequency of contraction-relaxation of the chromatophores per 10 sec was counted. All results (n = 3) are expressed as means ± standard error.

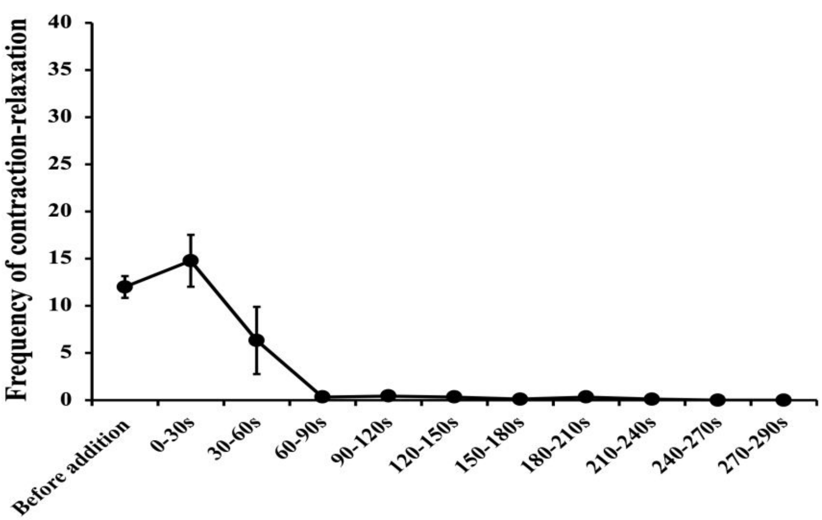

Fig. 5: Changes in frequency of contraction-relaxation by adding noradrenaline and yohimbine (each 1 mM) simultaneously. The frequency of contraction-relaxation of the chromatophores per 10 sec was counted. All results (n = 3) are expressed as means ± standard error.

Using our *in vitro* bioassay with squid epidermis, the influence of acetylcholine and α-melanocyte-stimulating hormone on chromatophore movement was investigated. Acetylcholine and α-melanocyte-stimulating hormone did not influence chromatophore contraction-relaxation movement under the present conditions in the bigfin reef squid. The chromatophore of the European common squid *Loligo vulgaris* was expanded by acetylcholine (1 μM) (Smotherman, 2002). The chromatophores of cuttlefish *Sepia officinalis* were also expanded with the treatment of α-melanocyte-stimulating hormone (100 μM) (Loi *et al.*, 1996). Different squid species may respond differently to chromatophores' movement. Notably, we found that noradrenaline promoted the contraction-relaxation movement of chromatophores in the bigfin reef squid. To the best of our knowledge, this is the first report showing that noradrenaline affects the contraction-relaxation movement of squid chromatophores. Moreover, this is the first

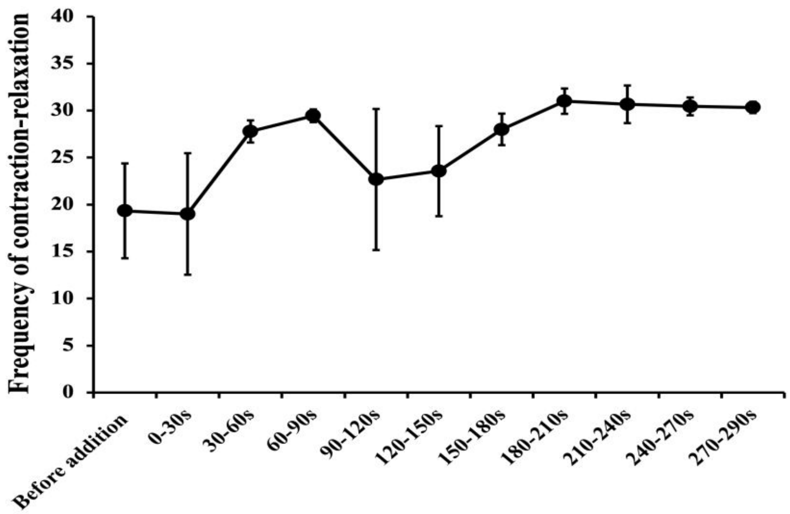

Fig. 6: Changes in frequency of contraction-relaxation by adding noradrenaline and propranolol (each 1 mM) simultaneously. The frequency of contraction-relaxation of the chromatophores per 10 sec was counted. All results (n = 3) are expressed as means ± standard error.

study demonstrating that yohimbine inhibits the chromatophore movement, whereas propranolol does not affect it (see videos in supplementary S3 and S4). Since yohimbine is known α2-adrenergic receptor blocker (Jabir *et al.*, 2022), this study is the first to determine that noradrenaline promotes contraction-relaxation movement of the chromatophore *via* α2 receptors in the bigfin reef squid.

As compared to other squids, the chromatophores of the bluefin reef squid more frequently moved and contraction-relaxation movements were repeated. The muscles of chromatophores in the bigfin reef squid are probably highly developed than those of other squids, and thus we plan to conduct a detailed analysis of the morphological structure of chromatophores in the bigfin reef squid in future.

Conclusion

In the bigfin reef squid *S. lessoniana*, noradrenaline promotes contraction and relaxation movements of chromatophores *via* α2-adrenergic receptors using the bioassay system developed in our laboratory by video-based analysis. This *in vitro* bioassay provided an effective model to investigate the mechanism of movement of squid chromatophores.

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References

DiPolo R. (1974) Effect of ATP on the calcium efflux in dialyzed squid giant axons. J Gen Physiol. 64: 503-517.

Griggs WSD, Hanyu Y and Matsumoto G. (1996) Cultured giant fiber lobe of squid expresses three distinct potassium channel activities in selective combinations. J Membr Biol. 152: 25-37.

Han SO, Pope R, Li S, Kishnani PS, Steet R and Koeberl DD. (2016) A beta-blocker, propranolol, decreases the efficacy from enzyme replacement therapy in Pompe disease. Mol Genet Metab. 117: 114-119.

Jabir NR, Firoz CK, Zughaibi TA, Alsaadi MA, Abuzenadah AM, Al-Asmari AI, Alsaieedi A, Ahmed BA, Ramu AK and Tabrez S. (2022) A literature perspective on the pharmacological applications of yohimbine. Ann Med. 54: 2861-2875.

Jozet-Alves C, Romagny S, Bellanger C and Dickel L. (2012) Cerebral correlates of visual lateralization in Sepia. Behav Brain Res. 234: 20-25.

Juorio AV and Barlow JJ. (1976) High noradrenaline content of a squid ganglion. Brain Res. 104: 379-383.

Katz B and Miledi R. (1967) A study of synaptic transmission in the absence of nerve impulses. J Physiol. 192: 407-436.

Lam DMK, Wiesel TN and Kaneko A. (1974) Neurotransmitter synthesis in cephalopod retina. Brain Res. 82: 365-368.

Loi PK, Saunders RG, Young DC and Tublitz NJ. (1996) Peptidergic regulation of chromatophore function in the European cuttlefish *Sepia officinalis*. J Exp Biol. 199: 1177-1187.

Mattiello T, Fiore G, Brown ER, d'Ischia M and Palumbo A. (2010) Nitric oxide mediates the glutamate-dependent pathway for neurotransmission in *Sepia officinalis* chromatophore organs. J Biol Chem. 285: 24154-24163.

Smotherman M. (2002) Acetylcholine mediates excitatory input to chromatophore motoneurons in the squid, *Loligo pealeii*. Biol Bull. 203: 231-232.

Suzuki N, Sato M, Nassar HF, Abdel-Gawad FKh, Bassem SM, Yachiguchi K, Tabuchi Y, Endo M, Sekiguchi T, Urata M, Hattori A, Mishima H, Shimasaki Y, Oshima Y, Hong CS, Makino F, Tang N, Toriba A and Hayakawa K. (2016) Seawater polluted with highly concentrated polycyclic aromatic hydrocarbons suppresses osteoblastic activity in the scales of goldfish, *Carassius auratus*. Zool Sci. 33: 407-413.

Thomas JT, Spady BL, Munday PL and Watson SA. (2021) The role of ligand-gated chloride channels in behavioural alterations at elevated CO_2 in a cephalopod. J Exp Biol. 224: 242335.

Williams TL, Senft SL, Yeo J, Martín-Martínez FJ, Kuzirian AM, Martin CA, DiBona CW, Chen CT, Dinneen SR, Nguyen HT, Gomes CM, Rosenthal JJC, MacManes MD, Chu F, Buehler MJ, Hanlon RT and Deravi LF. (2019) Dynamic pigmentary and structural coloration within cephalopod chromatophore organs. Nat Commun. 10: 1004.